IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK and QIAGEN SCIENCES, LLC,	)	
Plaintiffs,	)	Civil Action No. 19-1681-CFC
v.	)	
ILLUMINA, INC.,	)	
Defendant.	)	

JOINT CLAIM CONSTRUCTION APPENDIX

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US010407458B2

(12) United States Patent

Ju et al.

(10) Patent No.: US 10,407,458 B2

(45) Date of Patent: *Sep. 10, 2019

(54) **MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA**

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(72) Inventors: **Jingyue Ju**, Englewood Cliffs, NJ (US); **Zengmin Li**, Flushing, NY (US); **John Robert Edwards**, St. Louis, MO (US); **Yasuhiro Itagaki**, New York, NY (US)

(73) Assignee: **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(22) Filed: **Oct. 1, 2018**

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(60) Continuation of application No. 15/915,983, filed on Mar. 8, 2018, which is a continuation of application No. 14/670,748, filed on Mar. 27, 2015, which is a continuation of application No. 13/959,660, filed on Aug. 5, 2013, now Pat. No. 9,133,511, which is a continuation of application No. 13/672,437, filed on Nov. 8, 2012, now abandoned, which is a continuation of application No. 13/339,089, filed on Dec. 28, 2011, now abandoned, which is a continuation of application No. 12/804,284, filed on Jul. 19, 2010, now Pat. No. 8,088,575, which is a continuation of application No. 11/810,509, filed on Jun. 5, 2007, now Pat. No. 7,790,869, which is a continuation of application No. 10/702,203, filed on Nov. 4, 2003, now Pat. No. 7,345,159, which is a division of application No. 09/972,364, filed on Oct. 5, 2001, now Pat. No. 6,664,079, which is a continuation-in-part of application No. 09/684,670, filed on Oct. 6, 2000, now abandoned.

(60) Provisional application No. 60/300,894, filed on Jun. 26, 2001.

(51) **Int. Cl.**

C07H 19/14	(2006.01)
C12Q 1/68	(2018.01)
C07H 21/00	(2006.01)
C12Q 1/686	(2018.01)
C12Q 1/6874	(2018.01)
C12Q 1/6872	(2018.01)
C12Q 1/6869	(2018.01)
C07H 19/10	(2006.01)
C12Q 1/6876	(2018.01)
C40B 40/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 19/14*** (2013.01); ***C07H 19/10*** (2013.01); ***C07H 21/00*** (2013.01); ***C12Q 1/68*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6869*** (2013.01); ***C12Q 1/6872*** (2013.01); ***C12Q 1/6874*** (2013.01); ***C12Q 1/6876*** (2013.01); *C07B 2200/11* (2013.01); *C12Q 2525/117* (2013.01); *C12Q 2525/186* (2013.01); *C12Q 2535/101* (2013.01); *C12Q 2535/122* (2013.01); *C12Q 2563/107* (2013.01); *C12Q 2565/501* (2013.01); *C40B 40/00* (2013.01)

(58) **Field of Classification Search**

CPC C07H 19/04; C12Q 1/6869

USPC 536/4.1; 435/6.1

See application file for complete search history.

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(Continued)

Primary Examiner — Jezia Riley

(74) *Attorney, Agent, or Firm* — John P. White; Cooper & Dunham LLP

(57) **ABSTRACT**

This invention provides methods for attaching a nucleic acid to a solid surface and for sequencing nucleic acid by detecting the identity of each nucleotide analog after the nucleotide analog is incorporated into a growing strand of DNA in a polymerase reaction. The invention also provides nucleotide analogs which comprise unique labels attached to the nucleotide analog through a cleavable linker, and a cleavable chemical group to cap the —OH group at the 3'-position of the deoxyribose.

2 Claims, 28 Drawing Sheets

Specification includes a Sequence Listing.

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Patent Owner's Sur-reply filed by Columbia on Feb. 5, 2019, in connection with IPR No. IPR2018-00797.

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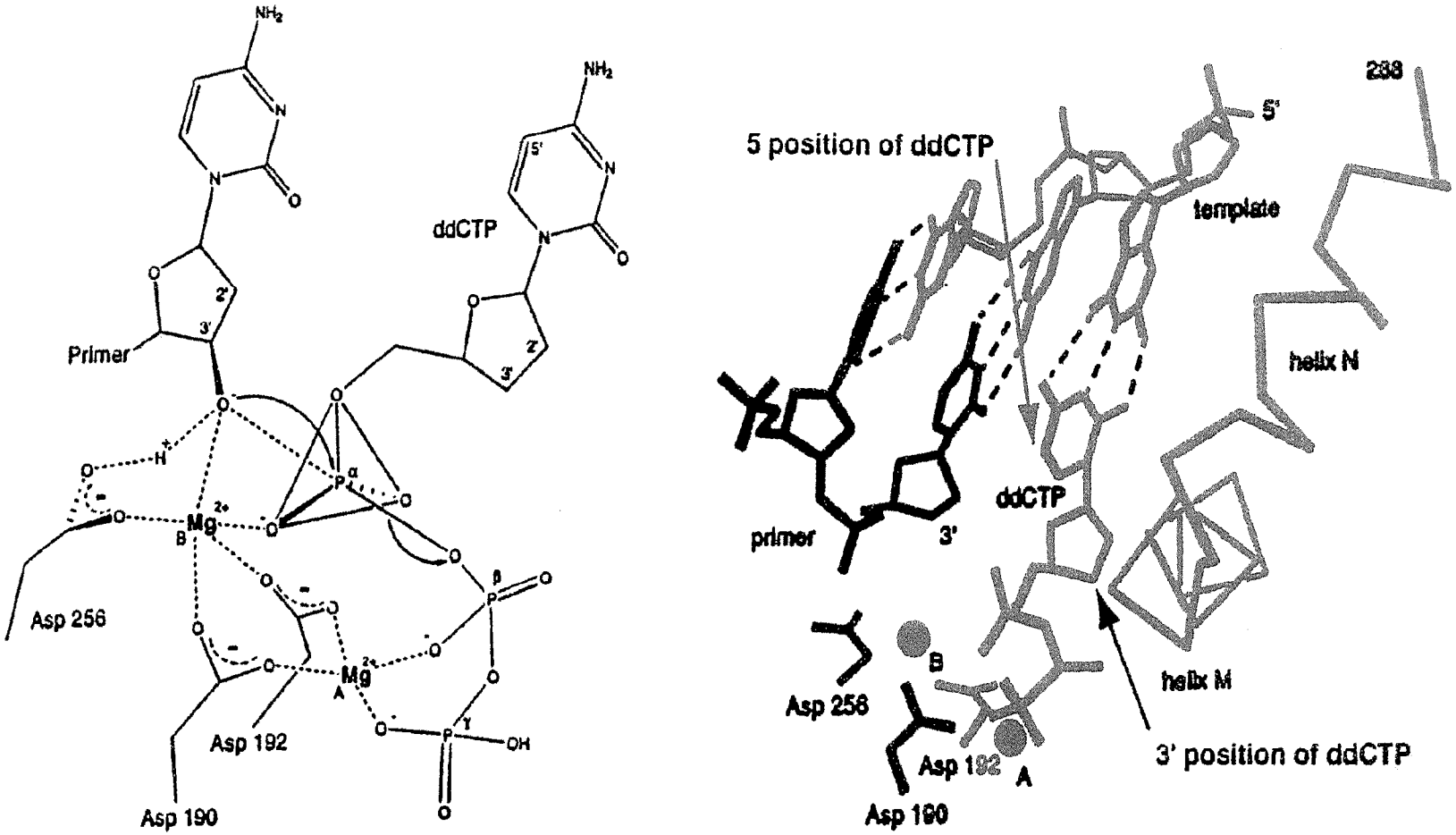
Final Written Decision dated Jun. 21, 2019, in connection with IPRs Nos. IPR2018-00291, IPR2018-00318, IPR2018-00322, and IPR2018-00385.

FIGURE 1



Asp = Aspartic Acid

FIGURE 7

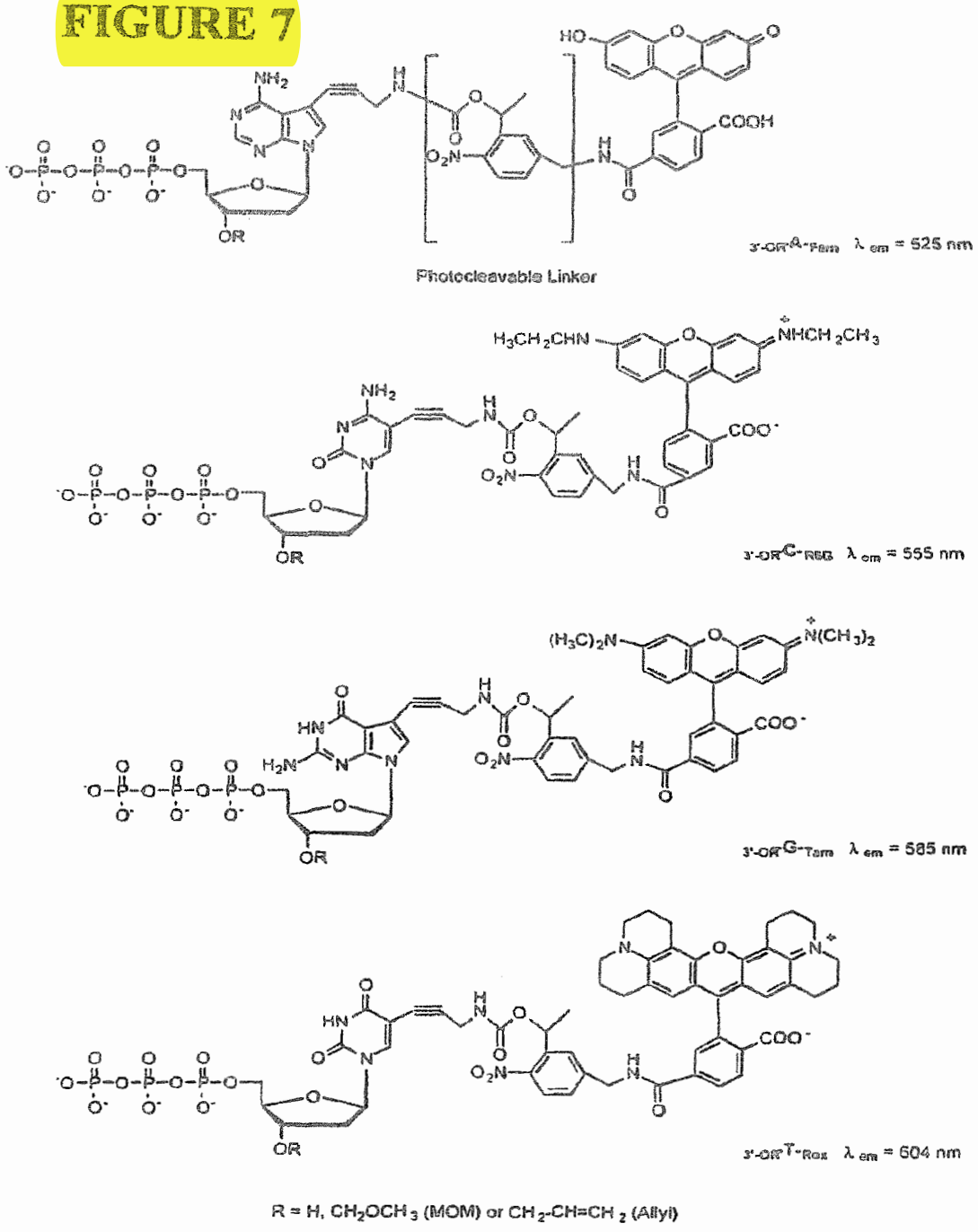

Photocleavable Linker
3'-OR^A-Fam λ em = 525 nm
3'-OR^C-RSG λ em = 555 nm
3'-OR^G-Tam λ em = 585 nm
3'-OR^T-Rox λ em = 604 nm
R = H, CH2OCH3 (MOM) or CH2-CH=CH2 (Allyl)

FIGURE 8

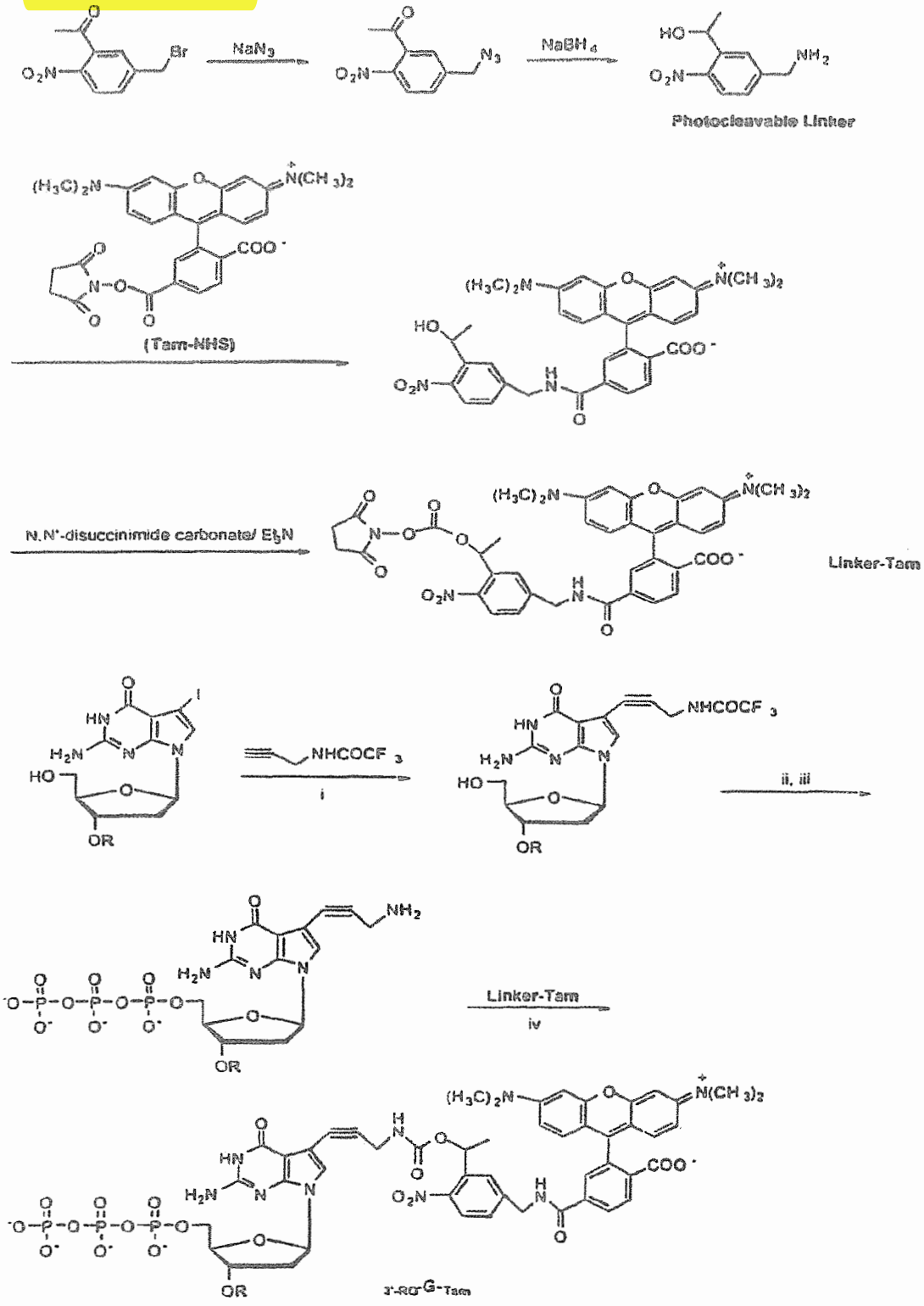

NaN3
NaBH4
Photocleavable Linker
(Tam-NHS)
N,N'-disuccinimide carbonate/ Et3N
Linker-Tam
i
ii, iii
iv
Linker-Tam
3'-RO-G-Tam

FIGURE 16

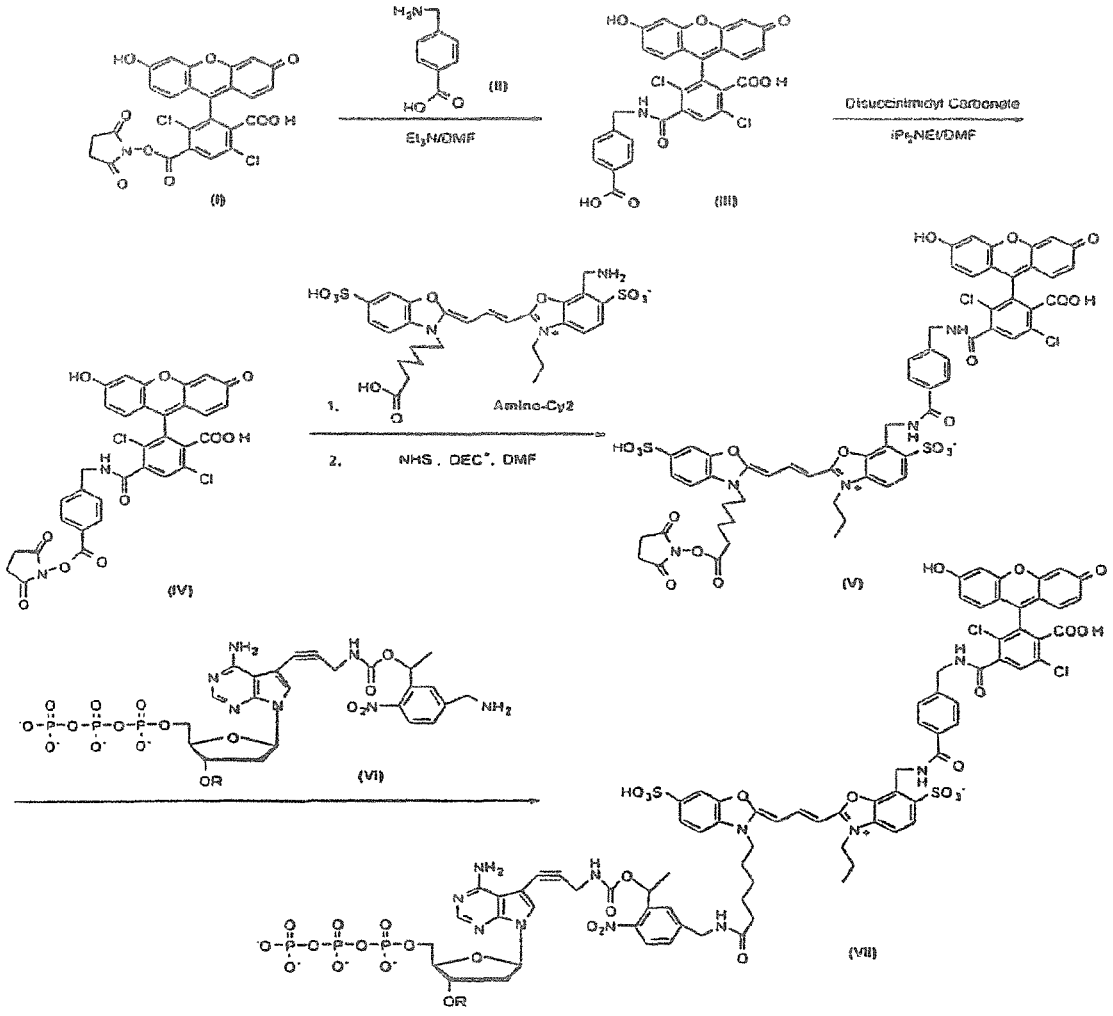
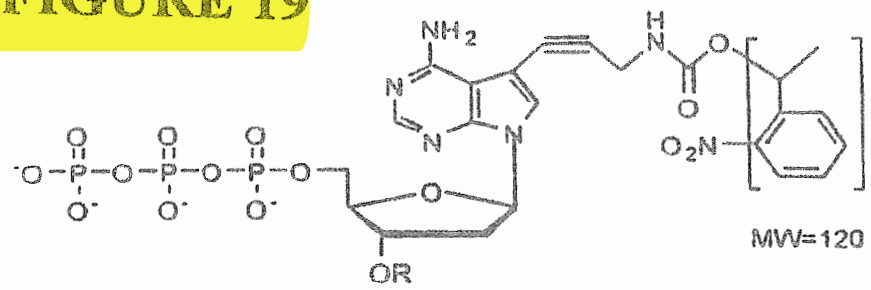
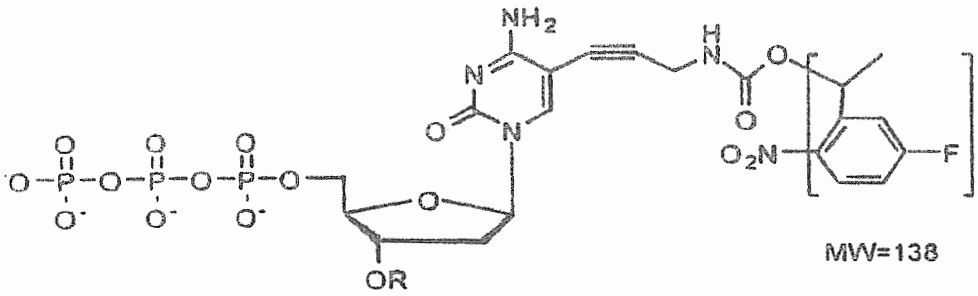

(I)
(II)
Et3N/DMF
(III)
Disuccinimidyl Carbonate
iPr2NEt/DMF
1.
Amino-Cy2
2.
NHS , DEC* , DMF
(IV)
(V)
OR
(VI)
(VII)

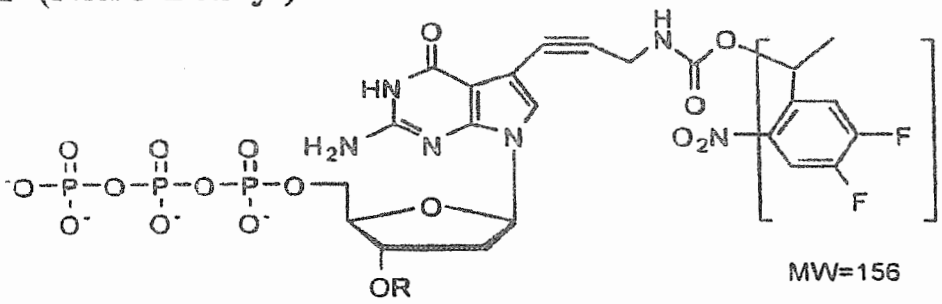
FIGURE 19



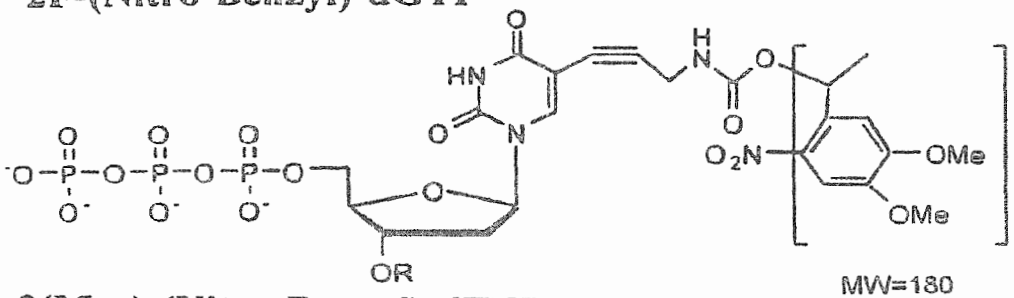
(Nitro-Benzyl)-dATP



F-(Nitro-Benzyl)-dCTP



2F-(Nitro-Benzyl)-dGTP



2(Meo)-(Nitro-Benzyl)-dTTP

R = H, MOM or Allyl

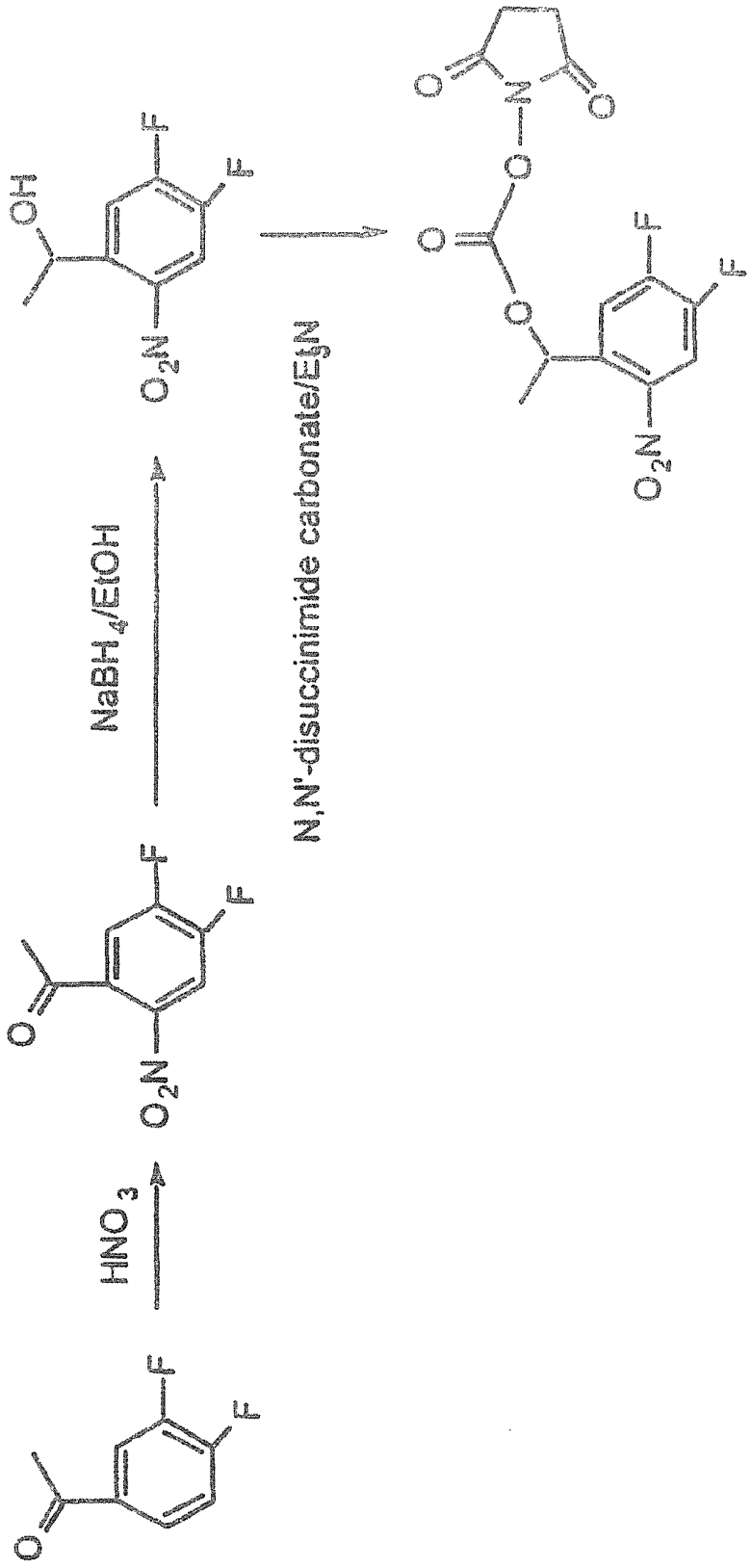


FIGURE 20

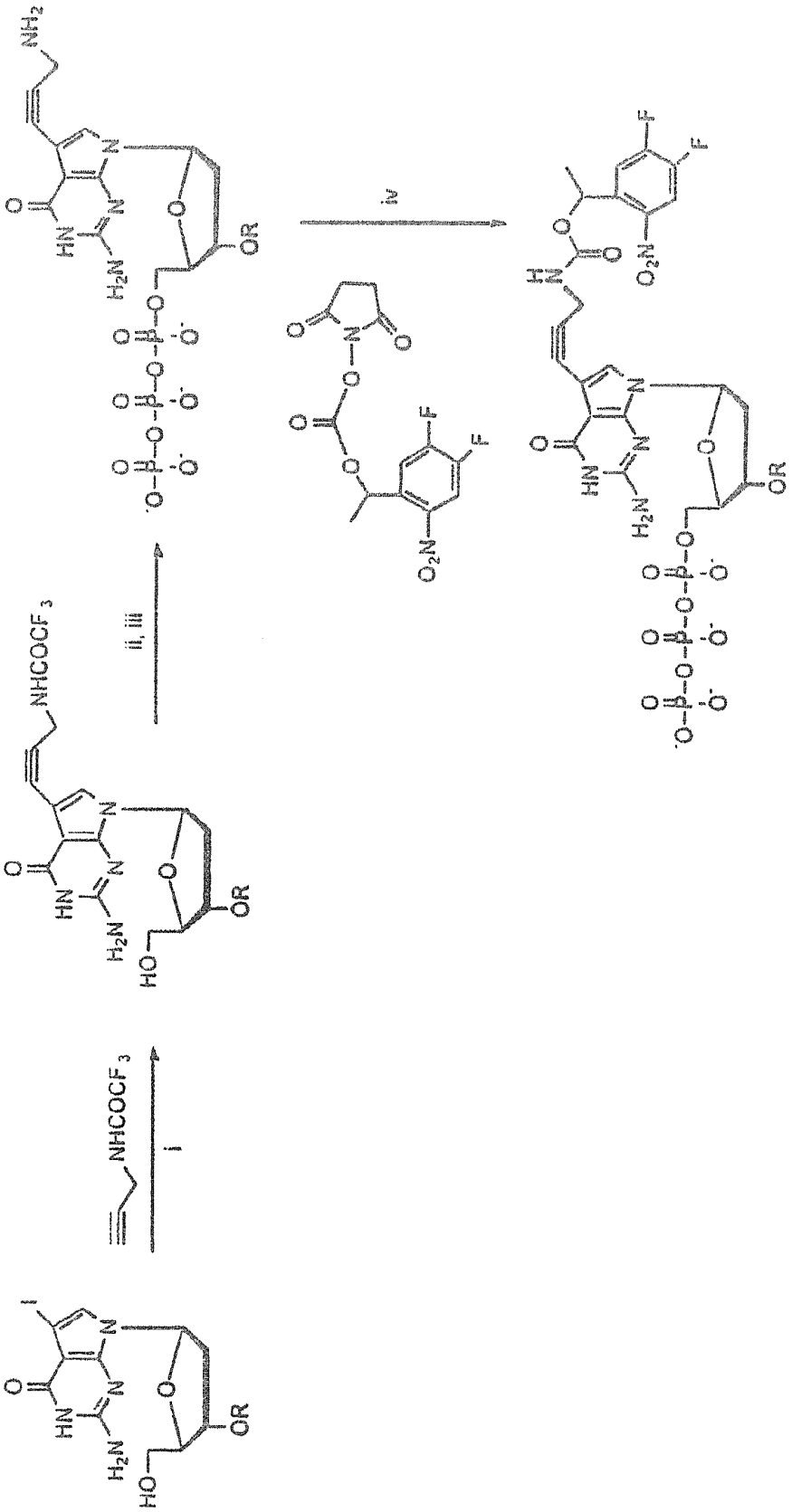


FIGURE 21

MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA

This application is a continuation of U.S. Ser. No. 15/915,983, filed Mar. 8, 2018, which is a continuation of U.S. Ser. No. 14/670,748, filed Mar. 27, 2015, which is a continuation of U.S. Ser. No. 13/959,660, filed Aug. 5, 2013, now U.S. Pat. No. 9,133,511, issued Sep. 15, 2015, which is a continuation of U.S. Ser. No. 13/672,437, filed Nov. 8, 2012, now abandoned, which is a continuation of U.S. Ser. No. 13/339,089, filed Dec. 28, 2011, now abandoned, which is a continuation of U.S. Ser. No. 12/804,284, filed Jul. 19, 2010, now U.S. Pat. No. 8,088,575, issued Jan. 3, 2012, which is a continuation of U.S. Ser. No. 11/810,509, filed Jun. 5, 2007, now U.S. Pat. No. 7,790,869, issued. Sep. 7, 2010, which is a continuation of U.S. Ser. No. 10/702,203, filed Nov. 4, 2003, now U.S. Pat. No. 7,345,159, issued Mar. 18, 2008, which is a divisional of U.S. Ser. No. 09/972,364, filed Oct. 5, 2001, now U.S. Pat. No. 6,664,079; issued Dec. 16, 2003, claiming the benefit of U.S. Provisional Application No. 60/300,894, filed Jun. 26, 2001, and is a continuation-in-part of U.S. Ser. No. 09/684,670, filed Oct. 6, 2000, now abandoned, the contents of each of which are hereby incorporated in its entirety into this application.

This invention was made with government support under grant no. BES0097793 awarded by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

The ability to sequence deoxyribonucleic acid (DNA) accurately and rapidly is revolutionizing biology and medicine. The confluence of the massive Human Genome Project is driving an exponential growth in the development of high throughput genetic analysis technologies. This rapid technological development involving chemistry, engineering, biology, and computer science makes it possible to move from studying single genes at a time to analyzing and comparing entire genomes.

With the completion of the first entire human genome sequence map, many areas in the genome that are highly polymorphic in both exons and introns will be known. The pharmacogenomics challenge is to comprehensively identify the genes and functional polymorphisms associated with the variability in drug response (Roses, 2000). Resequencing of polymorphic areas in the genome that are linked to disease development will contribute greatly to the understanding of diseases, such as cancer, and therapeutic development. Thus, high-throughput accurate methods for resequencing the highly variable intron/exon regions of the genome are needed in order to explore the full potential of the complete human genome sequence map. The current state-of-the-art technology for high throughput DNA sequencing, such as used for the Human Genome Project (Pennisi 2000), is capillary array DNA sequencers using laser induced fluorescence detection (Smith et al., 1986; Ju et al. 1995, 1996; Kheterpal et al. 1996; Salas-Solano et al. 1998). Improvements in the polymerase that lead to uniform termination efficiency and the introduction of thermostable polymerases have also significantly improved the quality of sequencing data (Tabor and Richardson, 1987, 1995). Although capillary array DNA sequencing technology to some extent addresses the throughput and read length requirements of large scale DNA sequencing projects, the throughput and accuracy required for mutation studies needs to be improved for a wide variety of applications ranging from disease gene discovery to forensic identification. For example, electrophoresis based DNA sequencing methods have difficulty detecting heterozygotes unambiguously and are not 100% accurate in regions rich in nucleotides comprising guanine or cytosine due to compressions (Bowling et al. 1991; Yamakawa et al. 1997). In addition, the first few bases after the priming site are often masked by the high fluorescence signal from excess dye-labeled primers or dye-labeled terminators, and are therefore difficult to identify. Therefore, the requirement of electrophoresis for DNA sequencing is still the bottleneck for high-throughput DNA sequencing and mutation detection projects.

The concept of sequencing DNA by synthesis without using electrophoresis was first revealed in 1988 (Hyman, 1988) and involves detecting the identity of each nucleotide as it is incorporated into the growing strand of DNA in a polymerase reaction. Such a scheme coupled with the chip format and laser-induced fluorescent detection has the potential to markedly increase the throughput of DNA sequencing projects. Consequently, several groups have investigated such a system with an aim to construct an ultra high-throughput DNA sequencing procedure (Cheeseman 1994, Metzker et al. 1994). Thus far, no complete success of using such a system to unambiguously sequence DNA has been reported. The pyrosequencing approach that employs four natural nucleotides (comprising a base of adenine (A), cytosine (C), guanine (G), or thymine (T)) and several other enzymes for sequencing DNA by synthesis is now widely used for mutation detection (Ronaghi 1998). In this approach, the detection is based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to adenosine triphosphate (ATP) by sulfurylase, and the subsequent production of visible light by firefly luciferase. This procedure can only sequence up to 30 base pairs (bps) of nucleotide sequences, and each of the 4 nucleotides needs to be added separately and detected separately. Long stretches of the same bases cannot be identified unambiguously with the pyrosequencing method.

More recent work in the literature exploring DNA sequencing by a synthesis method is mostly focused on designing and synthesizing a photocleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of deoxynucleoside triphosphates (dNTPs) (Welch et al. 1999). Limited success for the incorporation of the 3'-modified nucleotide by DNA polymerase is reported. The reason is that the 3'-position on the deoxyribose is very close to the amino acid residues in the active site of the polymerase, and the polymerase is therefore sensitive to modification in this area of the deoxyribose ring. On the other hand, it is known that modified DNA polymerases (Thermo Sequenase and Taq FS polymerase) are able to recognize nucleotides with extensive modifications with bulky groups such as energy transfer dyes at the 5-position of the pyrimidines (T and C) and at the 7-position of purines (G and A) (Rosenblum et al. 1997, Zhu et al. 1994). The ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP) have been determined (Pelletier et al. 1994) which supports this fact. As shown in FIG. **1**, the 3-D structure indicates that the surrounding area of the 3'-posi-

tion of the deoxyribose ring in ddCTP is very crowded, while there is ample space for modification on the 5-position the cytidine base.

The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye or a mass tag through a cleavable linker to the nucleotide base or an analogue of the nucleotide base, such as to the 5-position of the pyrimidines (T and C) and to the 7-position of the purines (G and A), to use a small cleavable chemical moiety to cap the 3'-OH group of the deoxyribose to make it nonreactive, and to incorporate the nucleotide analogues into the growing DNA strand as terminators. Detection of the unique label will yield the sequence identity of the nucleotide. Upon removing the label and the 3'-OH capping group, the polymerase reaction will proceed to incorporate the next nucleotide analogue and detect the next base.

It is also desirable to use a photocleavable group to cap the 3'-OH group. However, a photocleavable group is generally bulky and thus the DNA polymerase will have difficulty to incorporate the nucleotide analogues containing a photocleavable moiety capping the 3'-OH group. If small chemical moieties that can be easily cleaved chemically with high yield can be used to cap the 3'-OH group, such nucleotide analogues should also be recognized as substrates for DNA polymerase. It has been reported that 3'-O-methoxy-deoxynucleotides are good substrates for several polymerases (Axelrod et al. 1978). 3'-O-allyl-dATP was also shown to be incorporated by Ventr(exo-) DNA polymerase in the growing strand of DNA (Metzker et al. 1994). However, the procedure to chemically cleave the methoxy group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methoxy group to cap the 3'-OH group for sequencing DNA by synthesis. An ester group was also explored to cap the 3'-OH group of the nucleotide, but it was shown to be cleaved by the nucleophiles in the active site in DNA polymerase (Canard et al. 1995). Chemical groups with electrophiles such as ketone groups are not suitable for protecting the 3'-OH of the nucleotide in enzymatic reactions due to the existence of strong nucleophiles in the polymerase. It is known that MOM ($—CH_2OCH_3$) and allyl ($—CH_2CH═CH_2$) groups can be used to cap an —OH group, and can be cleaved chemically with high yield (Ireland et al. 1986; Kamal et al. 1999). The approach disclosed in the present application is to incorporate nucleotide analogues, which are labeled with cleavable, unique labels such as fluorescent dyes or mass tags and where the 3'-OH is capped with a cleavable chemical moiety such as either a MOM group ($—CH_2OCH_3$) or an allyl group ($—CH_2CH═CH_2$), into the growing strand DNA as terminators. The optimized nucleotide set ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$, where R denotes the chemical group used to cap the 3'-OH) can then be used for DNA sequencing by the synthesis approach.

There are many advantages of using mass spectrometry (MS) to detect small and stable molecules. For example, the mass resolution can be as good as one dalton. Thus, compared to gel electrophoresis sequencing systems and the laser induced fluorescence detection approach which have overlapping fluorescence emission spectra, leading to heterozygote detection difficulty, the MS approach disclosed in this application produces very high resolution of sequencing data by detecting the cleaved small mass tags instead of the long DNA fragment. This method also produces extremely fast separation in the time scale of microseconds. The high resolution allows accurate digital mutation and heterozygote detection. Another advantage of sequencing with mass spectrometry by detecting the small mass tags is that the compressions associated with gel based systems are completely eliminated.

In order to maintain a continuous hybridized primer extension product with the template DNA, a primer that contains a stable loop to form an entity capable of self-priming in a polymerase reaction can be ligated to the 3' end of each single stranded DNA template that is immobilized on a solid surface such as a chip. This approach will solve the problem of washing off the growing extension products in each cycle.

Saxon and Bertozzi (2000) developed an elegant and highly specific coupling chemistry linking a specific group that contains a phosphine moiety to an azido group on the surface of a biological cell. In the present application, this coupling chemistry is adopted to create a solid surface which is coated with a covalently linked phosphine moiety, and to generate polymerase chain reaction (PCR) products that contain an azido group at the 5' end for specific coupling of the DNA template with the solid surface. One example of a solid surface is glass channels which have an inner wall with an uneven or porous surface to increase the surface area. Another example is a chip.

The present application discloses a novel and advantageous system for DNA sequencing by the synthesis approach which employs a stable DNA template, which is able to self prime for the polymerase reaction, covalently linked to a solid surface such as a chip, and 4 unique nucleotides analogues ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$). The success of this novel system will allow the development of an ultra high-throughput and high fidelity DNA sequencing system for polymorphism, pharmacogenetics applications and for whole genome sequencing. This fast and accurate DNA resequencing system is needed in such fields as detection of single nucleotide polymorphisms (SNPs) (Chee et al. 1996), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), identification in forensics, and genetic disease association studies.

SUMMARY OF THE INVENTION

This invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) detecting the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the —OH group at the 3'-position of the deoxyribose to uncap the —OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (viii) so as to detect the identity of a newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and

wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose.

The invention provides a parallel mass spectrometry system, which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**: The 3D structure of the ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP). The left side of the illustration shows the mechanism for the addition of ddCTP and the right side of the illustration shows the active site of the polymerase. Note that the 3' position of the dideoxyribose ring is very crowded, while ample space is available at the 5 position of the cytidine base.

FIG. **2**A-**2**B: Scheme of sequencing by the synthesis approach. A: Example where the unique labels are dyes and the solid surface is a chip. B: Example where the unique labels are mass tags and the solid surface is channels etched into a glass chip. A, C, G, T; nucleotide triphosphates comprising bases adenine, cytosine, guanine, and thymine; d, deoxy; dd, dideoxy; R, cleavable chemical group used to cap the —OH group; Y, cleavable linker.

FIG. **3**: The synthetic scheme for the immobilization of an azido (N_3) labeled DNA fragment to a solid surface coated with a triarylphosphine moiety. Me, methyl group; P, phosphorus; Ph, phenyl.

FIG. **4**: The synthesis of triarylphosphine N-hydroxysuccinimide (NHS) ester.

FIG. **5**: The synthetic scheme for attaching an azido (N_3) group through a linker to the 5' end of a DNA fragment, which is then used to couple with the triarylphosphine moiety on a solid surface. DMSO, dimethylsulfonyl oxide.

FIG. **6**A-**6**B: Ligate the looped primer (B) to the immobilized single stranded DNA template forming a self primed DNA template moiety on a solid surface. P (in circle), phosphate.

FIG. **7**: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique fluorescent dye attached to the base through a photocleavable linker and the 3'-OH is either exposed or capped with a MOM group or an allyl group. FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **8**: A representative scheme for the synthesis of the nucleotide analogue $_{3'\text{-}RO}$-G-$_{Tam}$. A similar scheme can be used to create the other three modified nucleotides: $_{3'\text{-}RO}$-A-$_{Dye1}$, $_{3'\text{-}RO}$-C-$_{Dye2}$, $_{3'\text{-}RO}$-T-$_{Dye4}$. (i) tetrakis(triphenylphosphine)palladium(0); (ii) $POCl_3$, Bn_4N^+pyrophosphate; (iii) NH_4OH; (iv) $Na_2CO_3/NaHCO_3$ (pH=9.0)/DMSO.

FIG. **9**: A scheme for testing the sequencing by synthesis approach. Each nucleotide, modified by the attachment of a unique fluorescent dye, is added one by one, based on the complimentary template. The dye is detected and cleaved to test the approach. Dye1=Fam; Dye2=R6G; Dye3=Tam; Dye4=Rox.

FIG. **10**: The expected photocleavage products of DNA containing a photo-cleavable dye (Tam). Light absorption (300-360 nm) by the aromatic 2-nitrobenzyl moiety causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond located in the 2-position followed by cleavage and decarboxylation (Pillai 1980).

FIG. **11**: Synthesis of PC-LC-Biotin-FAM to evaluate the photolysis efficiency of the fluorophore coupled with the photocleavable linker 2-nitrobenzyl group.

FIG. **12**: Fluorescence spectra (λ_{ex}=480 nm) of PC-LC-Biotin-FAM immobilized on a microscope glass slide coated with streptavidin (a); after 10 min photolysis (λ_{irr}=350 nm; ~0.5 mW/cm^2) (b); and after washing with water to remove the photocleaved dye (c).

FIG. **13**A-**13**B: Synthetic scheme for capping the 3'-OH of nucleotide.

FIG. **14**: Chemical cleavage of the MOM group (top row) and the allyl group (bottom row) to free the 3'-OH in the nucleotide. CITMS=chlorotrimethylsilane.

FIG. **15**A-**15**B: Examples of energy transfer coupled dye systems, where Fam or Cy2 is employed as a light absorber (energy transfer donor) and Cl_2Fam, Cl_2R6G, Cl_2Tam, or Cl_2Rox as an energy transfer acceptor. Cy2, cyanine; FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine.

FIG. **16**: The synthesis of a photocleavable energy transfer dye-labeled nucleotide. DMF, dimethylformide. DEC=1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **17**: Structures of four mass tag precursors and four photoactive mass tags. Precursors: a) acetophenone; b) 3-fluoroacetophenone; c) 3,4-difluoroacetophenone; and d) 3,4-dimethoxyacetophenone. Four photoactive mass tags are used to code for the identity of each of the four nucleotides (A, C, G, T).

FIG. **18**: Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of mass tag precursors shown in FIG. **17**.

FIG. **19**: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique mass tag attached to the base through a photocleavable linker, and the 3'-OH is either exposed or capped with a MOM group or an allyl group. The square brackets indicated that the mass tag is cleavable. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **20**: Example of synthesis of NHS ester of one mass tag (Tag-3). A similar scheme is used to create other mass tags.

FIG. **21**: A representative scheme for the synthesis of the nucleotide analogue $_{3'\text{-}RO}$-G-$_{Tag3}$. A similar scheme is used to create the other three modified bases $_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-C-$_{Tag2}$, $_{3'\text{-}RO}$-G-$_{Tag4}$. (i) tetrakis(triphenylphosphine) palladium(0); (ii) $POCl_3$, Bn_4N^+pyrophosphate; (iii) NH_4OH; (iv) Na_2CO_3/$NaHCO_3$ (pH=9.0)/DMSO.

FIG. **22**: Examples of expected photocleavage products of DNA containing a photocleavable mass tag.

FIG. **23**: System for DNA sequencing comprising multiple channels in parallel and multiple mass spectrometers in parallel. The example shows 96 channels in a silica glass chip.

FIG. **24**: Parallel mass spectrometry system for DNA sequencing. Example shows three mass spectrometers in parallel. Samples are injected into the ion source where they are mixed with a nebulizer gas and ionized. A' turbo pump is used to continuously sweep away free radicals, neutral compounds and other undesirable elements coming from the ion source. A second turbo pump is used to generate a continuous vacuum in all three analyzers and detectors simultaneously. The acquired signal is then converted to a digital signal by the A/D converter. All three signals are then sent to the data acquisition processor to convert the signal to identify the mass tag in the injected sample and thus identify the nucleotide sequence.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are presented as an aid in understanding this invention.

As used herein, to cap an —OH group means to replace the "H" in the —OH group with a chemical group. As disclosed herein, the —OH group of the nucleotide analogue is capped with a cleavable chemical group. To uncap an —OH group means to cleave the chemical group from a capped —OH group and to replace the chemical group with "H", i.e., to replace the "R" in —OR with "H" wherein "R" is the chemical group used to cap the —OH group.

The nucleotide bases are abbreviated as follows: adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U).

An analogue of a nucleotide base refers to a structural and functional derivative of the base of a nucleotide which can be recognized by polymerase as a substrate. That is, for example, an analogue of adenine (A) should form hydrogen bonds with thymine (T), a C analogue should form hydrogen bonds with G, a G analogue should form hydrogen bonds with C, and a T analogue should form hydrogen bonds with A, in a double helix format. Examples of analogues of nucleotide bases include, but are not limited to, 7-deaza-adenine and 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom.

A nucleotide analogue refers to a chemical compound that is structurally and functionally similar to the nucleotide, i.e. the nucleotide analogue can be recognized by polymerase as a substrate. That is, for example, a nucleotide analogue comprising adenine or an analogue of adenine should form hydrogen bonds with thymine, a nucleotide analogue comprising C or an analogue of C should form hydrogen bonds with G, a nucleotide analogue comprising G or an analogue of G should form hydrogen bonds with C, and a nucleotide analogue comprising T or an analogue of T should form hydrogen bonds with A, in a double helix format. Examples of nucleotide analogues disclosed herein include analogues which comprise an analogue of the nucleotide base such as 7-deaza-adenine or 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom. Further examples include analogues in which a label is attached through a cleavable linker to the 5-position of cytosine or thymine or to the 7-position of deaza-adenine or deaza-guanine. Other examples include analogues in which a small chemical moiety such as —CH_2OCH_3 or —$CH_2CH{=}CH_2$ is used to cap the —OH group at the 3'-position of deoxyribose. Analogues of dideoxynucleotides can similarly be prepared.

As used herein, a porous surface is a surface which contains pores or is otherwise uneven, such that the surface area of the porous surface is increased relative to the surface area when the surface is smooth.

The present invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) detecting the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the —OH group at the 3'-position of the deoxyribose to uncap the —OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (viii) so as to detect the identity of a newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and

wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

In one embodiment of any of the nucleotide analogues described herein, the nucleotide base is adenine. In one embodiment, the nucleotide base is guanine. In one embodiment, the nucleotide base is cytosine. In one embodiment, the nucleotide base is thymine. In one embodiment, the nucleotide base is uracil. In one embodiment, the nucleotide base is an analogue of adenine. In one embodiment, the nucleotide base is an analogue of guanine. In one embodiment, the nucleotide base is an analogue of cytosine. In one embodiment, the nucleotide base is an analogue of thymine. In one embodiment, the nucleotide base is an analogue of uracil.

In different embodiments of any of the inventions described herein, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip. In one embodiment, the solid surface is glass. In one embodiment, the solid surface is silicon. In one embodiment, the solid surface is gold. In one embodiments, the solid surface is a magnetic bead. In one embodiment, the solid surface is a chip. In one embodiment, the solid surface is a channel in a chip. In one embodiment, the solid surface is a porous channel in a chip. Other materials can also be used as long as the material does not interfere with the steps of the method.

In one embodiment, the step of attaching the nucleic acid to the solid surface comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to the 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

In one embodiment, the nucleic acid that is attached to the solid surface is a single-stranded deoxyribonucleic acid (DNA). In another embodiment, the nucleic acid that is attached to the solid surface in step (i) is a double-stranded DNA, wherein only one strand is directly attached to the solid surface, and wherein the strand that is not directly attached to the solid surface is removed by denaturing before proceeding to step (ii). In one embodiment, the nucleic acid that is attached to the solid surface is a ribonucleic acid (RNA), and the polymerase in step (iii) is reverse transcriptase.

In one embodiment, the primer is attached to a 3' end of the nucleic acid in step (ii), and the attached primer comprises a stable loop and an —OH group at a 3'-position of a deoxyribose capable of self-priming in the polymerase reaction. In one embodiment, the step of attaching the primer to the nucleic acid comprises hybridizing the primer to the nucleic acid or ligating the primer to the nucleic acid. In one embodiment, the primer is attached to the nucleic acid through a ligation reaction which links the 3' end of the nucleic acid with the 5' end of the primer.

In one embodiment, one or more of four different nucleotide analogs is added in step (iii), wherein each different nucleotide analogue comprises a different base selected from the group consisting of thymine or uracil or an analogue of thymine or uracil, adenine or an analogue of adenine, cytosine or an analogue of cytosine, and guanine or an analogue of guanine, and wherein each of the four different nucleotide analogues comprises a unique label.

In one embodiment, the cleavable chemical group that caps the —OH group at the 3'-position of the deoxyribose in the nucleotide analogue is $—CH_2OCH_3$ or $—CH_2CH═CH_2$. Any chemical group could be used as long as the group 1) is stable during the polymerase reaction, 2) does not interfere with the recognition of the nucleotide analogue by polymerase as a substrate, and 3) is cleavable.

In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine. In one embodiment, the fluorescent moiety is 5-carboxyfluorescein. In one embodiment, the fluorescent moiety is 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the fluorescent moiety is 6-carboxy-X-rhodamine.

In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine. In one embodiment, the energy transfer acceptor is dichlorocarboxyfluorescein. In one embodiment, the energy transfer acceptor is dichloro-6-carboxyrhodamine-6G. In one embodiment, the energy transfer acceptor is dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the energy transfer acceptor is dichloro-6-carboxy-X-rhodamine.

In one embodiment, the unique label that is attached to the nucleotide analogue is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group consisting of a 2-nitro-α-methyl-benzyl group, a 2-nitro-α-methyl-3-fluorobenzyl group, a 2-nitro-α-methyl-3,4-difluorobenzyl group, and a 2-nitro-α-methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-benzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3-fluorobenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3,4-difluorobenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is detected using a parallel mass spectrometry system which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.

In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deazaguanine. The unique label could also be attached through a cleavable

linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

In one embodiment, the linker between the unique label and the nucleotide analogue is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical means. In one embodiment, the linker is cleaved by a chemical means. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

In one embodiment, the cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light.

In one embodiment, the chemical compounds added in step (vi) to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on the primer extension strand are a polymerase and one or more different dideoxynucleotides or analogues of dideoxynucleotides. In further embodiments, the different dideoxynucleotides are selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine 5'-triphosphase, and their analogues. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

In one embodiment, a polymerase and one or more of four different dideoxynucleotides are added in step (vi), wherein each different dideoxynucleotide is selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate or an analogue of 2',3'-dideoxyadenosine 5'-triphosphate; 2',3'-dideoxyguanosine 5'-triphosphate or an analogue of 2',3'-dideoxyguanosine 5'-triphosphate; 2',3'-dideoxycytidine 5'-triphosphate or an analogue of 2',3'-dideoxycytidine 5'-triphosphate; and 2',3'-dideoxythymidine 5'-triphosphate or 2',3'-dideoxyuridine 5'-triphosphase or an analogue of 2',3'-dideoxythymidine 5'-triphosphate or an analogue of 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

Another type of chemical compound that reacts specifically with the —OH group could also be used to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on an extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer.

The invention provides a method for simultaneously sequencing a plurality of different nucleic acids, which comprises simultaneously applying any of the methods disclosed herein for sequencing a nucleic acid to the plurality of different nucleic acids. In different embodiments, the method can be used to sequence from one to over 100,000 different nucleic acids simultaneously.

The invention provides for the use of any of the methods disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

In different embodiments, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in an chip, or a porous channel in a chip.

In different embodiments, the nucleic acid that is attached to the solid surface is a single-stranded or double-stranded DNA or a RNA. In one embodiment, the nucleic acid is a double-stranded DNA and only one strand is attached to the solid surface. In a further embodiment, the strand of the double-stranded DNA that is not attached to the solid surface is removed by denaturing.

The invention provides for the use of any of the methods disclosed herein for attaching a nucleic acid to a surface for gene expression analysis, microarray based gene expression analysis, or mutation detection, translational analysis, transcriptional analysis, or for other genetic applications.

The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique, label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose.

In one embodiment of the nucleotide analogue, the cleavable chemical group that caps the —OH group at the 3'-position of the deoxyribose is $—CH_2OCH_3$ or $—CH_2CH═CH_2$.

In one embodiment, the unique label is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine.

In one embodiment, the unique label is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy, transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine.

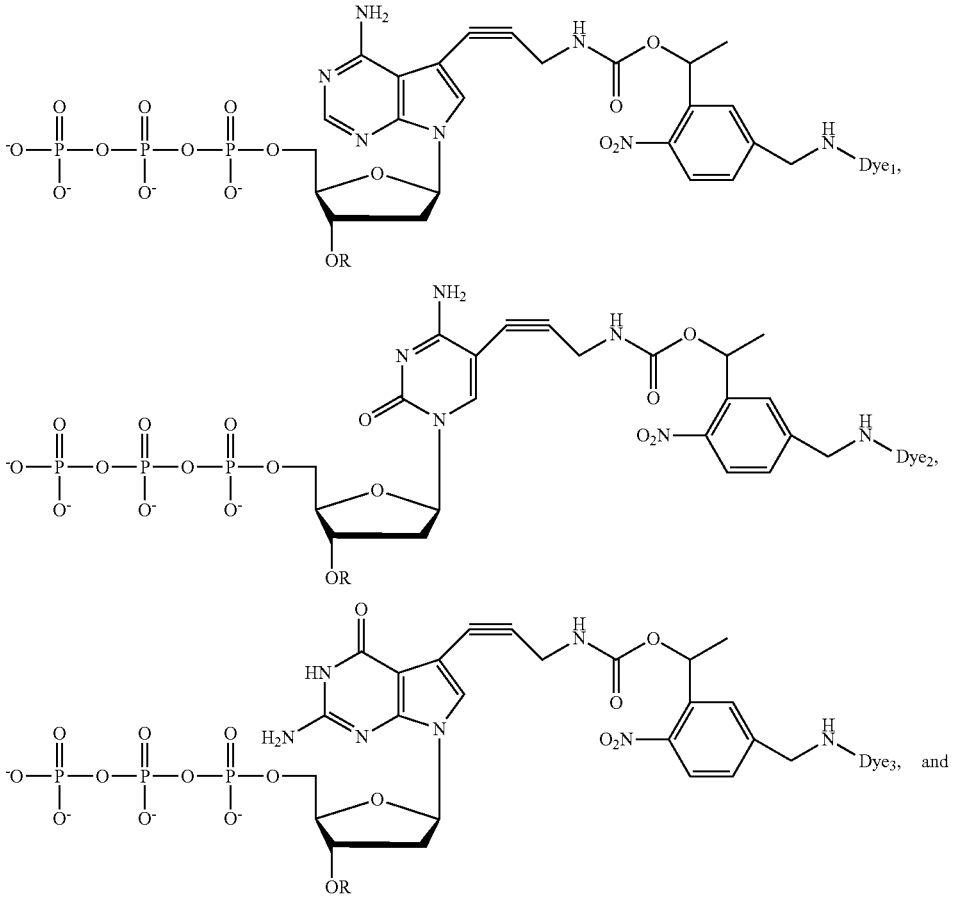
In one embodiment, the unique label is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group consisting of a 2-nitro-α-methyl-benzyl group, a 2-nitro-α-methyl-3-fluorobenzyl group, a 2-nitro-α-methyl-3,4-difluorobenzyl group, and a 2-nitro-α-methyl-3,4-dimethoxybenzyl group.

In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

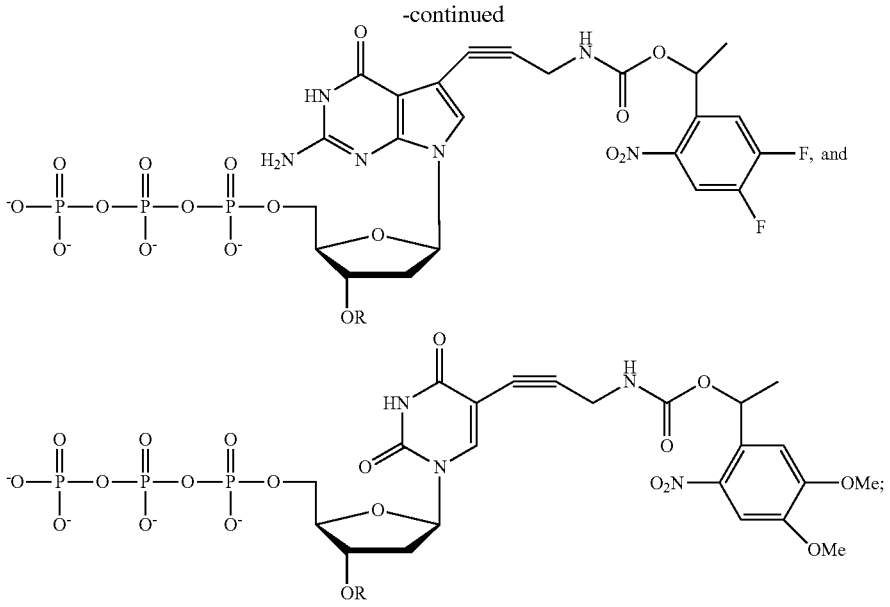
In one embodiment, the linker between the unique label and the nucleotide analogue is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

In one embodiment, the cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.

In different embodiments, the nucleotide analogue is selected from the group consisting of:



-continued



wherein R is —CH_2OCH_3 or —$CH_2CH{=}CH_2$.

The invention provides for the use any of the nucleotide analogues disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

The invention provides a parallel mass spectrometry system, which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags. In one embodiment, the mass spectrometers are quadrupole mass spectrometers. In one embodiment, the mass spectrometers are time-of-flight mass spectrometers. In one embodiment, the mass spectrometers are contained in one device. In one embodiment, the system further comprises two turbopumps, wherein one pump is used to generate a vacuum and a second pump is used to remove undesired elements. In one embodiment, the system comprises at least three mass spectrometers. In one embodiment, the mass tags have molecular weights between 150 daltons and 250 daltons. The invention provides for the use of the system for DNA sequencing analysis, detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

1. The Sequencing by Synthesis Approach

Sequencing DNA by synthesis involves the detection of the identity of each nucleotide as it is incorporated into the growing strand of DNA in the polymerase reaction. The fundamental requirements for such a system to work are: (1) the availability of 4 nucleotide analogues (aA, aC, aG, aT) each labeled with a unique label and containing a chemical moiety capping the 3'-OH group; (2) the 4 nucleotide analogues (aA, aC, aG, aT) need to be efficiently and faithfully incorporated by DNA polymerase as terminators in the polymerase reaction; (3) the tag and the group capping the 3'-OH need to be removed with high yield to allow the incorporation and detection of the next nucleotide; and (4) the growing strand of DNA should survive the washing, detection and cleavage processes to remain annealed to the DNA template.

The sequencing by synthesis approach disclosed herein is illustrated in FIG. **2**A-**2**B. In FIG. **2**A, an example is shown where the unique labels are fluorescent dyes and the surface is a chip; in FIG. **2**B, the unique labels are mass tags and the surface is channels etched into a chip. The synthesis approach uses a solid surface such as a glass chip with an immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$) each labeled with a unique label, e.g. a fluorescent dye or a mass tag, at a specific location on the purine or pyrimidine base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by the polymerase on each spot of the surface (step 1 in FIGS. **2**A and **2**B).

As shown in FIG. **2**A, where the unique labels are dyes, after removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, a detector is used to detect the unique label. For example, a four color fluorescence imager is used to image the surface of the chip, and the unique fluorescence emission from a specific dye on the nucleotide analogues on each spot of the chip will reveal the identity of the incorporated nucleotide (step 2 in FIG. **2**A). After imaging, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess dideoxynucleoside triphosphates (ddNTPs) (ddATP, ddGTP, ddTTP, and ddCTP) and DNA polymerase to avoid

interference with the next round of synthesis (step 3 in FIG. **2**A), a concept similar to the capping step in automated solid phase DNA synthesis (Caruthers, 1985). The ddNTPs, which lack a 3'-hydroxyl group, are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the dye-labeled nucleotides, and the excellent efficiency with which they are incorporated by DNA polymerase. The dye moiety is then cleaved by light (~350 nm), and the R group protecting the 3'-OH is removed chemically to generate free 3'-OH group with high yield (step 4 in FIG. **2**A). A washing step is applied to wash away the cleaved dyes and the R group. The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (step 5 in FIG. **2**A).

It is a routine procedure now to immobilize high density (>10,000 spots per chip) single stranded DNA on a 4 cm×1 cm glass chip (Schena et al. 1995). Thus, in the DNA sequencing system disclosed herein, more than 10,000 bases can be identified after each cycle and after 100 cycles, a million base pairs will be generated from one sequencing chip.

Possible DNA polymerases include Thermo Sequenase, Taq FS DNA polymerase, T7 DNA polymerase, and Vent (exo-) DNA polymerase. The fluorescence emission from each specific dye can be detected using a fluorimeter that is equipped with an accessory to detect fluorescence from a glass slide. For large scale evaluation, a multi-color scanning system capable of detecting multiple different fluorescent dyes (500 nm-700 nm) (GSI Lumonics ScanArray 5000 Standard Biochip Scanning System) on a glass slide can be used.

An example of the sequencing by synthesis approach using mass tags is shown in FIG. **2**B. The approach uses a solid surface, such as a porous silica glass channels in a chip, with immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues ($_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-C-$_{Tag2}$, $_{3'\text{-}RO}$-G-$_{Tag3}$, $_{3'\text{-}RO}$-T-$_{Tag4}$) each labeled with a unique photocleavable mass tag on the specific location of the base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by polymerase in each channel of the glass chip (step 1 in FIG. **2**B). After removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess ddNTPs (ddATP, ddGTP, ddTTP and ddCTP) and DNA polymerase to avoid interference with the next round of synthesis (step 2 in FIG. **2**B). The ddNTPs are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the labeled nucleotides, and their excellent efficiency to be incorporated by DNA polymerase. The mass tags are cleaved by irradiation with light (~350 nm) (step 3 in FIG. **2**B) and then detected with a mass spectrometer. The unique mass of each tag yields the identity of the nucleotide in each channel (step 4 in FIG. **2**B). The R protecting group is then removed chemically and washed away to generate free 3'-OH group with high yield (step 5 in FIG. **2**B). The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (step 6 in FIG. **2**B).

Since the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), mass spectrometry has become an indispensable tool in many areas of biomedical research. Though these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation are required for implementation of mass spectrometry for DNA sequencing applications. Since the approach disclosed herein uses small and stable mass tags, there is no need to detect large DNA sequencing fragments directly and it is not necessary to use MALDI or ESI methods for detection. Atmospheric pressure chemical ionization (APCI) is an ionization method that uses a gas-phase ion-molecular reaction at atmospheric pressure (Dizidic et al. 1975). In this method, samples are introduced by either chromatography or flow injection into a pneumatic nebulizer where they are converted into small droplets by a high-speed beam of nitrogen gas. When the heated gas and solution arrive at the reaction area, the excess amount of solvent is ionized by corona discharge. This ionized mobile phase acts as the ionizing agent toward the samples and yields pseudo molecular $(M+H)^+$ and $(M-H)^-$ ions. Due to the corona discharge ionization method, high ionization efficiency is attainable, maintaining stable ionization conditions with detection sensitivity lower than femtomole region for small and stable organic compounds. However, due to the limited detection of large molecules, ESI and MALDI have replaced APCI for analysis of peptides and nucleic acids. Since in the approach disclosed the mass tags to be detected are relatively small and very stable organic molecules, the ability to detect large biological molecules gained by using ESI and MALDI is not necessary. APCI has several advantages over ESI and MALDI because it does not require any tedious sample preparation such as desalting or mixing with matrix to prepare crystals on a target plate. In ESI, the sample nature and sample preparation conditions (i.e. the existence of buffer or inorganic salts) suppress the ionization efficiency. MALDI requires the addition of matrix prior to sample introduction into the mass spectrometer and its speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. These limitations are overcome by APCI because the mass tag solution can be injected directly with no additional sample purification or preparation into the mass spectrometer. Since the mass tagged samples are volatile and have small mass numbers, these compounds are easily detectable by APCI ionization with high sensitivity. This system can be scaled up into a high throughput operation.

Each component of the sequencing by synthesis system is described in more detail below.

2. Construction of a Surface Containing Immobilized Self-Primed DNA Moiety

The single stranded DNA template immobilized on a surface is prepared according to the scheme shown in FIG. **3**. The surface can be, for example, a glass chip, such as a 4 cm×1 cm glass chip, or channels in a glass chip. The surface is first treated with 0.5 M NaOH, washed with water, and then coated with high density 3-aminopropyltrimethoxysilane in aqueous ethanol (Woolley et al. 1994) forming a primary amine surface. N-Hydroxy Succinimidyl (NHS) ester of triarylphosphine (1) is covalently coupled with the primary amine group converting the amine surface to a novel triarylphosphine surface, which specifically reacts with DNA containing an azido group (2) forming a chip with immobilized DNA. Since the azido group is only located at the 5' end of the DNA and the coupling reaction is through the unique reaction of the triarylphosphine moiety with the

azido group in aqueous solution (Saxon and Bertozzi 2000), such a DNA surface will provide an optimal condition for hybridization.

The NHS ester of triarylphosphine (1) is prepared according to the scheme shown in FIG. **4**. 3-diphenylphosphino-4-methoxycarbonyl-benzoic acid (3) is prepared according to the procedure described by Bertozzi et al. (Saxon and Bertozzi 2000). Treatment of (3) with N-Hydroxysuccinimide forms the corresponding NHS ester (4). Coupling of (4) with an amino carboxylic acid moiety produces compound (5) that has a long linker (n=1 to 10) for optimized coupling with DNA on the surface. Treatment of (5) with N-Hydroxysuccinimide generates the NHS ester (1) which is ready for coupling with the primary amine coated surface (FIG. **3**).

The azido labeled DNA (2) is synthesized according to the scheme shown in FIG. **5**. Treatment of ethyl ester of 5-bromovaleric acid with sodium azide and then hydrolysis produces 5-azidovaleric acid (Khoukhi et al., 1987), which is subsequently converted to a NHS ester for coupling with an amino linker modified oligonucleotide primer. Using the azido-labeled primer to perform polymerase chain reaction (PCR) reaction generates azido-labeled DNA template (2) for coupling with the triarylphosphine-modified surface (FIG. **3**).

The self-primed DNA template moiety on the sequencing chip is constructed as shown in FIGS. **6** (A & B) using enzymatic ligation. A 5'-phosphorylated, 3'-OH capped loop oligonucleotide primer (B) is synthesized by a solid phase DNA synthesizer. Primer (B) is synthesized using a modified C phosphoramidite whose 3'-OH is capped with either a MOM ($—CH_2OCH_3$) group or an allyl ($—CH_2CH═CH_2$) group (designated by "R" in FIG. **6**) at the 3'-end of the oligonucleotide to prevent the self ligation of the primer in the ligation reaction. Thus, the looped primer can only ligate to the 3'-end of the DNA templates that are immobilized on the sequencing chip using T4 RNA ligase (Zhang et al. 1996) to form the self-primed DNA template moiety (A). The looped primer (B) is designed to contain a very stable loop (Antao et al. 1991) and a stem containing the sequence of M13 reverse DNA sequencing primer for efficient priming in the polymerase reaction once the primer is ligated to the immobilized DNA on the sequencing chip and the 3'-OH cap group is chemically cleaved off (Ireland et al. 1986; Kamal et al. 1999).

3. Sequencing by Synthesis Evaluation Using Nucleotide Analogues $_{3'\text{-}HO}$-A-$_{Dye1}$, $_{3'\text{-}HO}$-C-$_{Dye2}$, $_{3'\text{-}HO}$-G-$_{Dye3}$, $_{3'\text{-}HO}$-T-$_{Dye4}$

A scheme has been developed for evaluating the photocleavage efficiency using different dyes and testing the sequencing by synthesis approach. Four nucleotide analogues $_{3'\text{-}HO}$-A-$_{Dye1}$, $_{3'\text{-}HO}$-C-$_{Dye2}$, $_{3'\text{-}HO}$-G-$_{Dye3}$, $_{3'\text{-}HO}$-T=$_{Dye4}$ each labeled with a unique fluorescent dye through a photocleavable linker are synthesized and used in the sequencing by synthesis approach. Examples of dyes include, but are not limited to: Dye1=FAM, 5-carboxyfluorescein; Dye2=R6G, 6-carboxyrhodamine-6G; Dye3=TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; and Dye4=ROX, 6-carboxy-X-rhodamine. The structures of the 4 nucleotide analogues are shown in FIG. **7** (R═H).

The photocleavable 2-nitrobenzyl moiety has been used to link biotin to DNA and protein for efficient removal by UV light (~350 nm) (Olejnik et al. 1995, 1999). In the approach disclosed herein the 2-nitrobenzyl group is used to bridge the fluorescent dye and nucleotide together to form the dye labeled nucleotides as shown in FIG. **7**.

As a representative example, the synthesis of $_{3'\text{-}HO}$-G-$_{Dye3}$ (Dye3=Tam) is shown in FIG. **8**. 7-deaza-alkynylamino-dGTP is prepared using well-established procedures (Prober et al. 1987; Lee et al. 1992 and Hobbs et al. 1991). Linker-Tam is synthesized by coupling the Photocleavable Linker (Rollaf 1982) with NHS-Tam. 7-deaza-alkynylamino-dGTP is then coupled with the Linker-Tam to produce $_{3'\text{-}HO}$-G-$_{TAM}$. The nucleotide analogues with a free 3'-OH (i.e., R═H) are good substrates for the polymerase. An immobilized DNA template is synthesized (FIG. **9**) that contains a portion of nucleotide sequence ACGTACGACGT (SEQ ID NO: 1) that has no repeated sequences after the priming site. $_{3'\text{-}HO}$-A-$_{Dye1}$ and DNA polymerase are added to the self-primed DNA moiety and it is incorporated to the 3' site of the DNA. Then the steps in FIG. **2**A are followed (the chemical cleavage step is not required here because the 3'-OH is free) to detect the fluorescent signal from Dye-1 at 520 nm. Next, $_{3'\text{-}HO}$-C-$_{Dye2}$ is added to image the fluorescent signal from Dye-2 at 550 nm. Next, $_{3'\text{-}HO}$-G-$_{Dye3}$ is added to image the fluorescent signal from Dye-3 at 580 nm, and finally $_{3'\text{-}HO}$-T-$_{Dye4}$ is added to image the fluorescent signal from Dye-4 at 610 nm.

Results on Photochemical Cleavage Efficiency

The expected photolysis products of DNA containing a photocleavable fluorescent dye at the 3' end of the DNA are shown in FIG. **10**. The 2-nitrobenzyl moiety has been successfully employed in a wide range of studies as a photocleavable-protecting group (Pillai 1980). The efficiency of the photocleavage step depends on several factors including the efficiency of light absorption by the 2-nitrobenzyl moiety, the efficiency of the primary photochemical step, and the efficiency of the secondary thermal processes which lead to the final cleavage process (Turro 1991). Burgess et al. (1997) have reported the successful photocleavage of a fluorescent dye attached through a 2-nitrobenzyl linker on a nucleotide moiety, which shows that the fluorescent dye is not quenching the photocleavage process. A photoliable protecting group based on the 2-nitrobenzyl chromophore has also been developed for biological labeling applications that involve photocleavage (Olejnik et al. 1999). The protocol disclosed herein is used to optimize the photocleavage process shown in FIG. **10**. The absorption spectra of 2-nitro benzyl compounds are examined and compared quantitatively to the absorption spectra of the fluorescent dyes. Since there will be a one-to-one relationship between the number of 2-nitrobenzyl moieties and the dye molecules, the ratio of extinction coefficients of these two species will reflect the competition for light absorption at specific wavelengths. From this information, the wavelengths at which the 2-nitrobenzyl moieties absorbed most competitively can be determined, similar to the approach reported by Olejnik et al. (1995).

A photolysis setup can be used which allows a high throughput of monochromatic light from a 1000 watt high pressure xenon lamp (LX1000UV, ILC) in conjunction with a monochromator (Kratos, Schoeffel Instruments). This instrument allows the evaluation of the photocleavage of model systems as a function of the intensity and excitation wavelength of the absorbed light. Standard analytical analysis is used to determine the extent of photocleavage. From this information, the efficiency of the photocleavage as a function of wavelength can be determined. The wavelength at which photocleavage occurs most efficiently can be selected as for use in the sequencing system.

Photocleavage results have been obtained using a model system as shown in FIG. **11**. Coupling of PC-LC-Biotin-NHS ester (Pierce, Rockford Ill.) with 5-(aminoacetamido)-fluorescein (5-aminoFAM) (Molecular Probes, Eugene, Oreg.) in dimethylsulfonyl oxide (DMSO)/$NaHCO_3$

SEQUENCE LISTING

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<211> LENGTH: 11
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically Synthesized Template

<400> SEQUENCE: 1

acgtacgacg t 11

<210> SEQ ID NO 2
<211> LENGTH: 105
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically Synthesized Template

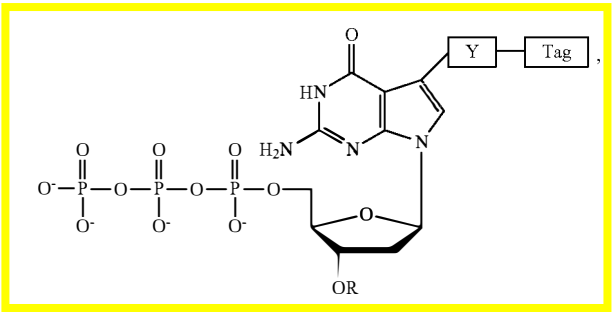
<400> SEQUENCE: 2

ttcctgcatg ggcggcatga acccgaggcc catcctcacc atcatcacac tggaagactc 60

cagtggtaat ctactgggac ggacggaaca gctttgaggt gcatt 105

What is claimed is:

1. A guanine deoxyribonucleotide analogue having the structure:



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, (d) does not contain a ketone group, and (e) is not a $—CH_2CH═CH_2$ group;

wherein OR is not a methoxy group or an ester group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction; and

wherein the guanine deoxyribonucleotide analogue:

i) is recognized as a substrate by a DNA polymerase,

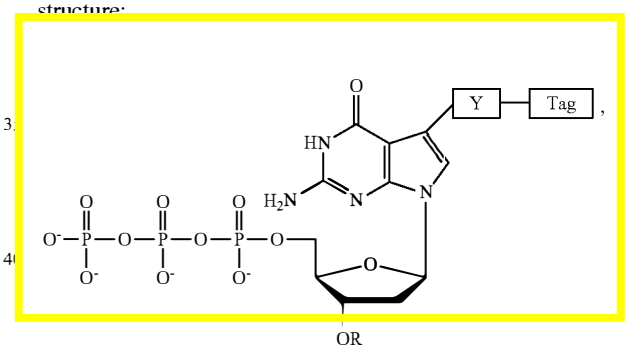
ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,

iii) produces a 3'-OH group on the deoxyribose upon cleavage of R,

iv) no longer includes a tag on the base upon cleavage of Y, and

v) is capable of forming hydrogen bonds with cytosine or a cytosine nucleotide analogue.

2. A guanine deoxyribonucleotide analogue having the structure:



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, and (d) does not contain a ketone group;

wherein OR is not a methoxy group, an ester group, or an allyl ether group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction; and

wherein the guanine deoxyribonucleotide analogue:

i) is recognized as a substrate by a DNA polymerase,

ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,

iii) produces a 3'-OH group on the deoxyribose upon cleavage of R,

iv) no longer includes a tag on the base upon cleavage of Y, and

v) is capable of forming hydrogen bonds with cytosine or a cytosine nucleotide analogue.

* * * * *

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Ju et al.

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(45) Date of Patent: *Oct. 1, 2019

(54) **MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(22) Filed: **Oct. 2, 2018**

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Related U.S. Application Data

(60) Continuation of application No. 15/915,983, filed on Mar. 8, 2018, which is a continuation of application No. 14/670,748, filed on Mar. 27, 2015, which is a continuation of application No. 13/959,660, filed on Aug. 5, 2013, now Pat. No. 9,133,511, which is a continuation of application No. 13/672,437, filed on Nov. 8, 2012, now abandoned, which is a continuation of application No. 13/339,089, filed on Dec. 28, 2011, now abandoned, which is a continuation of application No. 12/804,284, filed on Jul. 19, 2010, now Pat. No. 8,088,575, which is a continuation of application No. 11/810,509, filed on Jun. 5, 2007, now Pat. No. 7,790,869, which is a division of application No. 10/702,203, filed on Nov. 4, 2003, now Pat. No. 7,345,159, which is a division of application No. 09/972,364, filed on Oct. 5, 2001, now Pat. No. 6,664,079, which is a continuation-in-part of application No. 09/684,670, filed on Oct. 6, 2000, now abandoned.

(60) Provisional application No. 60/300,894, filed on Jun. 26, 2001.

(51) **Int. Cl.**

C12Q 1/68	(2018.01)
C07H 19/14	(2006.01)
C12Q 1/6869	(2018.01)
C07H 21/00	(2006.01)
C12Q 1/686	(2018.01)
C12Q 1/6874	(2018.01)
C12Q 1/6872	(2018.01)
C07H 19/10	(2006.01)
C12Q 1/6876	(2018.01)
C40B 40/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6869*** (2013.01); ***C07H 19/10*** (2013.01); ***C07H 19/14*** (2013.01); ***C07H 21/00*** (2013.01); ***C12Q 1/68*** (2013.01); ***C12Q 1/686*** (2013.01); ***C12Q 1/6872*** (2013.01); ***C12Q 1/6874*** (2013.01); ***C12Q 1/6876*** (2013.01); *C07B 2200/11* (2013.01); *C12Q 2525/117* (2013.01); *C12Q 2525/186* (2013.01); *C12Q 2535/101* (2013.01); *C12Q 2535/122* (2013.01); *C12Q 2563/107* (2013.01); *C12Q 2565/501* (2013.01); *C40B 40/00* (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/6869; C07H 19/14

USPC 435/6.1

See application file for complete search history.

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(57) **ABSTRACT**

This invention provides methods for attaching a nucleic acid to a solid surface and for sequencing nucleic acid by detecting the identity of each nucleotide analog after the nucleotide analog is incorporated into a growing strand of DNA in a polymerase reaction. The invention also provides nucleotide analogs which comprise unique labels attached to the nucleotide analog through a cleavable linker, and a cleavable chemical group to cap the —OH group at the 3'-position of the deoxyribose.

4 Claims, 28 Drawing Sheets

Specification includes a Sequence Listing.

tion of the deoxyribose ring in ddCTP is very crowded, while there is ample space for modification on the 5-position the cytidine base.

The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye or a mass tag through a cleavable linker to the nucleotide base or an analogue of the nucleotide base, such as to the 5-position of the pyrimidines (T and C) and to the 7-position of the purines (G and A), to use a small cleavable chemical moiety to cap the 3'-OH group of the deoxyribose to make it nonreactive, and to incorporate the nucleotide analogues into the growing DNA strand as terminators. Detection of the unique label will yield the sequence identity of the nucleotide. Upon removing the label and the 3'-OH capping group, the polymerase reaction will proceed to incorporate the next nucleotide analogue and detect the next base.

It is also desirable to use a photocleavable group to cap the 3'-OH group. However, a photocleavable group is generally bulky and thus the DNA polymerase will have difficulty to incorporate the nucleotide analogues containing a photocleavable moiety capping the 3'-OH group. If small chemical moieties that can be easily cleaved chemically with high yield can be used to cap the 3'-OH group, such nucleotide analogues should also be recognized as substrates for DNA polymerase. It has been reported that 3'-O-methoxy-deoxynucleotides are good substrates for several polymerases (Axelrod et al. 1978). 3'-O-allyl-dATP was also shown to be incorporated by Ventr(exo-) DNA polymerase in the growing strand of DNA (Metzker et al. 1994). However, the procedure to chemically cleave the methoxy group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methoxy group to cap the 3'-OH group for sequencing DNA by synthesis. An ester group was also explored to cap the 3'-OH group of the nucleotide, but it was shown to be cleaved by the nucleophiles in the active site in DNA polymerase (Canard et al. 1995). Chemical groups with electrophiles such as ketone groups are not suitable for protecting the 3'-OH of the nucleotide in enzymatic reactions due to the existence of strong nucleophiles in the polymerase. It is known that MOM ($—CH_2OCH_3$) and allyl ($—CH_2CH═CH_2$) groups can be used to cap an —OH group, and can be cleaved chemically with high yield (Ireland et al. 1986; Kamal et al. 1999). The approach disclosed in the present application is to incorporate nucleotide analogues, which are labeled with cleavable, unique labels such as fluorescent dyes or mass tags and where the 3'-OH is capped with a cleavable chemical moiety such as either a MOM group ($—CH_2OCH_3$) or an allyl group ($—CH_2CH═CH_2$), into the growing strand DNA as terminators. The optimized nucleotide set ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$, where R denotes the chemical group used to cap the 3'-OH) can then be used for DNA sequencing by the synthesis approach.

There are many advantages of using mass spectrometry (MS) to detect small and stable molecules. For example, the mass resolution can be as good as one dalton. Thus, compared to gel electrophoresis sequencing systems and the laser induced fluorescence detection approach which have overlapping fluorescence emission spectra, leading to heterozygote detection difficulty, the MS approach disclosed in this application produces very high resolution of sequencing data by detecting the cleaved small mass tags instead of the long DNA fragment. This method also produces extremely fast separation in the time scale of microseconds. The high resolution allows accurate digital mutation and heterozygote detection. Another advantage of sequencing with mass spectrometry by detecting the small mass tags is that the compressions associated with gel based systems are completely eliminated.

In order to maintain a continuous hybridized primer extension product with the template DNA, a primer that contains a stable loop to form an entity capable of self-priming in a polymerase reaction can be ligated to the 3' end of each single stranded DNA template that is immobilized on a solid surface such as a chip. This approach will solve the problem of washing off the growing extension products in each cycle.

Saxon and Bertozzi (2000) developed an elegant and highly specific coupling chemistry linking a specific group that contains a phosphine moiety to an azido group on the surface of a biological cell. In the present application, this coupling chemistry is adopted to create a solid surface which is coated with a covalently linked phosphine moiety, and to generate polymerase chain reaction (PCR) products that contain an azido group at the 5' end for specific coupling of the DNA template with the solid surface. One example of a solid surface is glass channels which have an inner wall with an uneven or porous surface to increase the surface area. Another example is a chip.

The present application discloses a novel and advantageous system for DNA sequencing by the synthesis approach which employs a stable DNA template, which is able to self prime for the polymerase reaction, covalently linked to a solid surface such as a chip, and 4 unique nucleotides analogues ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$). The success of this novel system will allow the development of an ultra high-throughput and high fidelity DNA sequencing system for polymorphism, pharmacogenetics applications and for whole genome sequencing. This fast and accurate DNA resequencing system is needed in such fields as detection of single nucleotide polymorphisms (SNPs) (Chee et al. 1996), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), identification in forensics, and genetic disease association studies.

SUMMARY OF THE INVENTION

This invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate, a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

FIG. **17**: Structures of four mass tag precursors and four photoactive mass tags. Precursors: a) acetophenone; b) 3-fluoroacetophenone; c) 3,4-difluoroacetophenone; and d) 3,4-dimethoxyacetophenone. Four photoactive mass tags are used to code for the identity of each of the four nucleotides (A, C, G, T).

FIG. **18**: Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of mass tag precursors shown in FIG. **17**.

FIG. **19**: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique mass tag attached to the base through a photocleavable linker, and the 3'-OH is either exposed or capped with a MOM group or an allyl group. The square brackets indicated that the mass tag is cleavable. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **20**: Example of synthesis of NHS ester of one mass tag (Tag-3). A similar scheme is used to create other mass tags.

FIG. **21**: A representative scheme for the synthesis of the nucleotide analogue $_{3'\text{-}RO}$-G-$_{Tag3}$. A similar scheme is used to create the other three modified bases $_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-C-$_{Tag2}$, $_{3'\text{-}RO}$-T-$_{Tag4}$. (i) tetrakis(triphenylphosphine)palladium(0); (ii) $POCl_3$, Bn_4N^+ pyrophosphate; (iii) NH_4OH; (iv) $Na_2CO_3/NaHCO_3$ (pH=9.0)/DMSO.

FIG. **22**: Examples of expected photocleavage products of DNA containing a photocleavable mass tag.

FIG. **23**: System for DNA sequencing comprising multiple channels in parallel and multiple mass spectrometers in parallel. The example shows 96 channels in a silica glass chip.

FIG. **24**: Parallel mass spectrometry system for DNA sequencing. Example shows three mass spectrometers in parallel. Samples are injected into the ion source where they are mixed with a nebulizer gas and ionized. A turbo pump is used to continuously sweep away free radicals, neutral compounds and other undesirable elements coming from the ion source. A second turbo pump is used to generate a continuous vacuum in all three analyzers and detectors simultaneously. The acquired signal is then converted to a digital signal by the A/D converter. All three signals are then sent to the data acquisition processor to convert the signal to identify the mass tag in the injected sample and thus identify the nucleotide sequence.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are presented as an aid in understanding this invention.

As used herein, to cap an —OH group means to replace the "H" in the —OH group with a chemical group. As disclosed herein, the —OH group of the nucleotide analogue is capped with a cleavable chemical group. To uncap an —OH group means to cleave the chemical group from a capped —OH group and to replace the chemical group with "H", i.e., to replace the "R" in —OR with "H" wherein "R" is the chemical group used to cap the —OH group.

The nucleotide bases are abbreviated as follows: adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U).

An analogue of a nucleotide base refers to a structural and functional derivative of the base of a nucleotide which can be recognized by polymerase as a substrate. That is, for example, an analogue of adenine (A) should form hydrogen bonds with thymine (T), a C analogue should form hydrogen bonds with G, a G analogue should form hydrogen bonds with C, and a T analogue should form hydrogen bonds with A, in a double helix format. Examples of analogues of nucleotide bases include, but are not limited to, 7-deaza-adenine and 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom.

A nucleotide analogue refers to a chemical compound that is structurally and functionally similar to the nucleotide, i.e. the nucleotide analogue can be recognized by polymerase as a substrate. That is, for example, a nucleotide analogue comprising adenine or an analogue of adenine should form hydrogen bonds with thymine, a nucleotide analogue comprising C or an analogue of C should form hydrogen bonds with G, a nucleotide analogue comprising G or an analogue of G should form hydrogen bonds with C, and a nucleotide analogue comprising T or an analogue of T should form hydrogen bonds with A, in a double helix format. Examples of nucleotide analogues disclosed herein include analogues which comprise an analogue of the nucleotide base such as 7-deaza-adenine or 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom. Further examples include analogues in which a label is attached through a cleavable linker to the 5-position of cytosine or thymine or to the 7-position of deaza-adenine or deaza-guanine. Other examples include analogues in which a small chemical moiety such as $—CH_2OCH_3$ or $—CH_2CH{=}CH_2$ is used to cap the —OH group at the 3'-position of deoxyribose. Analogues of dideoxynucleotides can similarly be prepared.

As used herein, a porous surface is a surface which contains pores or is otherwise uneven, such that the surface area of the porous surface is increased relative to the surface area when the surface is smooth.

The present invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) detecting the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

In one embodiment, the linker between the unique label and the nucleotide analogue is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical means. In one embodiment, the linker is cleaved by a chemical means. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

In one embodiment, the cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light.

In one embodiment, the chemical compounds added in step (vi) to permanently cap any unreacted —OH group on the primer, attached to the nucleic acid or on the primer extension strand are a polymerase and one or more different dideoxynucleotides or analogues of dideoxynucleotides. In further embodiments, the different dideoxynucleotides are selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine 5'-triphosphase, and their analogues. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

In one embodiment, a polymerase and one or more of four different dideoxynucleotides are added in step (vi), wherein each different dideoxynucleotide is selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate or an analogue of 2',3'-dideoxyadenosine 5'-triphosphate; 2',3'-dideoxyguanosine 5'-triphosphate or an analogue of 2',3'-dideoxyguanosine 5'-triphosphate; 2',3'-dideoxycytidine 5'-triphosphate or an analogue of 2',3'-dideoxycytidine 5'-triphosphate; and 2',3'-dideoxythymidine 5'-triphosphate or 2',3'-dideoxyuridine 5'-triphosphase or an analogue of 2',3'-0.5 dideoxythymidine 5'-triphosphate or an analogue of 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

Another type of chemical compound that reacts specifically with the —OH group could also be used to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on an extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer.

The invention provides a method for simultaneously sequencing a plurality of different nucleic acids, which comprises simultaneously applying any of the methods disclosed herein for sequencing a nucleic acid to the plurality of different nucleic acids. In different embodiments, the method can be used to sequence from one to over 100,000 different nucleic acids simultaneously.

The invention provides for the use of any of the methods disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

In different embodiments, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in an chip, or a porous channel in a chip.

In different embodiments, the nucleic acid that is attached to the solid surface is a single-stranded or double-stranded DNA or a RNA. In one embodiment, the nucleic acid is a double-stranded DNA and only one strand is attached to the solid surface. In a further embodiment, the strand of the double-stranded DNA that is not attached to the solid surface is removed by denaturing.

The invention provides for the use of any of the methods disclosed herein for attaching a nucleic acid to a surface for gene expression analysis, microarray based gene expression analysis, or mutation detection, translational analysis, transcriptional analysis, or for other genetic applications.

The invention provides a nucleotide analogue which comprises:

-continued

<211> LENGTH: 105
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically Synthesized Template

<400> SEQUENCE: 2

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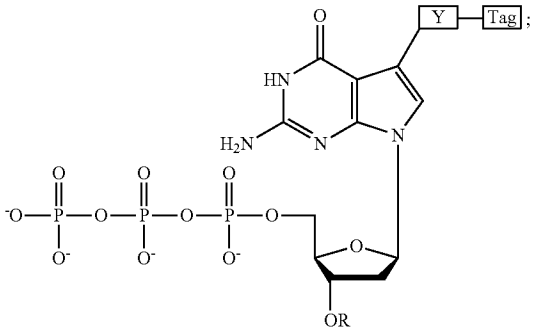
cagtggtaat ctactgggac ggacggaaca gctttgaggt gcatt 105

What is claimed is:

1. A method for sequencing a nucleic acid which comprises detecting the identity of a nucleotide analogue incorporated into the end of a growing strand of DNA in a polymerase reaction, wherein the nucleotide analogue is any of the following:

(A)



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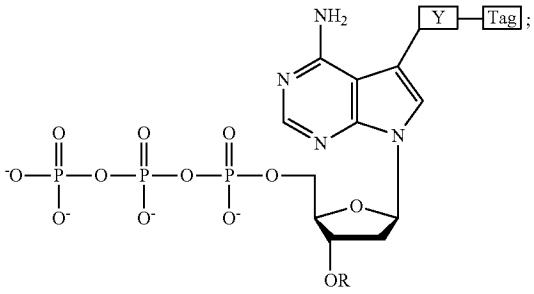
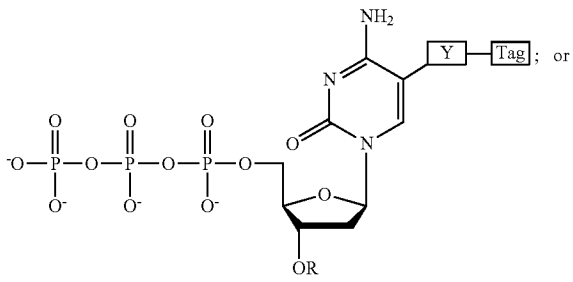


(C)



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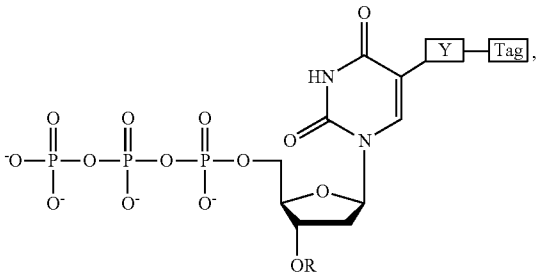
(D)



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of, the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, (d) does not contain a ketone group, and (e) is not a —$CH_2CH{=}CH_2$ group;

wherein OR is not a methoxy group or an ester group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction;

wherein the nucleotide analogue:

i) is recognized as a substrate by a DNA polymerase,

ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,

iii) produces a 3'-OH group on the deoxyribose upon cleavage of R, and

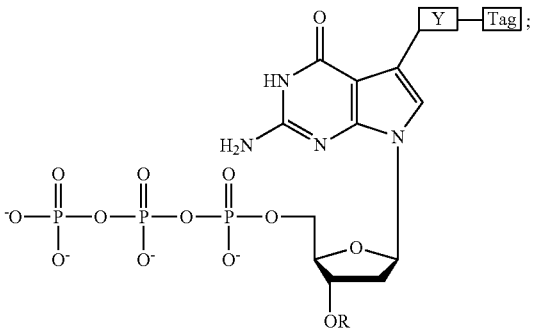
iv) no longer includes a tag on the base upon cleavage of Y;

and wherein if the nucleotide analogue is: (A), it is capable of forming hydrogen bonds with cytosine or a cytosine nucleotide analogue; (B), it is capable of forming hydrogen bonds with thymine or a thymine nucleotide analogue; (C), it is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue; or (D), it is capable of forming hydrogen bonds with adenine or an adenine nucleotide analogue.

2. A method for simultaneously sequencing a plurality of different nucleic acids which comprises simultaneously applying the method of claim **1** to the plurality of different nucleic acids.

3. A method for sequencing a nucleic acid which comprises detecting the identity of a nucleotide analogue incorporated into the end of a growing strand of DNA in a polymerase reaction, wherein the nucleotide analogue is any of the following:

(A)



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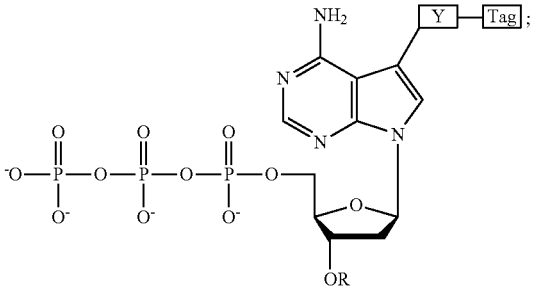
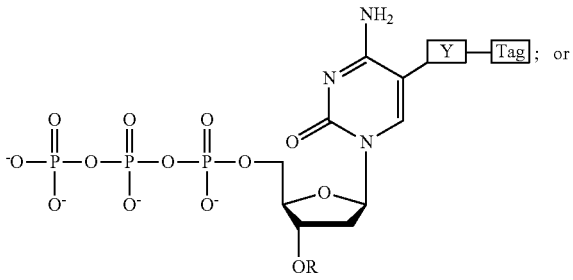


(C)



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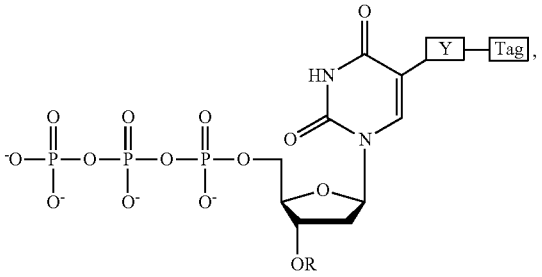
(D)



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, and (d) does not contain a ketone group;

wherein OR is not a methoxy group, an ester group, or an allyl ether group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction;

wherein the nucleotide analogue:

i) is recognized as a substrate by a DNA polymerase,

ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,

iii) produces a 3'-OH group on the deoxyribose upon cleavage of R, and

iv) no longer includes a tag on the base upon cleavage of Y;

and wherein if the nucleotide analogue is: (A), it is capable of forming hydrogen bonds with cytosine or a cytosine nucleotide analogue; (B), it is capable of forming hydrogen bonds with thymine or a thymine nucleotide analogue; (C), it is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue; or (D), it is capable of forming hydrogen bonds with adenine or an adenine nucleotide analogue.

4. A method for simultaneously sequencing a plurality of different nucleic acids which comprises simultaneously applying the method of claim **3** to the plurality of different nucleic acids.

* * * * *

Dkt. 62239-BZA6AA/JPW/BI

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	The Trustees of Columbia University in the City of New York	
Inventors :	Jingyue Ju et al.	
Serial No.:	16/149,098	Examiner: Jezia Riley
Filed :	October 1, 2018	Art Unit: 1637
Conf. No. :	3820	
For :	MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA	

30 Rockefeller Plaza
20th Floor
New York, New York 10112
May 9, 2019

BY EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL COMMUNICATION SUPPLEMENTING COMMUNICATION IN RESPONSE TO JANUARY 16, 2019 FIRST ACTION INTERVIEW PILOT PROGRAM PRE-INTERVIEW COMMUNICATION FILED FEBRUARY 12, 2019

This Supplemental Communication is submitted to supplement the Communication In Response To January 16, 2019 First Action Interview Pilot Program Pre-Interview Communication filed February 12, 2019 in connection with the above-identified application.

REMARKS

I. STATUS OF CLAIMS

The claims pending in this application are previously pending claims 1-2.

II. INTERVIEW SUMMARY

On May 1, 2019 the undersigned participated in a telephonic Interview with Primary Examiner Jezia Riley in connection with related U.S. Application No. 16/150,191. Ms. Brittany Internoscia, an associate of the undersigned, also participated.

Applicant acknowledges with appreciation the courtesy that Examiner Riley extended during the May 1, 2019 interview.

During the May 1, 2019 interview, the Examiner requested that applicant file a Supplemental Communication in Response to the First Action Communications mailed in connection with each of related U.S. Applications Nos. 16/149,098; 16/149,114; and 16/150,185 to address in the same way all of the issues addressed in the April 12, 2019 Communication in Response to the March 12, 2019 First Action Communication issued in U.S. Application No. 16/150,191, even though certain of these issues were inadvertently not raised in those First Action Communications. The issues not included in those First Action Communications include issues relating to 35 U.S.C. §112(a) and 35 U.S.C. §112(b) which were raised in U.S. Application No. 16/150,191. Subject to addressing in each of these three applications, each of these issues in the same way as they were addressed in connection with U.S. Application No. 16/150,191 Examiner Riley indicated the claims pending in those applications would be allowable.

III. OBVIOUSNESS-TYPE DOUBLE PATENTING

In response to the obviousness-type double patenting rejection of pending claims 1-2 over claim 1 of U.S. Patent No. 9,725,480 set forth in the January 16, 2019 First Action Interview Pilot Program Pre-Interview Communication, applicant, without conceding the correctness of the Examiner's rationale for this rejection, filed a Terminal Disclaimer with respect to U.S. Patent No. 9,725,480, the reference patent cited in the rejection. That Terminal Disclaimer was approved on February 16, 2019.

Accordingly, applicant maintains this rejection should be withdrawn.

IV. REJECTION FOR INDEFINITENESS

Although claims 1-2 were not rejected under 35 U.S.C. §112(b) as indefinite applicant responds as if such a rejection had been and the same issues had been raised as were raised in U.S. Application No. 16/150,191. Applicant's response follows:

A. The term "small"

The Examiner indicated that the term "small" in the claims is a relative term which renders the claim indefinite; that the term "small" is not defined by the claim; that the specification does not provide a standard for ascertaining the requisite degree and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. The Examiner further stated that the specification does not define "small" and provides only two examples, MOM ether and allyl, and a skilled artisan would not know which other groups meet the limitation "small".

Applicant notes that a relative term is not automatically indefinite [MPEP 2175.05(b)]. More importantly, applicant maintains that the specification of the subject application at page 4, lines 10-32; page 5, lines 1-32; page 6, lines 1-27; and page 13, lines 3-11, taken together

with FIG. 1 referred to at page 4, line 31 of the application, set forth a standard for assessing whether a 3'-O capping group is "small" based on its ability to fit into the active site of a polymerase. As of October 6, 2000, the person of ordinary skill in the art ("POSA") reading the specification would have understood that "small" referred to the ability to fit into the active site of the polymerase defined by reference to the three-dimensional structure shown in FIG. 1. The POSA would have further understood that FIG. 1 corresponds to FIG. 6 of previously published Pelletier et al. (*Science*, Vol. 264, June 24, 1994, 1891-1903) cited at page 4, line 30 of the application. The POSA would also have understood that Pelletier et al. disclosed, on page 1903, the precise coordinates of the structure of the polymerase in "References and Notes" 101 and, in Table 3 on page 1897, the distances between the sugar of the nucleotide analogue and the key amino acids in the active site of the polymerase. See also paragraphs 11-13 of the accompanying Declaration of Jingyue Ju, Ph.D. signed May 26, 2017 and submitted in connection with U.S. Application No. 15/167,917, now U.S. Patent No. 9,725,480. A copy of this Declaration is attached hereto as **Exhibit 1**, including copies of **Exhibits A-E** referred to therein and attached hereto. **Exhibit B** is a copy of Pelletier et al.

With the benefit of applicant's specification, a POSA in October 2000 could have readily determined whether any given R when present as OR (a 3'-O capping group) was small by this standard using the published coordinates and available software such as Chem3D Pro. More specifically, using this approach the POSA would have known that the space available around the 3' position of a deoxyribose in the active site of the polymerase was approximately 3.7Å in diameter. By this standard, R when present as OR would need to be less than 3.7Å in diameter. Consistently, the POSA would have known that the two examples in the application, MOM and Allyl with diameters of 2.1Å and 3.0Å, respectively, would fit in the active site of the polymerase and would be "small". [See also paragraphs 14-16 of the Declaration of Jingyue Ju, Ph.D. and the Analysis discussed therein and attached to the Declaration as **Exhibit C**.]

Using this standard the POSA also would have known which other 3'-O capping groups meet the definition "small" and have the other features recited in the claims and would, for example, have readily determined that groups such as Methylthiomethyl and Azidomethyl were "small" and would fit in the active site while a group such as a 2-Nitrobenzyl group which has a diameter of 5Å was not "small" and would not fit into the active site of the polymerase. [See also paragraph 17 of the Declaration of Jingyue Ju, Ph.D. and the Analysis attached thereto as **Exhibit C**.]

As Dr. Ju opines, the POSA reading the subject application and relying on information publicly known as of October 2000 would have known that the standard for assessing whether any specific 3'-O capping group in a nucleotide analogue was "small" was whether it has a diameter less than 3.7Å so that it would fit into the active site of the polymerase. [See also paragraph 18 of the Declaration of Jingyue Ju, Ph.D.]

Therefore, the meaning of "small" would not have been indefinite to the POSA. To the contrary, its meaning would have been reasonably certain to the POSA to the extent required by 35 U.S.C. §112.

B. The term "R"

The Examiner indicated that the definition of R in the claims is unclear. The Examiner acknowledged that the claims recite some functional characteristics of R but asserted that these functional limitations do not set forth well-defined boundaries of the invention because they only state a problem solved or a result achieved.

As an initial matter applicant points out that in the claims: (1) R is further defined as a small, chemically cleavable, chemical group capping the 3' oxygen of the sugar of a nucleotide analogue, (2) R does not contain a ketone group, or a $-CH_2CH=CH_2$ group, and (3) OR is not a methoxy group, an ester group, or an allyl ether group. Further, in the structures in the claims, R is shown as covalently bound to the 3' oxygen. With the meaning of "small" defined as indicated in the preceding section and the

two examples provided in the application, the POSA would readily know which chemical capping groups could be R since the size (diameter) and other properties required by the claims would be easily determined in the context of the claims as a whole. In this regard, applicant emphasizes that there is nothing wrong with using functional language to define features so long as the invention recited in the claims is not being defined entirely by functional features [MPEP 2173.05(g)]. Similarly, there is nothing wrong with using negative limitations [MPEP 2173.05(i)].

Moreover, prior art as of October 2000, including Tsien (WO 91/06678, May 16, 1991) and Stemple (WO 00/53805, September 14, 2000), identify numerous chemically cleavable, 3'-O capping chemical groups, each of which could be readily evaluated to determine whether it was "small" and also whether it met other structural requirements of the claims such as "is not a methoxy group or an ester group" [contrary to the teachings of Tsien that such groups could be used in sequencing by synthesis] and "does not contain a ketone group". The POSA would have understood, with reasonably certainty, the meaning of R and would not have found its meaning unclear. [See also paragraph 19 of the Declaration of Jingyue Ju, Ph.D.]

C. The term "Y"

The Examiner acknowledged that the claim recites some functional characteristics of Y but that these functional limitations do not set forth well-defined boundaries of the invention because they only state a problem solved or a result achieved.

Applicant initially points out that Y is also defined in the claims as a chemically cleavable, chemical linker and a POSA would have readily understood the meaning of Y, with reasonably certainty, as required by 35 U.S.C. §112. Applicant further points out that the scope of the claims should not be equated with indefiniteness [MPEP 2173.04].

Applicant further points out that Stemple and Tsien disclose examples of

chemically cleavable, chemical linkers. Therefore, the POSA would have readily understood the meaning of a chemically cleavable, chemical linker (Y). In addition, the structures in the claims show the structural features of covalent bonds joining Y to a specific position on the base at one end and to the fluorescent tag at the other end. Applicant also directs the Examiner's attention to FIGS. 7, 8, 10, and 15A of the application. The POSA would have been familiar with both the term chemically cleavable, chemical linker and numerous examples from the prior art and would have understood its meaning with reasonable certainty. [See also paragraph 20 of the Declaration of Jingyue Ju, Ph.D.]

D. Conclusion

Based on the preceding remarks and the accompanying Declaration of Jingyue Ju, Ph.D. including the Exhibits attached to the Declaration, applicant maintains that the pending claims satisfy the requirements of definiteness imposed by 35 U.S.C. §112(b) and requests that the Examiner reconsider and withdraw this ground of rejection.

V. REJECTION FOR FAILURE TO COMPLY WITH WRITTEN DESCRIPTION REQUIREMENT

Although the claims were not rejected under 35 U.S.C. §112(a) for failing to comply with the written description requirement applicant responds as if such a rejection had been and the same issues had been raised as were raised in U.S. Application No. 16/150,191. Applicant's response follows:

In support of this rejection the Examiner indicated that variables R and Y in the claims are defined functionally but lack a clear-cut indication of the scope of the subject matter embraced by the claims; and that in this case, the specification does not provide the particular structures that accomplish the functions recited in the claims with the exception of two examples of R and one example of Y. The Examiner further asserted that the skilled artisan would not be apprised that the inventors had possession of the full scope of the claimed invention at the time the

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Paper 67
Entered: June 21, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK,
Patent Owner.

Case IPR2018-00291 (Patent 9,718,852 B2)
Case IPR2018-00318 (Patent 9,719,139 B2)
Case IPR2018-00322 (Patent 9,708,358 B2)
Case IPR2018-00385 (Patent 9,725,480 B2)[1]

Before JAMES A. WORTH, MICHELLE N. ANKENBRAND, and BRIAN D. RANGE, *Administrative Patent Judges.*

Opinion for the Board *per curiam*.
Opinion Dissenting filed by Administrative Patent Judge WORTH.

Per curiam

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[1] The proceedings have not been consolidated. The parties are not authorized to use a combined caption unless an identical paper is being entered into each proceeding and the paper contains a footnote indicating the same.

FINAL WRITTEN DECISION
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

I. INTRODUCTION

This is a Final Written Decision addressing four *inter partes* reviews challenging each claim of U.S. Patent Nos. 9,718,852 B2 ("the '852 patent"), 9,719,139 B2 ("the '139 patent"), 9,708,358 B2 ("the '358 patent"), and 9,725,480 B2 ("the '480 patent"). We have jurisdiction under 35 U.S.C. § 6. For the reasons that follow, we determine that Illumina, Inc. ("Petitioner" or "Illumina") demonstrates, by a preponderance of the evidence, that the challenged claims are unpatentable.

A. Procedural History

Petitioner filed four Petitions (Paper 1,[2] "Pet.") requesting an *inter partes* review of the '852 patent, the '139 patent, the '358 patent, and the '480 patent. We instituted trial on the following grounds:[3]

Patent	References	Basis	Claim Challenged
'852	Tsien,[4] Prober[5]	§ 103(a)	1

[2] Unless this opinion otherwise indicates, all citations are to IPR2018-00291 ("the '291 IPR").

[3] *See* IPR2018-00291, Paper 16 (June 25, 2018); IPR2018-00318, Paper 16 (July 2, 2018); IPR2018-00322, Paper 16 (July 2, 2018); IPR2018-00385, Paper 20 (July 26, 2018).

[4] Tsien et al., WO 91/06678, May 16, 1991 ("Tsien") (Ex. 1013).

[5] James M. Prober et al., *A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides*, 238 SCIENCE 336–341 (Oct. 16, 1987) ("Prober") (Ex. 1014).

application. The parties agree that the priority date for the patent at issue is October 6, 2000. Tr. at 24:21–24.

C. Level of Ordinary Skill in the Art

We consider each asserted ground of unpatentability in view of the understanding of a person of ordinary skill in the art. Petitioner proposes a definition of the level of skill in the art (Pet. 7–8), and Patent Owner does not dispute this definition (Resp. 3). Petitioner's proposal is consistent with the evidence before us. *See* Findings of Fact, *infra*. We, therefore, adopt Petitioner's proposal and find that a person of ordinary skill in the art would have been a member of a team of scientists developing nucleotide analogues, researching DNA polymerases, and/or addressing DNA techniques. A person of ordinary skill in the art would have held a doctoral degree in chemistry, molecular biology, or a closely related discipline, and would have had at least five years of practical academic or industrial laboratory experience.

D. Claim Construction

The Board interprets claims in an unexpired patent using the "broadest reasonable construction in light of the specification of the patent." 37 C.F.R. § 42.100(b) (2017)[10]; *Cuozzo Speed Techs., LLC v. Lee*, 136 S. Ct.

[10] The Office recently changed the claim construction standard applicable to an *inter partes* review. *See* Changes to the Claim Construction Standard for Interpreting Claims in Trial Proceedings Before the Patent Trial and Appeal Board, 83 Fed. Reg. 51,340 (Oct. 11, 2018). The rule changing the claim construction standard, however, does not apply to this proceeding because Petitioner filed its Petition before the effective date of the final rule, i.e., November 13, 2018. *Id.* at 51,340 (rule effective date and applicability date), 51,344 (explaining how the Office will implement the rule).

2131, 2144–46 (2016). Under that standard, claim terms are given their ordinary and customary meaning in view of the specification, as would be understood by one of ordinary skill in the art at the time of the invention. *In re Translogic Tech., Inc.*, 504 F.3d 1249, 1257 (Fed. Cir. 2007). Any special definitions for claim terms must be set forth with reasonable clarity, deliberateness, and precision. *In re Paulsen*, 30 F.3d 1475, 1480 (Fed. Cir. 1994).

Here, Petitioner states that "no claim term requires express construction to evaluate patentability." Pet. 7. Patent Owner requests construction of "small" and "chemical linker." Resp. 9–11. We address these two issues below.

"*small*"

Each claim before us recites that the nucleotide capping group, R, "represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue." *See, e.g.*, Ex. 1001, 34:1–35:4. Patent Owner argues that "'small' means the group has a diameter less than 3.7 [Angstroms]." Resp. 9.

Here, each of Petitioner's asserted invalidity grounds relies upon the obviousness of using an allyl blocking group. Transcript, 14:2-11. The parties agree that an allyl blocking group is "small" within the context of the claims at issue. *Id.* at 14:9–11; Resp. 9. There is, therefore, no need for us to further construe "small." *See Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999) ("only those terms need be construed that are in controversy, and only to the extent necessary to resolve the controversy"); *see also Nidec Motor Corp. v. Zhongshan Broad Ocean*

Motor Co. Ltd., 868 F.3d 1013, 1017 (Fed. Cir. 2017) (quoting *Vivid Techs* when addressing an *inter partes* review proceeding on appeal).

"*chemical linker*"

Each challenged claim recites that the Y on the nucleotide structure "represents a chemically cleavable, chemical linker." *See, e.g.*, Ex. 1001, 34:30–31. Patent Owner argues that "'chemical linker' means a chemical moiety attached by covalent bonds at one end to a specified position on the base of a nucleotide and at the other end to a tag (detectable fluorescent moiety)." Resp. 10. Based on our review of the record, we determine that this term does not require express construction in order to resolve the parties' controversy. *Vivid Techs.*, 200 F.3d at 803.

E. Fact Findings

The fact findings below focus on issues that must be resolved in order to assess Petitioner's obviousness challenges. *Graham* 383 U.S. at 17–18. (1966). Each finding is based upon consideration of the record as a whole and is supported by the preponderance of the evidence.

1. Technology Overview

Deoxyribonucleotides make up the building blocks of DNA, and the chemical formula, nomenclature, and uses of deoxyribonucleotides were generally known before October 6, 2000. Ex. 1011, 46, 47, 58–60, 98–103. A strand of DNA consists of deoxyribonucleotides where the 5'-phosphate of one nucleotide is attached to the 3′-oxygen of the adjacent nucleotide. Ex. 1078 ¶¶ 33–36; Pet. 12–13.

Before October 6, 2000, persons having ordinary skill in the art would have been aware of several methods for determining the sequence of DNA, including Sanger sequencing and sequencing-by-synthesis ("SBS").

analogue having a tag attached through a cleavable linker at the 7-position. *E.g.*, Pet. 64 ("Dower in view of Prober . . . renders obvious a chemically cleavable linker at the 7-position of deaza-adenine."); *see In re Keller*, 642 F.2d 413, 525 (CCPA 1981) ("[T]he test [for obviousness] is what the combined teachings of the references would have suggested to those of ordinary skill in the art."). In that regard, Petitioner directs us to Dower's teaching of a fluorescent label as a removable moiety that can be "cleaved 'chemical[ly], using acid, base, or some other, preferably mild, reagent.'" Pet. 63–64 (quoting Ex. 1015, 21:32–40 and citing Ex. 1015, 5:35–37, 15:52–56, 25:35–40, Fig. 9); *see also* Ex. 1015, 15:52–53 ("One important functional property of the [dNTP] monomers is that the label be removable."); Ex. 1012 ¶ 121. Petitioner also points to Dower's citations to Prober for disclosing labeled nucleotide analogues, e.g., dATP, and Dr. Romesberg testifies that Prober discloses suitable reaction conditions for making such analogues. Pet. 63 (citing Ex. 1015, 20:39–42, 23:16–26, 25:4–12, 25:44–47); Ex. 1012 ¶¶ 122–123; *see* Ex. 2014, 337–338 (Prober's disclosure or nucleotide analogues having a fluorescent label linked to the 7-position of deaza-adenine).

Although we agree with Patent Owner that Prober's propargyl amine linker is not cleavable under DNA-compatible conditions, the evidence of record suggests that a person of ordinary skill in the art would have been able to identify and to use an appropriate chemically cleavable, chemical linker or linkers, and that using such a linker or linkers[33] was well within the

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[33] Patent Owner argues that claim 1 excludes a linker attached to a propargyl amine because the claim requires one linker, not two linkers. Surreply 24. We disagree. "As a general rule, the words 'a' or 'an' in a patent claim carry the meaning of 'one or more.'" *01 Communique Lab.,*

level of ordinary skill in the art. For example, during prosecution of the application that matured into the '852 patent, the patent applicant explained that a skilled artisan "would have been familiar with both the term chemically cleavable, chemical linker and numerous examples [of such linkers] from the prior art." Ex. 1009, 19. In addition, Petitioner directs us to Patent Owner's expert's testimony from IPR2012-00007 that it was known to add an alkeynlamino group (e.g., propargylamine) to a nucleotide base and to attach a fluorescent label to the alkynylamino group using a cleavable linker. Pet. 37 (citing Ex. 1028, 166:3–168:5, 170:7–171:5, 177:13–178:15, 179:7–23, 191:23–192:5, 342:19–343:9, 3875:–388:23), 73 (referring to Pet. § VIII.B.16 as explaining how the steps for preparing nucleotide analogues that the combination of Dower, Prober, and Metzker disclose "were within the level of ordinary skill"). And Dr. Romesberg testifies credibly that ordinary artisans would have known how to prepare 7-substituted 7-deaza-purine nucleotides (e.g., dATP) with chemically cleavable linkers. Ex. 1012 ¶ 97 (providing references that disclose how to prepare the nucleotides with alkynylamino linkers and allyl linkers and explaining that the cleavable linker that Seitz[34] discloses for attaching a fluorescent group to a molecule having an amine group would have been

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Inc. v. LogMeln, Inc., 687 F.3d 1292, 1297 (Fed. Cir. 2012) (quoting *TiVo, Inc. v. EchoStar Commc'ns Corp.*, 516 F.3d 1290, 1303 (Fed. Cir. 2008)). The exceptions to the rule are "extremely limited" and require that a patentee "evince a clear intent to limit 'a' or 'an' to 'one.'" *Id.* (quoting *Baldwin Graphic Sys., Inc. v. Siebert, Inc.*, 512 F.3d 1338, 1342 (Fed. Cir. 2008)). Patent Owner's bare argument does not establish such a clear intent.

[34] Oliver Seitz & Horst Kunz, *HYCRON, an Allylic Anchor for High-Efficiency Solid Phase Synthesis of Protected Peptides and Glycopeptides*, 62 J. ORGANIC CHEM. 813, 815 (1997).

US009718852B2

(12) **United States Patent**
Ju et al.

(10) **Patent No.: US 9,718,852 B2**
(45) **Date of Patent: *Aug. 1, 2017**

(54) **MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA**

(71) Applicant: **The Trustees of Columbia University in the City of New York**, New York, NY (US)

(72) Inventors: **Jingyue Ju**, Englewood Cliffs, NJ (US); **Zengmin Li**, Flushing, NY (US); **John Robert Edwards**, St. Louis, MO (US); **Yasuhiro Itagaki**, New York, NY (US)

(73) Assignee: **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/380,270**

(22) Filed: **Dec. 15, 2016**

(65) **Prior Publication Data**

US 2017/0088575 A1 Mar. 30, 2017

Related U.S. Application Data

(60) Continuation of application No. 14/670,748, filed on Mar. 27, 2015, which is a continuation of application No. 13/959,660, filed on Aug. 5, 2013, now Pat. No. 9,133,511, which is a continuation of application No. 13/672,437, filed on Nov. 8, 2012, now abandoned, which is a continuation of application No. 13/339,089, filed on Dec. 28, 2011, now abandoned, which is a continuation of application No. 12/804,284, filed on Jul. 19, 2010, now Pat. No. 8,088,575, which is a continuation of application No. 11/810,509, filed on Jun. 5, 2007, now Pat. No. 7,790,869, which is a continuation of application No. 10/702,203, filed on Nov. 4, 2003, now Pat. No. 7,345,159, which is a division of application No. 09/972,364, filed on Oct. 5, 2001, now Pat. No. 6,664,079, which is a continuation-in-part of application No. 09/684,670, filed on Oct. 6, 2000, now abandoned.

(60) Provisional application No. 60/300,894, filed on Jun. 26, 2001.

(51) **Int. Cl.**
C07H 19/14 (2006.01)
C12Q 1/68 (2006.01)

(52) **U.S. Cl.**
CPC ***C07H 19/14*** (2013.01); ***C12Q 1/6872*** (2013.01); ***C12Q 1/6874*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(Continued)

Primary Examiner — Layla Berry
(74) *Attorney, Agent, or Firm* — John P. White; Gary J. Gershik; Cooper & Dunham LLP

(57) **ABSTRACT**

This invention provides methods for attaching a nucleic acid to a solid surface and for sequencing nucleic acid by detecting the identity of each nucleotide analog after the nucleotide analog is incorporated into a growing strand of DNA in a polymerase reaction. The invention also provides nucleotide analogs which comprise unique labels attached to the nucleotide analog through a cleavable linker, and a cleavable chemical group to cap the —OH group at the 3'-position of the deoxyribose.

1 Claim, 28 Drawing Sheets

Tabor S, Richardson C. C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 84: 4767-4771.

Tabor S. & Richardson, C C. (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 92: 6339-6343.

Turro N J. (1991) Modern Molecular Photochemistry; University Science Books, Mill Valley, Calif.

Velculesu V E, Zhang, I, Vogelstein, B. and Kinzler K W (1995) Serial Analysis of Gene Expression. *Science* 270: 484-487.

Welch M B, Burgess K, (1999) Synthesis of fluorescent, photolabile 3'-O-protected nucleoside triphosphates for the base addition sequencing scheme. *Nucleosides and Nucleotides* 18:197-201.

Woolley A T, Mathies R A. (1994) Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. *Proc. Natl. Acad. Sci. USA.* 91: 11348-11352.

Woolley A T, Sensabaugh G F and Mathies R A. (1997) High-Speed DNA Genotyping Using Microfabricated Capillary Array Electrophoresis Chips, *Anal. Chem.* 69(11); 2181-2186.

Yamakawa H, Ohara O. (1997) A DNA cycle sequencing reaction that minimizes compressions on automated fluorescent sequencers. *Nucleic. Acids. Res.* 25: 1311-1312.

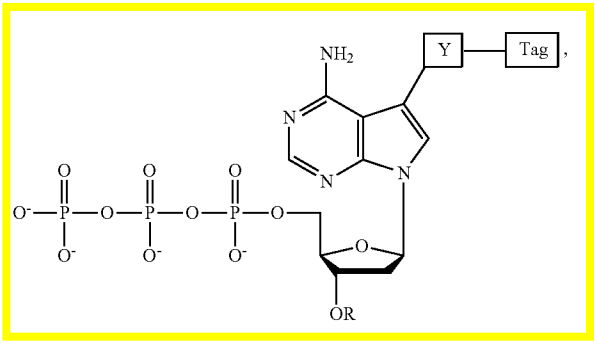
Zhang X H, Chiang V L, (1996) Single-stranded DNA ligation by T4 RNA ligase for PCR cloning of 5'-noncoding fragments and coding sequence of a specific gene. *Nucleic Acids Res.* 24: 990-991.

Zhang B., Liu H. Karger B L. Foret F. (1999) Microfabricated devices for capillary electrophoresis-electrospray mass spectrometry. *Anal. Chem.* 71:3258-3264.

Zhu Z, Chao J, Yu H, Waggoner A S. (1994) Directly labeled DNA probes using fluorescent nucleotides with different length linkers. *Nucleic Acids Res.* 22: 3418-3422.

What is claimed is:

1. An adenine deoxyribonucleotide analogue having the structure:



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, and (d) does not contain a ketone group;

wherein OR is not a methoxy group or an ester group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction; and

wherein the adenine deoxyribonucleotide analogue:

i) is recognized as a substrate by a DNA polymerase,

ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,

iii) produces a 3'-OH group on the deoxyribose upon cleavage of R,

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1
<211> LENGTH: 11
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically Synthesized Template

<400> SEQUENCE: 1

acgtacgacg t 11

<210> SEQ ID NO 2
<211> LENGTH: 105
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Chemically Synthesized Template

<400> SEQUENCE: 2

ttcctgcatg ggcggcatga acccgaggcc catcctcacc atcatcacac tggaagactc 60

cagtggtaat ctactgggac ggacggaaca gctttgaggt gcatt 105

iv) no longer includes a tag on the base upon cleavage of Y, and

v) is capable of forming hydrogen bonds with thymine or a thymine nucleotide analogue.

* * * * *

Filed December 8, 2017

Filed on behalf of:
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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner.

Case No. IPR2018-00291
U.S. Patent No. 9,718,852

PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 9,718,852

3. Limitation R(b): "wherein R ... (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase"

Tsien discloses that a "criteria for successful use of 3′-blocking groups include[s]: (1) the ability of a polymerase enzyme to accurately and effectively incorporate the dNTPs carrying the 3′-blocking groups into the cDNA chain." Ex-1013 at 20:28-32; *id.* at 19:4-18, 12:11-18, 24:1-2. A POSA would have known that Tsien's 3′-O-allyl group does not interfere with recognition by a DNA polymerase because Metzker demonstrated polymerase recognition in 1994. Ex-1016 at 4263; Ex-1012 ¶¶63, 66. Columbia's specification concedes that the 3′-O-allyl group had been "shown to be incorporated by Ventr(exo-) DNA polymerase in the growing strand of DNA (Metzker et al. 1994)." Ex-1001 at 3:28-30; *id.* at 3:22-26.

Dr. Ju admitted during prosecution that Tsien's 3′-O-allyl meets this limitation and all other claimed limitations of the "R" group. Ex-1022 ¶¶22a (admitting allyl is one of "[o]nly a limited number of 3′-O capping groups [that] meet the ... structural and functional features recited in the claim"). Columbia's witness in IPR2012-00007, Dr. Trainor, admitted Tsien discloses polymerase incorporation of 3′-capped and base-labeled nucleotides. Ex-1013 at 27:33-28:18; Ex-1028 at 449:3-20. Columbia admitted Tsien "explicitly disclosed" this limitation during prosecution of related U.S. 9,725,480 ("'480 patent"). Ex-1038 at 10.

Tsien's disclosure of this limitation was settled when the Board held unpatentable Claim 28 from Columbia's '869 patent. Ex-1005 at 11. Claim 28 recited "wherein said cleavable chemical group does not interfere with the recognition of the nucleotide by a polymerase." Ex-1010 at 34:40-42; Ex-1008 at 33. Tsien therefore discloses limitation R(b).

4. Limitations "wherein R ... (c) is stable during a DNA polymerase reaction" and "wherein the covalent bond between the 3′-oxygen and R is stable during a DNA polymerase reaction"

Tsien discloses the stability of 3′-capping groups during DNA polymerase incorporation. Ex-1013 at 19:4-18, 12:11-13:13, 23:28-32. Tsien discloses that the allyl capping group is particularly advantageous because "deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized." *Id.* at 24:24-25:3. Tsien's stated "incorporation" refers to DNA polymerase incorporation. *Id.* at 20:28-32; Ex-1012 ¶68. The overall stability disclosed by Tsien for the 3′-O-allyl group includes stability at the bond between the 3′-oxygen and the rest of the blocking group. Ex-1012 ¶68.

Columbia admitted Tsien discloses these two limitations during prosecution of the related '480 patent. Ex-1038 at 10-11 (citing Tsien at 23:28-32). Dr. Ju admitted the 3′-O-allyl group meets all claimed "R" limitations. Ex-1022 ¶¶22a.

Tsien therefore discloses limitation R(c) and that the 3′-oxygen to R bond is stable during a polymerase reaction.

5. Limitations "wherein R ... (d) does not contain a ketone group" and "wherein OR is not a methoxy group or an ester group"

Tsien discloses the advantages of the 3′-O-allyl capping group (Ex-1013 at 24:24-25:3, 24:5-7), and this group does not contain a ketone group, as shown below:

$-CH_2-CH=CH_2$ allyl Ex-1039 at 502-504	-C(=O)- ketone Ex-1039 at 43

Tsien's 3′-O-allyl capping group is not a methoxy or an ester group:

$-O-CH_2-CH=CH_2$ -O-allyl	$-O-CH_3$ methoxy	$-O-C(=O)-CH_3$ an ester

Ex-1012 ¶70. Tsien's disclosure of a 3′-O-allyl capping group meets and render obvious limitations R(d) and wherein OR is not a methoxy group or an ester group.

6. Limitation "wherein tag represents a detectable fluorescent moiety"

Tsien discloses an exemplary adenine analogue: "3′-BLOCKED dA'TP." Ex-1013 at Fig. 2; *id.* at 10:4-15. The apostrophe indicates a fluorescent tag. *Id.* at 10:7-10 ("When [the dNTPs] are each tagged or labeled with different reporter groups, such as different fluorescent groups, they are represented as dA'TP, dC"TP, dG'"TP and dT""TP."). The "fluorescent tag [is] attached to the base moiety" and

10. Limitation i): "wherein the adenine deoxyribonucleotide analogue: i) is recognized as a substrate by a DNA polymerase"

Tsien discloses polymerase recognition of a nucleotide analogue having a small chemically cleavable 3′-capping group (such as Tsien's 3′-O-allyl group) and a chemically cleavable linker attached to the base. *Supra* Sections VIII.B.3 and 8.

Tsien further discloses a "3′-BLOCKED dA'TP" analogue is added to a reaction zone with the three other labeled and blocked dNTPs where a polymerase brings "about addition of the one, and only the one, of the four labeled blocked dNTPs which is complementary to the first available template nucleotide following the primer." Ex-1013 at 12:22-27, Fig. 2. This polymerase addition discloses recognition of an adenine deoxyribonucleotide having a 3′-OH capping group and a detectable tag attached to the base. Ex-1012 ¶¶81-82; *see also* Ex-1013 at 19:4-18, 20:28-32. Tsien's cleavable linkers "do not interfere with the binding of the dNTP to the polymerase" (Ex-1013 at 28:31-35) and the fluorescent tag and tethers are "innocuous" to the polymerase. *Id.* at 26:2-12. It was known that Vent polymerase incorporates 3′-O-allyl nucleotide analogues (Ex-1016 at 4263), as well as 7-deaza-purine nucleotide analogues. Ex-1040 at 3228, 3230; Ex-1041 at 4832-33; Ex-1059 at 632; Ex-1012 ¶82. Tsien's disclosures therefore meet limitation i).

3. Motivation to use a cleavable allyl linker

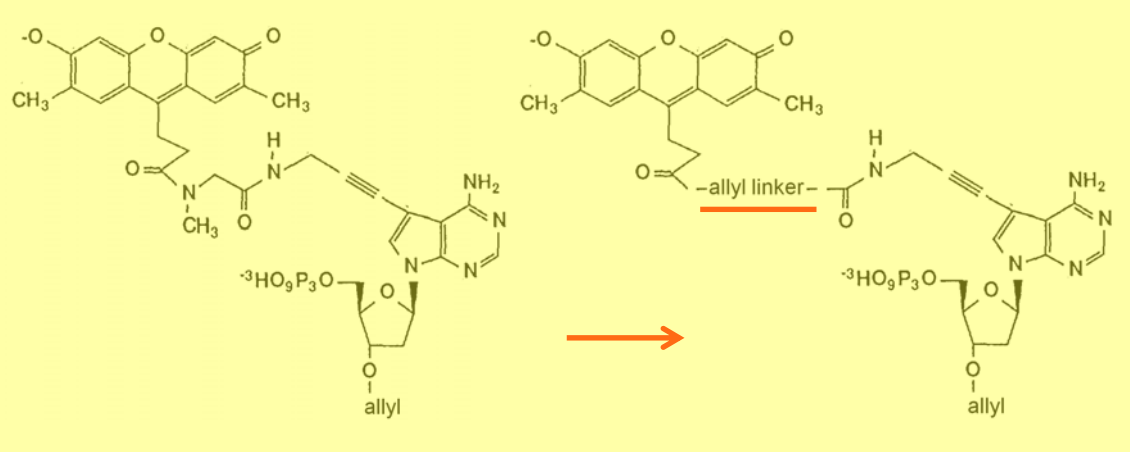
A POSA would have been motivated to use a cleavable allyl linker to attach the fluorescent label to the 7-position of a deaza-adenine nucleotide analogue. The Board and Federal Circuit determined that Tsien provides an anticipatory disclosure of a nucleotide having a cleavable linker attaching a fluorescent label to the base and a 3′-OH capping group. Ex-1005 at 10-11; Ex-1008 at 31. The Board determined that a POSA "would have been guided to use the cleavable linker [described in Tsien] to attach the fluorescent label to the base on the nucleotide." Ex-1005 at 10.

Columbia relied on Tsien's disclosure of chemically cleavable linkers to fulfill its §112 requirements. Ex-1009 at 19; Ex-1022 ¶20. Tsien discloses a cleavable allyl linker as one of three exemplary cleavable linkers for attaching the fluorescent label to the base. Ex-1013 at 28:19-29; Ex-1005 at 10; Ex-1008 at 31; *supra* Section VIII.B.7. The Federal Circuit agreed "that an embodiment comprising a 3′-OH-capped nucleotide, base-label, and cleavable linker could be envisaged clearly by one of ordinary skill in the art upon reading the Tsien disclosure." Ex-1008 at 31.

A POSA would have been motivated to place Tsien's cleavable allyl linker on Prober's 7-deaza-adenine following Tsien's guidance to use a cleavable allyl linker. Allyl linkers were common in the prior art. Ex-1051 at 4062-63; Ex-1052

at 813-15; Ex-1053 at 2132-33; Ex-1012 ¶¶110-113. Seitz discloses an allyl linker for attaching a fluorescent Fmoc group to a molecule having an amine group (Ex-1052 at 815) that is compatible with the amine used by Prober at the 7-position of deaza-adenine. Ex-1014 at 337; Ex-1012 ¶109.

Following Tsien's direction to use an allyl linker, a POSA would have arrived at a deaza-adenine deoxyribonucleotide analogue having a fluorescent group attached through a cleavable allyl linker to the 7-position of the base and a 3′-O-allyl capping group:



This compound meets all limitations of Columbia's claim for the same reasons discussed in Section VIII.B.

4. <u>There was a reasonable expectation of success</u>

The Board previously determined that a POSA would have had a reasonable expectation of success in combining Tsien and Prober to make 3′-capped 7-substituted deaza-adenine nucleotide analogues with cleavable linkers. Ex-1005 at 26-35. The Federal Circuit affirmed. Ex-1008 at 31. The steps for preparing

Paper No. _
Filed: October 26, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK
Patent Owner.

IPR2018-00291 (Patent 9,718,852)
IPR2018-00318 (Patent 9,719,139)
IPR2018-00322 (Patent 9,708,358)
IPR2018-00385 (Patent 9,725,480)[1]

PATENT OWNER'S RESPONSE

[1] An identical Paper is being entered into each listed proceeding.

Illumina has not argued otherwise. Ex. 2116 ¶18; Ex. 2035 at 63-65 (Dr. Romesberg testifying that even in 2002, "people did not believe that you could"); *see also* Ex. 2126 at 252-53.

IV. Claim Construction

Illumina asserts claim term construction is unnecessary. Columbia disagrees. To properly evaluate the differences between the challenged claim[7] and the cited art, the following claim terms need to be construed.

A. "Small"

The term "small" refers to the ability of the capping group to fit into the active site of the polymerase whose three-dimensional structure is shown in Figure 1 of the patent-at-issue. More specifically, "small" means the group has a diameter less than 3.7Å. This construction is based on the specification of the patent-at-issue. Exs. 1001-1004, each at 2:63-3:54, 5:52-59, Fig. 1, 7:51-8:28. As explained during prosecution of the challenged claim, "[a]s of October 6, 2000, the POSA reading the specification would have understood that 'small' referred to the ability to fit into the active site of the polymerase defined by reference to the three-

[7] Unless otherwise indicated, the "challenged claim" refers to the claim at issue in each of IPR2018-00291, -00318, -00322, and 00385. Similarly, the "patent-at-issue" refers to the patent at issue in those proceedings.

dimensional structure shown in FIG. 1", and capping group R must have "a diameter less than 3.7Å so that it would fit into the active site of the polymerase." Exs. 1009, 1062, 1065, each at 16, 17, 27-29; Ex. 1068 at 12, 13, 23-25.[8] The Examiner adopted this meaning in the Notice of Allowability. Ex. 1009 at 4, 12 ("The declaration of Jingyue Ju . . . is sufficient to explain what is meant by 'small.'"); Ex. 1062 at 4, 12 (same); Ex. 1065 at 4, 11 (same); Ex. 1068 at 7 ("The declaration of Jingyue Ju . . . explaining what is meant by 'small' . . . [is] persuasive.").

B. "Chemical Linker"

In the context of claimed feature Y, "chemical linker" means a chemical moiety attached by covalent bonds at one end to a specified position on the base of a nucleotide and at the other end to a tag (detectable fluorescent moiety). Ex. 2116 ¶20. It does not mean merely a covalent bond between the base and the label as disclosed in Dower. *Id*. The specification of the patent-at-issue requires this construction (Exs. 1001-1004, each at 10:64-66, 14:8-10, the structures shown at columns 13-20, and Figs. 7, 8, 10, and 15A), which was expressly addressed

[8] Citations to Ex. 1009, Ex. 1062, Ex. 1065 and Ex. 1068 are only applicable to IPR2018-00291, IPR2018-00318, IPR2018-00322 and IPR2018-00385, respectively.

during prosecution of the challenged claim. Exs. 1009, 1062, 1065, each at 18-19, 30; Ex. 1068 at 14-15, 26; Ex. 2116 ¶20. Dr. Romesberg agrees that "Y represents a component that is between the fluorophore and the base that is cleavable . . . [the fluorophore is] not directly attached . . . to the base[.]" Ex. 2113 at 117.

During prosecution, Columbia also directed the Examiner to Tsien and Stemple for examples of chemical linkers. Exs. 1009, 1062, 1065, each at 19, 30; Ex. 1068 at 14, 26; Ex. 2116 ¶21. The linkers in those references are chemical moieties. *See* Ex. 1013 at 28:23-29:2; Ex. 2116 ¶21. The Examiner found Columbia's definition "persuasive." Ex. 1009 at 4, 12; Ex. 1062 at 4, 12; Ex. 1065 at 4, 11; Ex. 1068 at 7; Ex. 2116 ¶21.

V. Illumina's Ground 1 Challenge For Obviousness Over Tsien In View Of Prober Fails[9]

Illumina's Ground 1 challenge relies on a single premise—that it would have been obvious to make a base-labeled nucleotide with the 3-carbon, 5-hydrogen allyl capping group ($-CH_2CH=CH_2$) ("the allyl capping group"). Illumina's Ground 1 challenge fails because:

[9] In IPR2018-00318 and -00322, Illumina's Ground 1 challenge is based solely on Tsien.

(A) Illumina's references provided no motivation to select the allyl capping group because it had been described as being incapable of achieving the efficient incorporation requirement of SBS;

(B) Illumina's references provided no motivation to select the allyl capping group because they do not teach quantitative, rapid cleavage of the allyl capping group under mild, aqueous conditions;

(C) A POSA weighing the allyl capping group's numerous disadvantages would have concluded that it was incompatible with SBS;

(D) Illumina's references do not teach that capping groups had to be small or that there was any advantage to selecting small capping groups, and a POSA would not have reviewed Pelletier because it related to an entirely different field from SBS;

(E) There was no reasonable expectation of success in achieving the claimed invention;

(F) Tsien does not disclose the allyl capping group, instead it discloses vinyl ethers, and neither does Prober, and Tsien provides no motivation to select the allyl capping group because its cleavage conditions are incompatible with SBS; and

(G) Application of the lead compound analysis demonstrates that Illumina selected the wrong lead compounds.

"[t]etrahydrothiofuranyl ethers" and "2,4-[d]initrobenzenesulfenyl groups[.]" Ex. 1013 at 24-25; Ex. 2116 ¶85. If a POSA were motivated by such a benefit, the POSA would pursue other capping groups disclosed in Tsien that were not understood (based on experimental data) to be incompatible with SBS. Ex. 2116 ¶85.

D. A POSA Would Not Have Been Motivated To Select Small Capping Groups

Contrary to Illumina's assertions (*e.g.*, IPR2018-00291, Petition at 11-12 (Dec. 8, 2017)), a POSA before the Priority Date would not have been motivated to select a capping group that was "small," Ex. 2116 ¶86, which in the claim means less than 3.7Å in diameter (*see* Section IV(A)).

First, the prior art reported that a nucleotide with a larger, 2-nitrobenzyl capping group was efficiently incorporated by a polymerase. Ex. 1016 at 4263. The 2-nitrobenzyl capping group is 5.0Å in diameter (not small). Exs. 1009, 1062, 1065, each at 23, 29; Ex. 1068 at 18, 27-28. As such, a POSA had no reason to believe that SBS required capping groups smaller than 3.7Å in diameter.

Second, Illumina's reliance on Pelletier to argue that a POSA would be motivated to use small capping groups is misplaced. Ex. 2116 ¶87. A POSA would not have reviewed Pelletier because it related to HIV drug development, an entirely different field from SBS. *Id.*; Ex. 1021. Additional evidence distinguishing Pelletier from the relevant field is that there is no evidence of any

researchers developing nucleotides for SBS between 1994 and the Priority Date who cited Pelletier. Ex. 2116 ¶87. Illumina's reliance on Pelletier represents hindsight, driven by Columbia's citation to that reference in the specification and prosecution history to explain that a POSA ***reading the specification of the patent-at-issue*** would be able to determine whether a capping group was suitably small. Contrary to Illumina's assertions, Columbia did not concede that a POSA without the benefit of the patent-at-issue's specification would have consulted Pelletier. Moreover, "[t]he inventor's own path itself never leads to a conclusion of obviousness; that is hindsight." *Otsuka Pharm. Co. v. Sandoz, Inc.*, 678 F.3d 1280, 1296 (Fed. Cir. 2012).

Third, Drs. Romesberg and Menchen agree that a POSA would not have expected a capping group to possess the characteristics necessary for SBS (*e.g.*, efficient incorporation of the capped nucleotide) simply because it was small. Ex. 2007 ¶58; Ex. 2126 at 81-84; Ex. 2116 ¶88.

Fourth, contrary to Illumina's assertions that "Dower disclosed the desirability of nucleotides having 'small blocking groups' on the 3'-OH," *e.g.*, IPR2018-00291, Petition at 11 (Dec. 8, 2017), Dower's use of the term "small" to describe several capping groups (Ex. 1015 at 25:48-51) does not support a conclusion that Dower teaches that "small" capping groups are "desirable." Ex. 2116 ¶89. Dower does not state that the four capping groups it characterizes as

small are desirable because of their size. *Id*. Regardless, Dower's use of "small" when referring to capping groups does not equate to "small" as defined by the patent-at-issue (*i.e.*, smaller than 3.7Å in diameter). For example, the NBOC capping group referred to as small by Dower contains "nitrobenzyl" ("2-***nitrobenzyl***oxycarbonyl"; Ex. 1015 at 18:54), which as explained above, is not less than 3.7Å in diameter. Similarly, the tBOC and NVOC are not smaller than 3.7Å in diameter and Metzker showed that the acetyl was not incorporated at all by any polymerase. Ex. 1016 at 4263.

E. A POSA Would Not Have Had A Reasonable Expectation Of Success In Achieving The Claimed Invention

Contrary to Illumina's assertion, a POSA would not have had a reasonable expectation of success with regard to each of the features in the challenged claim, including "each of the recited functional limitations[.]" *E.g.*, IPR2018-00291, Petition at 38 (Dec. 8, 2017). Illumina alleges that a single claimed species is obvious: a nucleotide with the allyl capping group and a base label. As explained below, a POSA would not have had a reasonable expectation that this nucleotide would achieve the challenged claim's limitations. Ex. 2116 ¶90.

1. There Was No Reasonable Expectation That A 3'-O-Allyl Thymine, Cytosine, Or Guanine Nucleotide Would Be Incorporated

In IPR2018-00318, -00322, and -00385, the challenged claim requires that the claimed ***thymine*** nucleotide, ***cytosine*** nucleotide, or ***guanine*** nucleotide,

(B) Illumina's references provided no motivation to select the allyl capping group because they do not teach quantitative, rapid cleavage of the allyl capping group under mild, aqueous conditions (*see* Section V(B));

(C) A POSA weighing the allyl capping group's numerous disadvantages would have concluded that it was incompatible with SBS (*see* Section V(C));

(D) Illumina's references do not teach that capping groups had to be small or that there was any advantage to selecting small capping groups, and a POSA would not have reviewed Pelletier because it related to an entirely different field from SBS (*see* Section V(D));

(E) There was no reasonable expectation of success in achieving the claimed invention (*see* Section V(E)); and

(F) Application of the lead compound analysis demonstrates that Illumina selected the wrong lead compounds (*see* Section V(G)). Ex. 2116 ¶109.

B. Illumina's Ground 2 Challenge Fails Because None Of Its References Disclose The Claimed "Chemically Cleavable, Chemical Linker"

Illumina's Ground 2 challenge also fails because none of its references (Dower, Prober, or Metzker) disclose a "chemically cleavable, chemical linker" as required by the challenged claim. Ex. 2116 ¶110. A claim cannot be rendered obvious by a combination of references none of which discloses or suggests a feature of the claim. *See* Section V(F).

Docket No. 62239-BZA7/JPW/AC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : The Trustees of Columbia University in the City of New York

Inventors : Jingyue Ju et al.

Serial No.: 15/167,917 Examiner: Jezia Riley

Filed : May 27, 2016 Art Unit: 1637

For : MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA

30 Rockefeller Plaza
20th Floor
New York, New York 10112

BY EFS
Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132 OF JINGYUE JU, PH.D.

I, Jingyue Ju, Ph.D., declare as follows:

1. I am the first named inventor on the above-identified patent application.

2. I am a Professor of Chemical Engineering and Pharmacology and the Director of the Center for Genome Technology and Biomolecular Engineering at Columbia University in the City of New York ("Columbia").

3. Columbia is the owner (assignee) of rights in the above-identified patent application and has granted a license to the patent application and any patent issued from it to Qiagen.

4. I have been a professor at Columbia since 1999 and my research at Columbia has focused on developing new molecules and methods for DNA sequencing.

5. Prior to 1999, I worked at Incyte Genomics, Inc. and my work there focused on developing and improving DNA sequencing methods for the discovery and sequence identification of genes within the human genome.

Applicant: The Trustees of Columbia University in the City of New York
U.S. Serial No.: 16/149,098
Filed: October 1, 2018
Exhibit 1

6. A copy of my curriculum vitae is attached hereto as **Exhibit A**. As indicated therein, I am a named inventor on 39 issued U.S. patents and an author on 96 scientific publications.

7. Based on my extensive experience and expertise in the research and development of DNA sequencing and related technologies, including the design and synthesis of nucleotide analogues and the preparation and use of labeled nucleotide analogues, I am very well familiar with the level of education, knowledge and experience of persons working in these areas.

8. Based on legal advice concerning the meaning of the phrase a "person of ordinary skill in the art" or "POSA", I think I am well qualified to comment on the appropriate definition of a POSA in the field of DNA sequencing and on issues in this field from the perspective of a POSA as of October 6, 2000. In this regard, my opinion is that a POSA would have been a person with a Ph.D. degree in chemistry, chemical biology, or a related discipline and at least two years postdoctoral experience in the area of DNA sequencing, particularly the design and synthesis of nucleotide analogues for use in DNA sequencing.

9. This Declaration sets forth my opinions as to what a POSA's understanding would have been as of October 2000 concerning various issues raised by the Examiner with respect to the single claim pending in the above-identified patent application.

I. Indefiniteness

A. Meaning of "small"

10. I understand that the Examiner has raised a concern that the term "small", which appears in the claim, is indefinite and that a POSA would not have had an understanding of the meaning of the term in the context of the claim because the

specification does not provide a standard for assessing whether a 3'-O capping group R on the sugar of the claimed nucleotide analogue is "small".

11. In my opinion, a POSA reading the application, particularly paragraphs 6-8 of the published version of the application and Fig. 1 of the application including the brief description of Fig. 1 in paragraph 35, would readily have understood that the application indicates that the standard for assessing whether a 3'-O capping group R is "small" is the ability of the group to fit into the active site of the DNA polymerase shown in Fig. 1 of the patent application.

12. The POSA would further have understood that the 3D structure shown in Fig. 1 is the same as Fig. 6 previously published by Pelletier et al. (*Science*, Vol. 264, June 24, 1994, 1891-1903) and referred to in paragraph 6 of the published version of this patent application. A copy of Pelletier et al. is attached hereto as **Exhibit B.**

13. The POSA would also have known the precise coordinates of the polymerase structure based on the publication of Pelletier et al. (see "References and Notes" 101, page 1903) and the distances between the sugar of the nucleotide analogue and the key amino acids located in the active site of the polymerase (see Table 3, page 1897 of Pelletier et al.).

14. With the information in the patent application and the information available in Pelletier et al. and software available in October 2000, such as Chem3D Pro, the POSA could have readily calculated the space available around the 3' position of a deoxyribose of a nucleotide analogue in the active site of the polymerase.

15. I have performed an analysis of the space available within the active site of the polymerase to accommodate a 3'-O capped

dNTP and prepared a written summary of the result of this analysis attached hereto as **Exhibit C**. As explained in greater detail in the attached Analysis, the available space is approximately 3.7Å in diameter. Given this available space, the POSA would have understood that the term "small", in the context of the claimed nucleotide analogue, assessed by the standard provided in the patent application meant a 3'-O capping group with a diameter less than 3.7Å.

16. Using this standard the POSA could have readily determined that the two examples of 3'-O capping groups listed in the application, Allyl and Methoxymethyl, had diameters of 3.0Å and 2.1Å, respectively, and therefore were "small." (see attached Analysis, Exhibit C)

17. Using this standard, the POSA could also have readily determined which of the many known 3'-O capping groups were "small". Thus, the POSA would have determined that Methylthiomethyl and Azidomethyl, with diameters of 2.4Å and 2.1Å, respectively, were "small" while 2-Nitrobenzyl with a diameter of 5Å was not small (see attached Analysis, Exhibit C).

18. In summary, a POSA reading the above-identified application and relying on information publicly known as of October 2000 would have known that the standard for assessing whether any specific 3'-O capping group in a nucleotide analogue was "small" was whether it had a diameter less than 3.7Å so that it could fit into the active site of the polymerase. Therefore, the meaning of "small" would not have been indefinite.

B. Definition of R is clear

19. With the meaning of "small" well defined, the POSA looking at the structure shown in the pending claim and the definition of

R in the claim would have been able to readily know whether any given 3'-O capping group in a nucleotide analogue came within the scope of the claim. In this regard, prior art references as of October 2000, such as Tsien (WO 91/06678, May 16, 1991) and Stemple (WO 00/53805, September 14, 2000), identify numerous, chemically cleavable 3'-O capping groups which could be readily evaluated to determine whether they were "small" and also meet other conditions of the claim such as the structural features "OR is not a methoxy or an ester group" and R "does not contain a ketone group". Thus, the POSA would have understood with reasonable certainty the definition of R in the context of the patent application as a whole.

C. The scope of Y

20. Y is defined as a chemically cleavable, chemical linker and as shown in the structure shown in the pending claim, Y is attached by covalent bonds at one end to the base of a nucleotide analogue at a specific position and at the other end to a detectable fluorescent moiety. A POSA would have been familiar with many such chemical linkers from the prior art as of October 2000 including such linkers described by Tsien and Stemple. Therefore, a POSA would have readily understood the meaning of Y in the context of the pending claim as a whole read in light of the patent application.

D. Other functional characteristics

21. In the context of the pending claim as a whole read in light of the patent application, a POSA would have readily understood the meaning of the functional characteristics recited in the claim with reasonable certainty. To the POSA who had read the patent application, the scope of the structures encompassed by the claimed nucleotide analogue would have been clear and not indefinite.

II. Written Description

22. Contrary to the Examiner's assertion a POSA would have understood that the inventors of the claimed invention had possession of the invention for at least the following reasons:

a. Only a limited number of 3'-O capping groups meet the standard of "small" along with the other structural and functional features recited in the claim. I estimate the number of such groups would be less than 10 and 2 examples of such groups were provided.

b. The application contains a detailed description, including figures and examples, which shows that the invention was complete.

c. There is literal support in the application for the claim language.

d. The invention was ready for patenting as evidenced by the previous grant of patents from the same specification cited in the rejection for obviousness-type double patenting.

23. Based on the application, including the drawings and examples, a POSA would have immediately envisioned the scope of compounds encompassed by the specific chemical structure recited in the claim, most of which is fixed and with only a few variables which are well defined and not indefinite, for the reasons set forth above.

Stemple's 2-Nitrobenzyl Group And Obviousness

24. As discussed in paragraph 17 above and in the attached Analysis (Exhibit C), the 2-Nitrobenzyl 3'-O capping group referred to in Stemple has a diameter of 5Å and therefore is

not "small" by the standard provided in the patent application.

25. Without the insight into the importance of the 3'-O capping group being small, a POSA, reading Stemple's hypothetical examples and the Metzker 1994 paper (Metzker et al., *Nucleic Acids Research*, Vol. 22, October 11, 1994, 4259-4267) relied upon by Stemple on pages 13, 29 and 31, would have expected that a nucleotide analogue with a 2-Nitrobenzyl 3'-O capping group would be incorporated by a polymerase and therefore must fit in the active site of the polymerase. A copy of the Metzker 1994 paper is attached hereto as **Exhibit D**.

26. Therefore, from Stemple it would not have been obvious to a POSA that the 3'-O capping group must be smaller than a 2-Nitrobenzyl group for a nucleotide analogue containing the group to be incorporated by a polymerase. The subject patent application teaches for the first time that Stemple's prediction based on the Metzker 1994 paper that a nucleotide analogue with a 2-Nitrobenzyl 3'-O capping group would be incorporated by a polymerase was incorrect because the 2-Nitrobenzyl group is not "small". Subsequently Metzker, the senior author of Wu et al., (*Nucleic Acids Research*, Vol. 35, September 18, 2007, 6339-6349) acknowledged in 2007 that the compound actually made and reported in the Metzker 1994 paper was not, in fact, a 3'-O 2-Nitrobenzyl capped nucleotide analogue. Wu et al. further indicated that they had actually made the 3'-O 2-Nitrobenzyl capped nucleotide analogue described in the Metzker 1994 paper (but not, in fact, made in 1994) and had tested it for incorporation by polymerase. The result was that the compound was inactive in DNA polymerase incorporation assays. (see page 6339, lower right, and page 6340, left column, of Wu et al. attached hereto as **Exhibit E**.)

27. Thus, the insight into the critical importance of using a "small" 3'-O capping group for a nucleotide analogue where

"small" means that the 3'-O capping group must fit in the active site of the polymerase and has a diameter less than 3.7Å, would not have been appreciated by a POSA prior to having this patent application available. It would not have been obvious to a POSA in October 2000 from the prior art, such as Metzker and Stemple, which mistakenly teach that a nucleotide analogue with a larger 3'-O capping group, i.e. a 2-Nitrobenzyl group, could be incorporated by polymerase, that it was required to use a nucleotide analogue with a "small" 3'-O capping group R having the additional features recited in the sole pending claim.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Dated: 5/26/2017

Jingyue Ju, Ph.D.

RESEARCH ARTICLE

Structures of Ternary Complexes of Rat DNA Polymerase β, a DNA Template-Primer, and ddCTP*

Huguette Pelletier, Michael R. Sawaya, Amalendra Kumar, Samuel H. Wilson, Joseph Kraut

Two ternary complexes of rat DNA polymerase β (pol β), a DNA template-primer, and dideoxycytidine triphosphate (ddCTP) have been determined at 2.9 Å and 3.6 Å resolution, respectively. ddCTP is the triphosphate of dideoxycytidine (ddC), a nucleoside analog that targets the reverse transcriptase of human immunodeficiency virus (HIV) and is at present used to treat AIDS. Although crystals of the two complexes belong to different space groups, the structures are similar, suggesting that the polymerase-DNA-ddCTP interactions are not affected by crystal packing forces. In the pol β active site, the attacking 3′-OH of the elongating primer, the ddCTP phosphates, and two Mg^{2+} ions are all clustered around Asp^{190}, Asp^{192}, and Asp^{256}. Two of these residues, Asp^{190} and Asp^{256}, are present in the amino acid sequences of all polymerases so far studied and are also spatially similar in the four polymerases—the Klenow fragment of *Escherichia coli* DNA polymerase I, HIV-1 reverse transcriptase, T7 RNA polymerase, and rat DNA pol β—whose crystal structures are now known. A two-metal ion mechanism is described for the nucleotidyl transfer reaction and may apply to all polymerases. In the ternary complex structures analyzed, pol β binds to the DNA template-primer in a different manner from that recently proposed for other polymerase-DNA models.

DNA replication (*1*) is a highly complex biological process, even for a relatively simple organism such as *Escherichia coli*. During replication, the double helical DNA molecule is unwound, and the two resultant single strands of DNA act as templates to guide the synthesis, one complementary base at a time, of antiparallel primer strands. Although many auxiliary proteins such as ligases, helicases, and topoisomerases are usually involved, the chemical reaction at the core of DNA replication, the nucleotidyl transfer reaction, is catalyzed by DNA polymerases and may be depicted as follows:

$$\text{Template—primer}_n + \text{dNTP} \rightarrow \text{template-primer-dNMP}_{n+1} + \text{PP}_i$$

where dNTP (2′-deoxyribonucleoside 5′-triphosphate) represents any one of four deoxynucleotides (dATP, dGTP, dCTP, and dTTP), and dNMP and PP_i represent 2′-deoxyribonucleoside 5′-monophosphate and pyrophosphate, respectively (Fig. 1).

Inhibition of a polymerase that effects genomic replication can be fatal to an organism. In a common type of polymerase inhibition, 2′,3′-dideoxynucleotides (ddNTPs) act as chain terminators of the primer strand. The ddNTPs differ from their cellular dNTP counterparts by the absence of an attacking 3′-hydroxyl group (3′-OH) (Fig. 2) and therefore, once a dideoxynucleotide is successfully incorporated into a growing primer strand, there can be no further incorporation of subsequent nucleotides. A well-known example of this kind of inhibition involves HIV-1 reverse transcriptase (RT), which is the polymerase responsible for the replication of the HIV genome. 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxycytidine (ddC) are all anti-HIV drugs (*2, 3*) that become potent chain termination inhibitors of RT after they are converted by cellular kinases (*4, 5*), in vivo, to their corresponding nucleoside 5′-triphosphates, AZT-TP, ddATP (*6*), and ddCTP, respectively. In that all polymerases probably share a common catalytic mechanism, it is not surprising that some toxic effects of these drugs have been attributed to inhibition of host-cell polymerases, perhaps including the pol β described here (*7–9*). Therefore, a detailed understanding of the nucleotidyl transfer reaction, as well as the mechanism of inhibition of viral and host cell polymerases by nucleoside analogs, may lead to the design of more potent and less toxic HIV-1 RT inhibitors for use in the treatment of AIDS.

Despite limited sequence similarity to the Klenow fragment (KF) of *E. coli* DNA pol I [the only other polymerase for which a crystal structure (*10*) was known at the time], the crystal structure determinations of HIV-1 RT (*11, 12*) revealed a common polymerase fold consisting of three distinct subdomains (designated fingers, palm, and thumb because of the resemblance to a hand) forming an obvious DNA binding channel. The strongest structural overlap between KF and RT comprised a trio of carboxylic acid residues located in the palm subdomain (*11, 12*). These observations led to the hypothesis that perhaps all polymerases share a common nucleotidyl transfer mechanism centered around the highly conserved carboxylic acid residues (*11*). Strengthening this argument somewhat was the subsequent crystal structure determination of an RNA polymerase (RNAP) from bacteriophage T7, which showed strong structural similarities with KF (*13*). How-

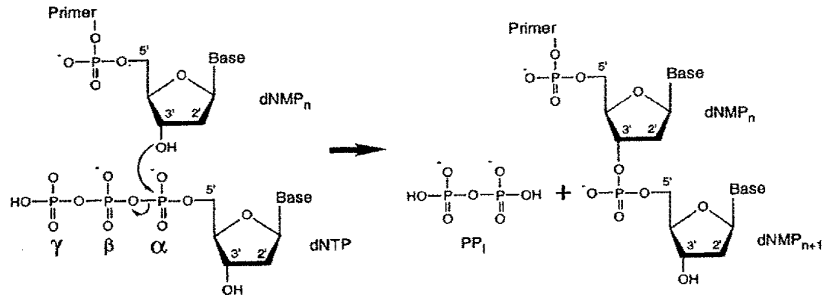


Fig. 1. The nucleotidyl transfer reaction. The 3′-OH group of the terminal dNMP on the primer strand attacks the 5′–α phosphate of an incoming dNTP, and a newly formed phosphodiester linkage results in elongation of the primer strand by one dNMP. After release of pyrophosphate (PP_i), the catalytic cycle is complete and the 3′-OH group of the newly incorporated dNMP is now ready to attack yet another incoming dNTP. Only the 3′ end of a primer is extended so that DNA polymerization is said to proceed in a 5′ to 3′ direction. If the polymerase molecule does not release the template-primer before incorporation of a second dNMP, the mode of DNA synthesis is said to be "processive", but if the polymerase releases the template-primer after each successive incorporation of a dNMP, the mode of DNA synthesis is said to be "distributive".

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ever, because structural evidence for a common nucleotidyl transfer mechanism has so far been limited to comparisons among polymerases from a bacterium (KF), a virus (RT), and a phage (RNAP), perhaps the most convincing evidence for this hypothesis is provided by the crystal structure determination of a eukaryotic polymerase, rat DNA pol β (*14*). Sequence alignments show that pol β is so distantly related, even from its eukaryotic relatives, polymerases α, γ, δ, and ε, that it stands in a class of its own along with only one other polymerase, terminal deoxynucleotidyltransferase (TdT) (*15*). The crystal structure of pol β nevertheless revealed a polymerase fold consisting of palm, fingers, and thumb (along with an additional 8-kD domain attached to the fingers), and the most striking structural similarity with its distant relatives, KF, RT, and RNAP, is a portion of the palm that bears the highly conserved carboxylic acid residues (*14*). This suggests that despite the large differences in size (pol β, at 39 kD, is the smallest polymerase known), in function [although pol β may play a role in DNA replication (*16, 17*), its primary function is in DNA repair (*18–20*)], and in fidelity [pol β is the most error prone eukaryotic polymerase studied to date (*21, 22*)], pol β probably shares a common nucleotidyl transfer catalytic mechanism with all other polymerases.

Taking advantage of the chain termination method of polymerase inhibition with ddNTPs, we have succeeded in growing crystals of rat pol β complexed with two pseudo substrates, namely, (i) a DNA template-primer in which the 3′ end of the primer has been "terminated" by ddCMP, and (ii) ddCTP. In preparation for crystallization experiments, we mixed pol β with the DNA template-primer shown below and a large excess of ddCTP on the assumption that, prior to crystallization, the following reaction would occur:

```
3′GCCGCGGGAAA5′
5′CGGCGC      3′ + ddCTP →

    3′GCCGCGGGAAA5′
    5′CGGCGCC_dd   3′ + PP_i
```

where C_{dd} is the newly incorporated ddCMP. If pol β then were to try to incorporate another nucleotide onto the primer terminus, a second nucleotidyl transfer reaction could not occur because the recently incorporated ddCMP lacks a 3′-OH group. This should result in a pseudo Michaelis-Menten ternary complex in which both "substrates" are present, namely, a nonreactive template-primer and a nucleoside triphosphate. Crystals were obtained, and the subsequent structure determinations revealed that this must have been what happened. Electron density maps showed a primer strand that was seven nucleotides long, although we started with a primer that was only six nucleotides in length, and the 3′ (deoxy) terminus of the primer was positioned next to strong electron density resembling a nucleoside triphosphate, presumably ddCTP.

Such a detailed view of the active site in the ternary complex allows us to propose a two-metal ion mechanism for the nucleotidyl transfer reaction that is similar, in many ways, to the two-metal ion mechanism previously proposed for another type of phosphoryl transfer reaction—the exonuclease reaction of the 3′→5′ exonuclease of *E. coli* DNA pol I (*23, 24*). Our proposed nucleotidyl transfer mechanism probably applies to all polymerases, but when we attempt to extend that mechanism to the other three polymerases—KF, RT, and RNAP—for which the crystal structures are known, a problem arises: in our structures, pol β is bound to the DNA in a manner that differs from the recently proposed polymerase-DNA models for all three of these polymerases.

Crystallizations and preliminary diffraction studies. Recombinant rat DNA pol β (*25*) was expressed in *E. coli* and purified as described (*26*). After purification, the protein was washed three times in a microconcentrator (Centricon-10, Amicon) with a buffer solution (10 mM ammonium sulfate, 0.1 M tris, pH 7.0), then concentrated to 20 mg/ml and stored at −80°C in sealed Eppendorf tubes (120-μl portions). Prior to crystallization, a protein-DNA-ddCTP sample was prepared at room temperature; approximately 1.2 mg of the 11-nucleotide (nt) template and 0.8 mg of the 6-nt primer (*27*) were dissolved in 240 μl of a buffer solution (20 mM $MgCl_2$, 0.1 M MES, pH 6.5) and the mixture was left in a sealed Eppendorf tube for 1 hour to allow annealing of the template-primer (*28*). Two portions of pol β (240-μl of a solution containing 20 mg/ml) were then thawed and added, and the protein-template-primer sample was allowed to stand for an additional hour. A 4-μmol sample of ddCTP (in 40 μl of H_2O) (*29*) was the last component to be added, resulting in a reaction mixture containing pol β at approximately 10 mg/ml, 10 mM $MgCl_2$, and an excess of template:primer:ddCTP in molar ratios of 3:4:30, respectively, relative to the amount of protein. The reaction, nucleotidyl transfer of ddCMP to the primer 3′ terminus, was allowed to proceed for 2 hours before crystallization trays were set up (*30*).

Two different crystal forms were observed, depending on the concentration of lithium sulfate in the reservoir solution. One crystal form, obtained with lithium sulfate concentrations from 40 to 75 mM, was hexagonal and grew to dimensions of 0.8 by 0.8 by 0.6 mm in about 2 weeks. These crystals belong to space group $P6_1$ ($a = b = 94.9$, $c = 117.6$ Å), with one complex molecule per asymmetric unit. Under similar conditions, but at lithium sulfate concentrations from 75 to 150 mM, platelike crystals grew to dimensions of

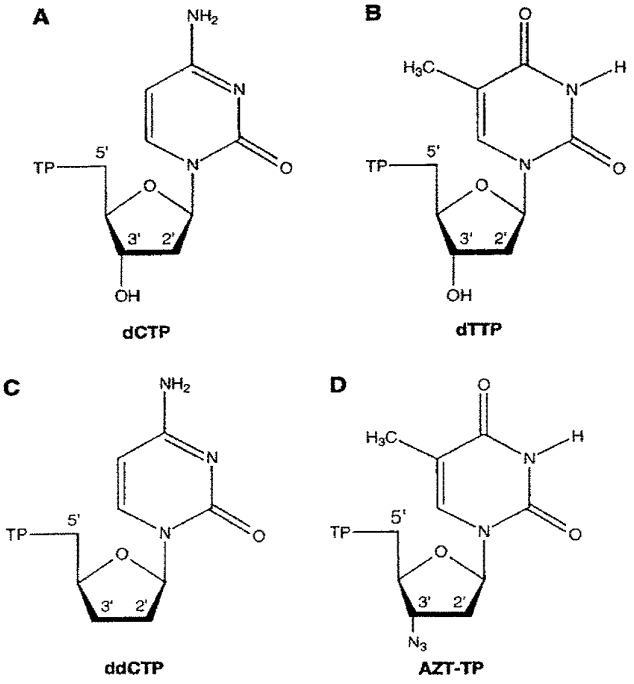

Fig. 2. Two normal cellular nucleotides, (**A**) 2′-deoxycytidine 5′-triphosphate (dCTP) and (**B**) 2′-deoxythymidine 5′-triphosphate (dTTP), and their anti-HIV (drug) counterparts (**C**) 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) and (**D**) 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate (AZT-TP). The triphosphate moiety, which is linked via a phosphoester bond to the 5′ carbon of the ribose, is designated TP.

1.0 by 0.6 by 0.2 mm in a few days. These crystals belong to space group $P2_1$ (a = 106.3, b = 56.8, c = 86.7 Å, and β = 106.4°) and there are two complex molecules per asymmetric unit. Often both crystal forms grew in the same drop, and the crystals on which data were collected (Table 1) were grown at the same concentration of lithium sulfate, 75 mM. The unusually large excess of ddCTP (1:30 molar ratio) was required in order to obtain the $P6_1$ crystals, but the $P2_1$ crystals could be grown under much lower ddCTP excesses (1:10 molar ratio). Extreme purity of all components in the crystallization medium, particularly the DNA samples (*27*), seemed to be an absolute requirement for growing both types of ternary complex crystals.

Attempts were made to obtain ternary complex crystals of rat pol β, a DNA template-primer, and AZT-TP (Fig. 2D) (*31*) under similar conditions, even though incorporation of AZT-MP would result in a mismatched base pair (of a G-T type) at the primer terminus (*22*). Orthorhombic crystals grew in space group $P2_12_12$ (a = 188.4, b = 67.7, c = 39.1 Å) with one pol β molecule in the asymmetric unit. A 4.0 Å data set was collected and preliminary structural studies (*32*) showed that, because of crystal packing, it was not possible for the template-primer to occupy the pol β binding channel. Failure of pol β to form a tight complex with the DNA template-primer under these conditions might be attributed to steric hindrance by the azido group of a newly incorporated AZT-MP on the primer terminus.

Efforts to obtain a binary complex of pol β and a DNA template-primer alone (neither ddCTP nor AZT-TP) resulted in crystals that grew under much different conditions, but were nonetheless isomorphous with the $P2_12_12$ (AZT-TP) crystals mentioned above. Failure of pol β to bind to the DNA in this case could be due to the higher salt concentration of the crystallization medium (about 250 mM salt compared to 75 mM). Because one crystallization medium contained AZT-TP and the other did not, we calculated $F_{O(AZT\text{-}TP)} - F_{O(apo)}$, α_c, difference Fourier maps to see whether an AZT-TP binding site could be located. Strong electron density was observed in an area of the map adjacent to Arg^{149}, which is near the catalytically important residues, Asp^{190}, Asp^{192}, and Asp^{256}. This pol β–AZT-TP binary complex which, as discussed below, is probably not catalytically relevant, is similar to a KF-dNTP binary complex in which the dNTP bound to Arg residues near the catalytically important carboxylic acid residues of KF (*33*).

Human pol β, which has been cloned and expressed (*34, 35*) in a manner similar to that of rat pol β, shares more than 95 percent sequence similarity with rat pol β, so it was somewhat surprising when attempts to obtain ternary complex crystals of human pol β, a DNA template-primer, and ddCTP under the same conditions described above for the rat enzyme resulted in crystals that grew in two previously unobserved orthorhombic crystal forms. One form has unit cell parameters a = 158, b = 108, c = 60 Å, with probably two complex molecules in the asymmetric unit, but the crystals diffract only to about 5 Å resolution. In contrast, the second crystal form diffracts quite well (to about 3.3 Å), but its unit cell parameters of a = 465, b = 168, c = 56 Å are so large that special data collection techniques would be required.

Structure determination and refinement. Data collection and refinement statistics for the structure determinations of the two ternary complexes of rat pol β, a DNA template-primer, and ddCTP are listed in Table 1. Structure solutions utilized the refined atomic coordinates of the high resolution (2.3 Å) structure of the 31-kD domain of rat pol β (*14*). The molecular replacement programs of XPLOR (*36*) gave clear rotation solutions for the 31-kD domain of both ternary complexes, but only after results from classical cross-rotation searches had been filtered through the Patterson-correlation (PC) refinement steps (*37*). PC refinement techniques were particularly powerful for our structure determinations because independent rigid body movements of the fingers, palm, and thumb subdomains of the 31-kD domain could be allowed during PC refinement of the rotation searches. Results from subsequent translation searches gave solutions for the $P6_1$ complex structure that were, in general, at higher peak height to background ratios than translation solutions for the $P2_1$ complex structure, but the highest translation peaks in both cases nevertheless were the correct solutions (*38*).

The 31-kD partial structure solutions obtained by molecular replacement techniques were subjected to rigid body refinement by XPLOR (*36*), where the entire 31-kD domain was first allowed to move as a rigid body, then later, the fingers, palm, and thumb subdomains were allowed to move as independent rigid bodies simultaneously. Typical R factors at this stage were about 50 percent. After subsequent positional refinement with the least squares program package TNT (*39*) had lowered the R factors of the partial solutions to about 45 percent, we calculated $F_o - F_c$, α_c, difference Fourier maps that revealed clear electron density for many of the backbone phosphates of a double-stranded DNA molecule as well as the three phosphates of a ddCTP nucleotide, and even portions of the 8-kD domain were evident at this early stage. Cycles of model building and least squares refinement improved the electron density for the rest of the DNA as well as the 8-kD domain for both complex structures, and once the R factors had dropped below 30 percent, refinement of individual isotropic temperature factors also improved the maps and facilitated refinement.

Although we were unable to discern the DNA base sequences at these resolutions, the directionality (5′ → 3′) of the DNA strands was evident early in our modeling efforts, hence we knew that the 3′ terminus of either the template strand or the primer strand was positioned at the pol β active site. What ultimately distinguished the template from the primer was that we were

Table 1. Data collection and refinement statistics. X-ray diffraction data were collected on a multiwire area detector (*98*) (San Diego Multiwire Systems) with monochromatized CuKα radiation (Rigaku rotating anode x-ray generator), and intensity observations for each data set were processed with a local UCSD Data Collection Facility software package (*99*). Reflections from 20 Å to the maximum resolution were included in all least squares refinement steps. The final structures for both complexes include all residues, with the exception of residues 1 to 8 of the disordered NH_2-terminus and residues 246 to 248 of a disordered surface loop. There are a few missing side chain atoms in both coordinate sets that are mainly in lysine and arginine residues of the 8-kD domain. Omit maps were used to confirm the modeling of the DNA template-primer, the ddCTP nucleotide, and the cis-peptide bond between Gly^{274} and Ser^{275}.

Data collection							Refinement			
Space group	d_{min} (Å)	$\frac{I}{\sigma}$*	Reflections		Completeness (%)	R_{sym}†	Atoms‡	rms deviation§		Final R‖
			Total	Unique				Bond (Å)	Angle (°)	
$P6_1$	2.9	1.8	53,583	13,281	99	0.087	2914	0.020	2.9	0.193
$P2_1$	3.6	1.8	25,046	10,650	96	0.059	5753	0.018	2.9	0.199

*Average ratio of observed intensity to background in the highest resolution shell of reflections. †$R_{sym} = \Sigma|I_{obs} - I_{avg}|/\Sigma I_{avg}$. ‡The number of nonhydrogen atoms includes 31 and 4 water oxygens for the $P6_1$ and the $P2_1$ structures, respectively. §The rms bond and rms angle values are the deviations from ideal values of the bond lengths and bond angles in the final model. ‖Final $R = \Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$, including all data between 20 Å and the maximum resolution.

able to model in seven nucleotides for one of the DNA strands and at least 8 nt for the second DNA strand. Provided that no unexpected side reactions had occurred during crystallization, we knew that the primer could be no longer than 7 nt, and therefore the DNA strand containing 8 nt was designated the template. We then concluded that the first three bases of the template (AAA) are disordered in both crystal structures. This interpretation of the data is in agreement with the idea that the 3′ terminus of the primer should be positioned at the polymerase active site. Analysis of the DNA in our refined structures with the program CURVES II (*40*) indicated that the DNA is predominantly B form. Our DNA may have some A-DNA characteristics, however, in that the minor groove width appears to increase as the DNA approaches the pol β active site; the section of the double-stranded DNA that is removed from the active site and protrudes into solution is characteristic of B-DNA with a minor groove width of 11 Å, whereas nearer to the active site, the minor groove width is almost 15 Å (typical A-DNA has a minor groove width of about 17 Å).

Description of the structures. When the pol β ternary complex structures are compared with the structure of the apo enzyme (Fig. 3), the most apparent differences consist of large movements of the 8-kD NH_2-terminal domain relative to the fingers, palm, and thumb of 31-kD COOH-terminal domain. The 8-kD domain is tethered to a proteolytically sensitive hinge region (residues 80–90) and changes from an open conformation in the apo structure to more closed conformations in the complex structures. Because of the precarious position of the 8-kD domain in the pol β apo structure, this type of conformational change seemed inevitable even before the structures of the ternary complexes were determined. The only other significant conformational changes on complex formation were noticeable rigid-body movements of the thumb and, to a lesser degree, the fingers, resulting in a somewhat more tightly closed hand in the ternary com- plexes. A greater degree of flexibility on the part of the thumb subdomain has also been observed in other polymerase-DNA structures (*12, 23, 24, 41*). A least squares superposition of the 31-kD domain of the apo structure (*14*) on the 31-kD domain of one of the ternary complex structures ($P6_1$) resulted in a root-mean-square (rms) deviation in α carbon positions of 2.5 Å, whereas when the fingers, palm, and thumb subdomains were treated separately, the rms deviations in α carbon positions were only 0.71, 0.69, and 0.82 Å, respectively.

The 8-kD domain has a net charge of +10 (assuming neutral histidines) and binds to single stranded DNA with an association constant of $2 \times 10^5\ M^{-1}$ (*26*). It has no obvious structural equivalent in any of the other polymerases for which crystal structures have been determined, and crosslinking studies with gapped DNA substrates (*42*) suggest that the 8-kD domain is probably responsible for the highly

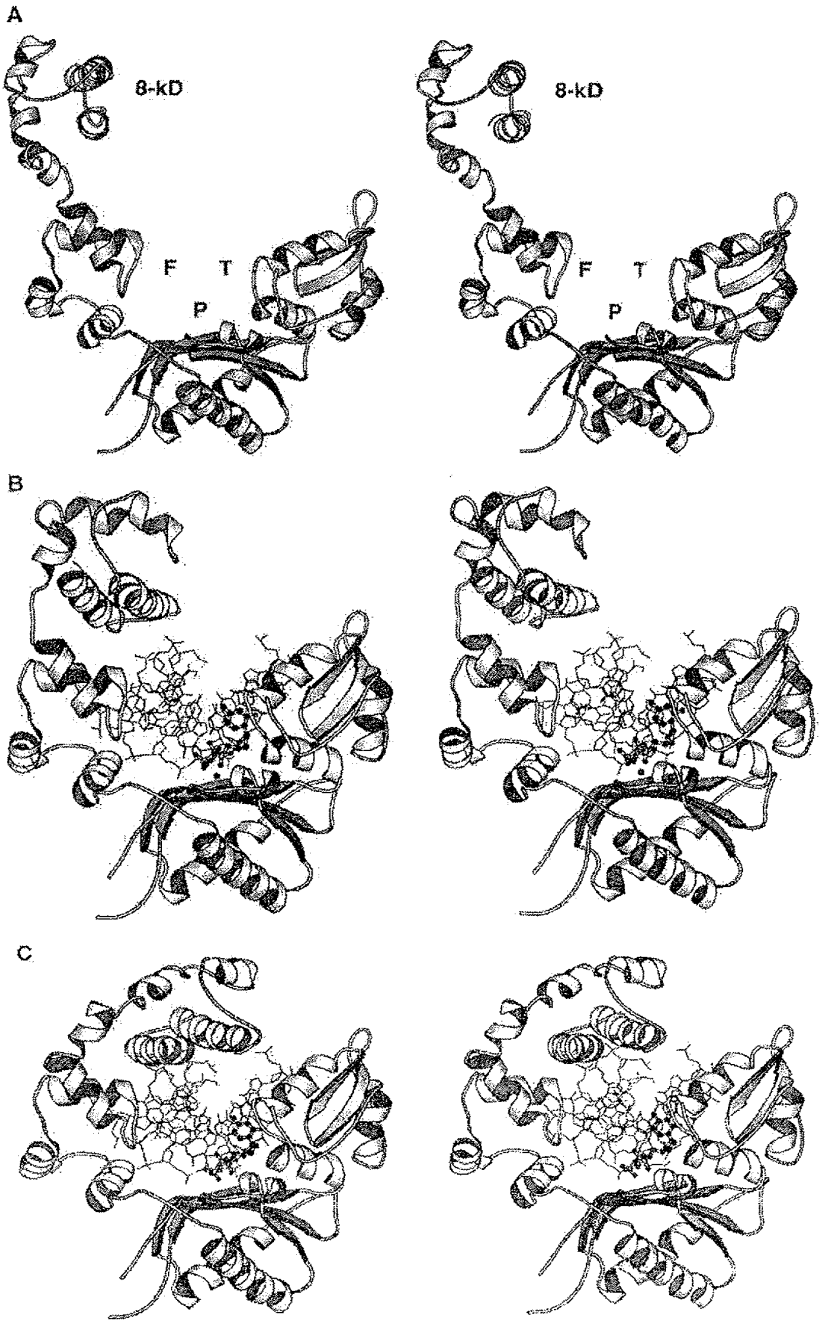

Fig. 3. Stereoview ribbon diagrams (*100*) of (**A**) rat DNA pol β, apo structure (*14*) and (**B** and **C**) ternary complexes of rat DNA pol β with a DNA template-primer and ddCTP in space groups $P6_1$ and $P2_1$, respectively. In (A) the 8-kD domain is designated 8-kD, and the fingers, palm, and thumb subdomains of the 31-kD domain are represented by F, P, and T, respectively. A ball-and-stick representation highlights the ddCTP nucleotide in (B and C). In (B) the positions of the two Mg^{2+} ions are marked with black spheres. These metals ions are not shown in (C) because we were unable to see the Mg^{2+} ions in electron density maps of the lower resolution *P2*, ternary complex structure.

processive short-gap filling activities found exclusively in pol β (*43*). It has been proposed that the 31-kD domain binds to the double-stranded segment of the template-primer, and the 8-kD domain binds to the single-stranded template overhang (*44*)—or in the case of binding to a short gap in the DNA, to the 5′-phosphate of the downstream oligonucleotide (*42*). We see some evidence of this in that the 31-kD domain clearly uses its palm, fingers, and thumb to grasp the double-stranded segment of the template-primer while the 8-kD domain, although positioned quite differently in the two complex structures, is nevertheless close to where an extended template would be. Unfortunately, our template overhang was probably a little too short (only four bases—GAAA) to interact strongly with the 8-kD domain, causing the first three bases of the template to be disordered in both crystal structures. It is possible that, because the highly flexible 8-kD domain had no template on which to anchor in our crystallization experiments, its position was determined almost entirely by crystal packing forces, and probably neither of the two conformations of the 8-kD domain seen in Fig. 3, B and C, is correct for template binding in vivo. Nevertheless, kinetic studies of the 31-kD fragment alone showed that pol β can still function as a polymerase without the 8-kD domain, albeit at only about 5 percent of its normal activity (*44*).

Table 2. Hydrogen bond interactions of 3.3 Å or less between pol β and the DNA template-primer.

Residue	Subdomain	Atom	Base*	Atom	Distance (Å)
		Protein to DNA phosphate H bonds			
Gly^{105}	Fingers	N	P-6C	O2P	2.9
Gly^{107}	Fingers	N	P-5G	O2P	2.7
Ser^{109}	Fingers	N	P-5G	O1P	2.9
Ala^{110}	Fingers	N	P-5G	O2P	3.1
Arg^{254}	Fingers	NH2	P-7C	O2P	2.7
Lys^{230}	Palm	N	T-9C	O2P	3.0
Thr^{233}	Palm	N	T-8G	O2P	3.1
Lys^{234}	Palm	N	T-8G	O2P	2.7
Thr^{292}	Thumb	OG1	T-5G	O2P	2.7
Tyr^{296}	Thumb	OH	T-5C	O2P	2.6
		Protein to DNA base H bonds			
Lys^{234}	Palm	NZ	T-7C	O2	2.9
Tyr^{271}	Thumb	OH	P-7C	O2	2.7
Arg^{283}	Thumb	NH1	T-4G	N3	3.2

*DNA bases are designated T or P to distinguish template bases from primer bases, respectively. Starting from the 5′ terminus of each strand, bases are numbered 1 to 11 for the template and are numbered 1 to 7 for the primer. C and G represent cytosine and guanine, respectively, and atom designations follow Protein Data Bank nomenclature.

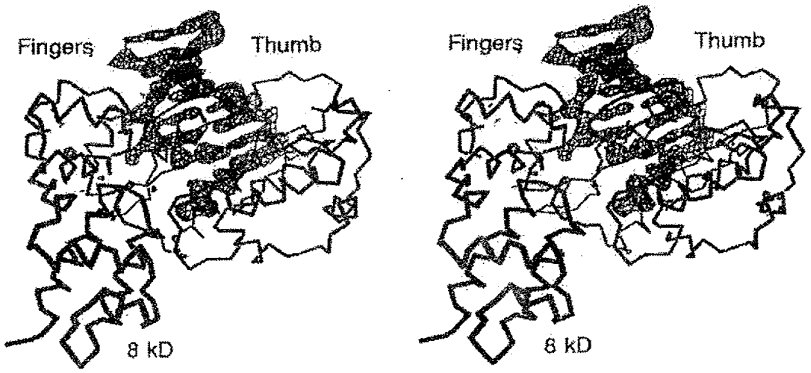

Fig. 4. Omit map of the DNA template-primer and ddCTP overlayed on an α carbon diagram of the refined *P6*, pol β structure. The view is that of Fig. 3 rotated by 90° about a horizontal axis in the plane of the page so that the DNA binding channel is now vertical. The template-primer sits on top of the palm subdomain, which is not labeled. Before all omit maps were calculated, the part of the structure in question was deleted from the coordinate file and the remaining partial structure was subjected to 200 cycles of least squares positional refinement in XPLOR (*36*) in order to remove bias from the phases.

In contrast to the 8-kD domain, the rest of the structure (the fingers, palm, and thumb of 31-kD domain, as well as the template-primer and ddCTP substrate) is virtually identical in both crystal forms of the ternary complex. This provides support for the physiological relevance of our complex crystals, at least with respect to the polymerase-DNA-ddCTP interactions. Also strengthening the argument is that, unlike other reported crystals of polymerase-DNA complexes (*12*, *41*), our crystals were grown at low, near physiological salt concentrations. Finally, the fact that that a ddCMP was incorporated into our template-primer shows that the nucleotidyl transfer reaction did proceed, at least for one turnover, in the same medium from which crystals were eventually obtained. In that the following discussions do not apply to the 8-kD domain of pol β and will be limited mostly to the 31-kD domain's interactions with DNA and ddCTP, we will henceforth refer only to the complex structure that has been refined to the highest resolution, the $P6_1$ crystal structure.

The DNA binding channel in pol β, just as in KF, RT, and RNAP, is lined with positively charged lysine and arginine side chains, and it has always been a reasonable assumption that their function is to stabilize the negatively charged backbone phosphates of the DNA (*45*). Therefore it was quite surprising that, except for Arg^{254}, which is hydrogen bonded to the phosphate of the newly incorporated ddCMP of the primer strand, there are no direct lysine or arginine interactions with the backbone phosphates of the DNA in our complex (Table 2). Instead, nearly all of the interactions of protein with DNA involve two different clusters of protein backbone nitrogens located at the entrance to the DNA binding channel (Table 2). One cluster, consisting of four of the backbone nitrogens between Gly^{105} and Ala^{110}, is located at the NH_2-terminal end of helix G in the fingers subdomain of pol β and interacts with the phosphates of the primer strand. The second cluster, comprising three of the five backbone nitrogens between Lys^{230} and Lys^{234}, is located in a beta turn, which connects beta strands 3 and 4 of the palm subdomain and interacts with backbone phosphates of the template strand. The only other hydrogen bonded interactions (3.3 Å or less) between pol β and DNA phosphates are between the side chains of Thr^{292} and Tyr^{296}, located on a loop between beta strands 6 and 7 of the thumb subdomain, and the backbone phosphates of the template strand (Table 2).

In addition to our observations that there seemed to be fewer hydrogen bond interactions between pol β and DNA than expected (Table 2), we were also initially

surprised to see that the DNA sits in the binding channel at a slight angle and appears to "run into" alpha helices M and N of the thumb subdomain (Fig. 4). It is possible that the angle between the DNA axis and the apparent axis of the pol β binding channel would change considerably if a longer template were used and, as proposed above, the 8-kD domain participated in the positioning of the template-primer. However, the aesthetically pleasing observation that the base pairs of the DNA are parallel to the beta strands of the palm subdomain (Fig. 5) encourages us to believe that interactions of pol β with the double-stranded segment of any DNA template-primer will not vary much from what is seen in the present ternary complex structures.

Perhaps one of the most unvarying characteristics of B-DNA is that it has a spine of well-ordered water molecules which interacts with the O2 of pyrimidines and the N3's of purines in the minor groove, and it has been proposed that the disruption of this particular water structure is the first step in the B-DNA to A-DNA transition (*46*, *47*). In our complex structure, only three protein side chains come within 3.3 Å of the DNA bases, and they are all located in the shallow minor groove of the template-primer (Table 2). Two of these (Lys^{234} and Tyr^{271}) are hydrogen bonded to the O2 of a template cytidine and the O2 of a primer cytidine, respectively, while another (Arg^{283}) is hydrogen bonded to the N3 of a template guanine. This leads us to propose that perhaps Lys^{234}, Tyr^{271}, and Arg^{283} all function to break up the water structure in the minor groove of the template-primer upon complex formation, resulting in a larger minor groove width, characteristic of A-DNA, at the pol β active site.

Unlike transcription factors and other gene regulatory DNA-binding proteins, polymerases must bind to DNA with little regard for sequence specificity. This is evident from Table 2 where, as discussed above, most of the protein to DNA interactions are nonsequence-specific hydrogen bonds between pol β backbone nitrogens and DNA phosphate oxygens. Even what appear to be sequence-specific interactions between protein side chains and DNA bases, upon closer inspection turn out to be rather nonspecific in that each of the three protein side chains mentioned above—Lys^{234}, Tyr^{271}, and Arg^{283}—that are in contact with DNA bases can act as an unbiased hydrogen bond donor to either the O2 of pyrimidines or the N3 of purines in the minor groove.

Description of the active site. Much of the ddCTP binding pocket in the ternary complex is made up of the 3′ terminus of the primer strand (Fig. 6A, toward the left) and the template overhang (at the top), where the base pairing of ddCTP with a complementary base, guanine, is evident in the crystal structure (Fig. 6A and Table 3). The idea that the template-primer makes up a large part of the binding site in pol β for the incoming ddCTP is consistent with kinetic studies showing that there is a strict kinetic order of binding for the substrates in nucleotidyl transfer reactions catalyzed by pol β (*48*), *E. coli* pol I (*49*), and RT (*50*), the polymerase binds to the template-primer first, and then nucleotide binding takes place. Supporting this view, structural studies of a pol β–dATP binary complex (*14*) and of a KF-dATP binary complex (*33*), both of which were crystallized in the absence of a template-primer, revealed nucleotide binding sites that differed somewhat from that seen in our ternary complex.

Making up most of the right side of the nucleotide binding pocket and interacting primarily with the base and ribose moieties of the ddCTP is a structural motif consisting of a sharp kink, made possible by a cis-peptide bond, between α helices M and N in the thumb subdomain of pol β (Fig. 6A). This was the only cis-peptide found in the pol β apo structure (between Gly^{274} and Ser^{275}) (*14*) and even though it appears to have remained as a cis-peptide upon complex formation with DNA, its proximity to the active site nevertheless suggests that it may play a dynamic role in catalysis. For instance, it is evident that a cis- to trans-peptide bond transition, perhaps occurring during or just after the incorporation of a nucleotide, could result in a large displacement of one or both helices (M and N), which in turn could push the enzyme off the template-primer. Thus, we propose that the cis-peptide in pol β may function to facilitate the product-off step of catalysis, which is typically the steady-state rate-limiting step for polymerases when in a distributive mode of synthesis (*21*, *51*, *52*). Another possibility is that the cis-peptide bond, which links the active site to the template overhang via helix N (Fig. 6A), functions only to facilitate the translation of the enzyme along the template during processive polymerization.

It is quite appropriate that the protein side chain that interacts specifically with the base of ddCTP, Asn^{279} of helix N (Table 3), is unbiased toward all four possible incoming nucleotides in that it can act either as a hydrogen bond acceptor or a hydrogen bond donor. The only other interactions (of 4 Å or less) between pol β and the base moiety of ddCTP are nonspecific van der Waals contacts between the side chain carbon (CB) of Asp^{276} and ring carbon atoms (C4 and C5) of the cytidine base (Table 3).

Pol β interacts intimately with the sugar moiety of ddCTP as is evident from the close van der Waals contacts between the protein backbone atoms of Tyr^{271}, Phe^{272}, and Gly^{274} (*53*), and the ribose ring carbons, C2′ and C3′, of ddCTP (Fig. 6A and Table 3). Because the only difference between a ribonucleotide and a deoxyribonucleotide is a hydroxyl at the C2′ of the ribose ring, the protein backbone segment Tyr^{271}-Gly^{274} may participate in nucleotide selectivity of DNA over RNA for pol β. For example, a DNA polymerase can be converted to a relatively efficient RNA polymerase by changing the metal ion in the reaction medium from Mg^{2+} to Mn^{2+} (*54*). The two metal ions in the pol β active site together bind to all three phosphates (α, β, and γ) of ddCTP (Table 3), and therefore the metal ions probably participate to some extent in the positioning of the incoming nucleotide. As seen in our structure, only a slight change in ddCTP orientation, which could be induced by a change from Mg^{2+} to Mn^{2+} ions in the active site, would be required to reduce steric hindrance at the C2′ ribose of ddCTP, possibly making

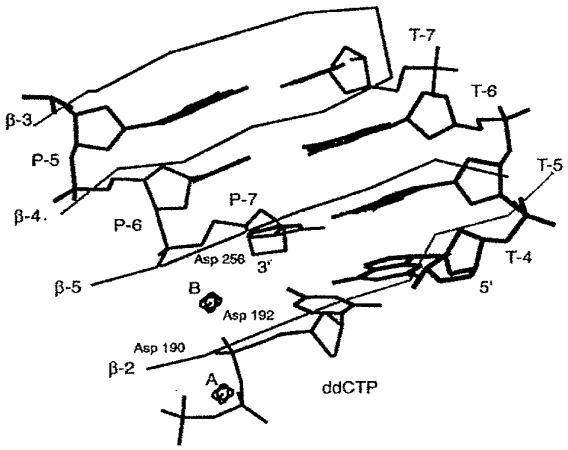

Fig. 5. Alignment of the template-primer base pairs with the beta strands of the palm subdomain. The view is the same as that in Fig. 4. Beta strands are labeled β-2 through β-5. The α carbon positions of the catalytically important residues, Asp^{190}, Asp^{192}, and Asp^{256}, are shown, as well as the nucleotide substrate, ddCTP. The two Mg^{2+} ions in metal sites A and B are labeled A and B, respectively. Base designations defined in Table 2.

RNA just as good a substrate as DNA.

Another example of nucleotide selectivity, this time dTTP over AZT-TP, might also be explained by steric hindrance in the pol β active site, particularly at the ribose C3′. The cellular nucleotide dTTP, which has a hydroxyl group attached to its ribose C3′ (Fig. 2B), binds more tightly to a pol β-DNA complex than does its analog AZT-TP, which has a bulky azido group at C3′ (Fig. 2D) (*55*). Such observations might explain why a drug like AZT specifically targets the HIV-1 RT and not host cell polymerases like pol β; in contrast to pol β, RT shows no selectivity in binding dTTP compared to AZT-TP during reverse transcription (*56*), and thus, perhaps RT lacks the structural equivalent of the pol β "selective" Tyr^{271} to Gly^{274} backbone segment, making RT more susceptible to AZT-TP inhibition.

Three of the six hydrogen bonds between the protein and the negatively charged phosphate moiety of ddCTP involve nitrogen backbone atoms of pol β (Table 3). Although this is reminiscent of the nitrogen clusters mentioned above that help to stabilize the template and primer phosphates in the DNA binding channel, the geometry of the nitrogen backbones differ in that they more closely resemble the mononucleotide binding motifs found in other enzymes. This and other aspects of the interactions between protein and ddCTP phosphates in the pol β active site are discussed in (*14*).

The strongest interactions with ddCTP in the pol β active site, however, are not with protein side chains directly, but rather with two Mg^{2+} ions that in turn coordinate the side chain oxygens of Asp^{190}, Asp^{192}, and Asp^{256} (Fig. 6 and Table 3). These three carboxylic acids are present in all DNA polymerases for which amino acid sequences are known (*15*), and mutagenesis studies show that they are critical for catalysis in pol β (*57*), KF (*58, 59*), and RT (*60*). However, we avoid referring to these three aspartic acids as a "catalytic triad" because only two of the three (Asp^{190} and Asp^{256}) are present in all known RNA polymerase amino acid sequences (*15*). As discussed above, converting a DNA polymerase to a relatively efficient RNA polymerase can be as simple as changing the metal ions in the active site from Mg^{2+} to Mn^{2+} (*54*), and consequently there is not much reason to suppose that the RNA polymerases utilize a nucleotidyl transfer mechanism that differs drastically from the mechanism proposed below for a DNA polymerase such as pol β. In support of this view, mutagenesis experiments that targeted catalytically important residues in KF (*59*) showed that Asp^{882} and Asp^{705} (the equivalents of Asp^{190} and Asp^{256} in pol β) are much more critical to catalysis than the third carboxylic acid of the trio, Glu^{883} (equivalent to Asp^{192} in pol β), which is not conserved in the RNA polymerases. In agreement with these observations and as discussed below, the primary function of Asp^{256} in the pol β active site appears to aid in stabilizing the pentacoordinated α phosphate of the transition state, whereas the primary functions of Asp^{190} and Asp^{192} are to aid in positioning the nucleotide substrate. This suggests that pol β could quite possibly still function in the absence of one of the two latter carboxylic acids, such as Asp^{192}, which would be the case for an RNA polymerase. Perhaps also significant is the observation that the metal-to-oxygen bonds are longer for Asp^{192} than they are for Asp^{190} in the pol β active site (Table 3).

Table 3. Interatomic distances of interest for the pol β active site.

ddCTP moiety	Atom	Base* or residue	Atom	Distance (Å)
		Contacts between ddCTP and DNA or protein		
Base	N4	T-4 G	O6	3.0
	N3	T-4 G	N1	2.7
	O2	T-4 G	N2	2.7
	C4	Asp^{276}	CB	3.5
	C5	Asp^{276}	CB	3.7
	O2	Asn^{279}	ND2	3.0
Ribose	C2′	Tyr^{271}	O	3.5
	C3′	Phe^{272}	O	3.2
	C2′	Gly^{274}	CA	3.2
α Phosphate	PA	P-7 C	O3′	4.3†
β Phosphate	O1B	Ser^{180}	N	3.0
	O1B	Arg^{183}	NH2	2.7
	O3B	Ser^{180}	OG	2.8
γ Phosphate	O1G	Gly^{189}	N	2.8
	O1G	Asp^{190}	N	3.0
	O2G	Arg^{149}	NH2	2.5
		Ligand	Atom	Distance (Å)
Mg^{2+} site A		Asp^{190}	OD1	2.0
		Asp^{192}	OD2	2.7
		ddCTP, β phosphate	OD2	1.7
		ddCTP, γ phosphate	O1G	2.3
		Water	O	2.6
Mg^{2+} site B		Asp^{190}	OD2	2.0
		Asp^{192}	OD1	2.6
		Asp^{256}	OD1	3.0
		ddCTP, α phosphate	O1A	2.7
		P-7 C	O3′	2.9†
		Water	O	—‡

*Base designations are in Table 2. †These distances were obtained with a O3′ atom that had to be modeled into the active site because the newly incorporated ddCMP primer terminus lacks this group. ‡This water is not seen in our crystal structures, but because the Mg^{2+} ion in site B has octahedral geometry, we propose that a water might occupy this empty sixth-ligand position.

Mechanism of nucleotidyl transfer. In 1979, with results from kinetic experiments on *E. coli* pol I that utilized phosphorothioate dATP analogs, Burgers and Eckstein proposed that the pol I catalyzed nucleotidyl transfer reaction had the following properties (*61*): (i) A divalent metal ion (Mg^{2+}) is bound specifically to the β and γ phosphates of the nucleotide (dATP) as a β,γ-bidentate; (ii) the negative charge on the α-phosphate of the nucleotide is neutralized by a positive group on the enzyme; and (iii) attack by the 3′-OH group of the primer on the α phosphate and subsequent release of the PP_i (or Mg-PP_i) proceeds in an in-line fashion.

All three of these properties are in good agreement with the geometry seen in the active site of our ternary complex, and in fact, our active site (Fig. 6) does not differ significantly from that proposed by Burgers and Eckstein [figure 4 of (*61*)]. In agreement with criterion number (i), the pol β active site shows a Mg^{2+} ion (metal site A) bound as a bidentate to the β and γ phosphates of ddCTP (Table 3). As for criterion number (ii), we now know that the originally undefined "positive group on the enzyme" that stabilizes the α phosphate of the nucleotide is simply the second Mg^{2+} ion in metal site B (Fig. 6). In fact, the geometry of the Mg^{2+} ion in site B of our

structure can explain why polymerases are highly selective for only the S, as opposed to the R, absolute configuration of dATPαS phosphorothioate analogs whenever Mg^{2+} is the metal ion in the reaction mixture (*61*). Mg^{2+} coordinates oxygen much more strongly than sulfur, and in our active site the Mg^{2+} ion in metal site B coordinates a specific, nonesterified oxygen of the α-phosphate of the nucleotide. If this particular oxygen were replaced by a sulfur, as would be the case for the *R* configuration of dATPαS, coordination by the Mg^{2+} ion in site B should be weakened considerably, at least according to the geometry our structure. Finally, the geometry of the active site is also in accord with an in-line mechanism, criterion number (iii), for the nucleotidyl transfer reaction. An in-line mechanism, proposed because the polymerase reaction proceeds with inversion of configuration at the α phosphate (*61*), restricts the possible orientation of the attacking group with respect to the leaving group. The attacking and leaving groups must be opposite one another, relative to the α phosphate, in order to occupy the two apical positions of the pentacoordinated α phosphate in the transition state. In the pol β active site, the 3′ carbon of the primer strand (which normally possesses the attacking 3′-OH) lies just opposite, relative to the α phosphate, to the scissile oxygen of the PP_i leaving group.

It is not too surprising that the active site of pol β is similar to the active site of the 3′ → 5′ exonuclease domain of *E. coli* pol I, which is known, through extensive structural (*23, 24*) and mutagenesis (*62, 63*) studies, to employ a two-metal ion mechanism for phosphoryl transfer, and like pol β, proceeds with inversion of configuration at the scissile phosphate (*64*). Like the 3′ → 5′ exonuclease, pol β has three carboxylic acids that position two divalent metal ions about 4 Å apart in the active site and, although the orientations of the carboxylic acids are quite different in the two structures, the geometries of the metal sites are strikingly similar. The metal ion in site A in both cases tightly coordinates four oxygen ligands with highly distorted tetragonal geometry and has a fifth, weakly bound water ligand (Table 3). In fact, a better description of the unusual geometry around metal site A in our structure would be that of a square pyramid in which the Mg^{2+} is at the apex, rising about 1.8 Å out of the square plane described by four oxygen ligands (one from Asp^{190}, one from Asp^{192}, one from the β phosphate, and one from the γ phosphate) (Fig. 6) (Table 3). In contrast, the metal ion in site B (for both pol β and the 3′ → 5′ exonuclease) is not as tightly bound as the metal ion in site A and has slightly distorted octahedral geometry (*65*). Just as with the 3′ → 5′ exonuclease, we propose that the primary function of the metal ion in site A is to aid in binding and positioning of the substrate, and the primary function of the metal ion in site B is to help stabilize the pentacoordinated phosphate of the transition state, though both metal sites probably participate in both of these functions to some degree. Finally, as is the case with the 3′ → 5′ exonuclease, we propose that a metal ion activates the attacking oxygen by acting as a Lewis acid, while a protein side chain acts as a proton acceptor (Fig. 6B). For the 3′ → 5′ exonuclease, the metal ion in site A activates an attacking water molecule, while Glu^{357} acts as the proton acceptor and, in the case of pol β, the metal ion in site B activates the attacking 3′-OH of the primer, while Asp^{256} acts as the proton acceptor. All of these features of the transition state of the nucleotidyl transfer reaction of pol β are evident from the crystal structure, and only the missing 3′-OH of the primer strand was added in order to draw the schematic in Fig. 6B.

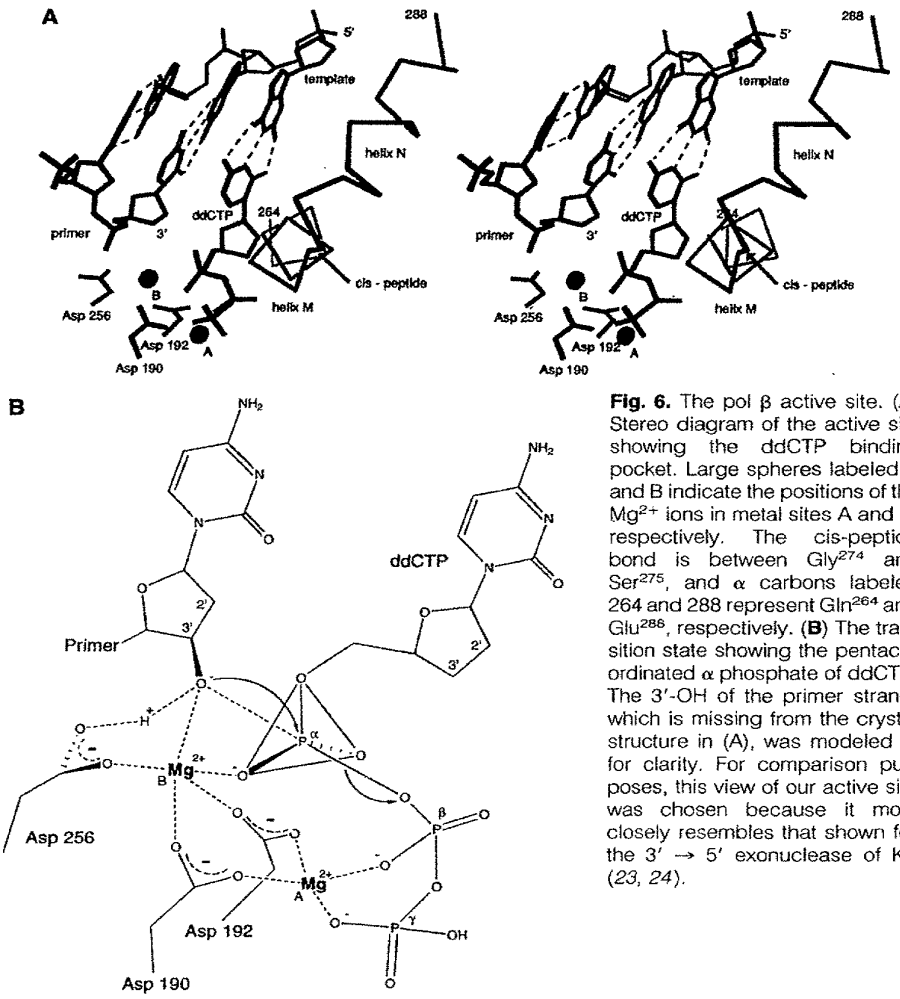

Fig. 6. The pol β active site. (**A**) Stereo diagram of the active site showing the ddCTP binding pocket. Large spheres labeled A and B indicate the positions of the Mg^{2+} ions in metal sites A and B, respectively. The cis-peptide bond is between Gly^{274} and Ser^{275}, and α carbons labeled 264 and 288 represent Gln^{264} and Glu^{288}, respectively. (**B**) The transition state showing the pentacoordinated α phosphate of ddCTP. The 3′-OH of the primer strand, which is missing from the crystal structure in (A), was modeled in for clarity. For comparison purposes, this view of our active site was chosen because it most closely resembles that shown for the 3′ → 5′ exonuclease of KF (*23, 24*).

Despite all the similarities, we caution against thinking of the 3′ → 5′ exonuclease reaction as "the polymerase reaction in reverse." In fact, the polymerase reaction in reverse is pyrophosphorolysis and differs from the exonuclease reaction as follows:

3′ → 5′ Exonuclease reaction:
primer-dNMP + H_2O→primer + dNMP

Pyrophosphorolysis:
primer-dNMP + PP_i→primer + dNTP

so that the metal ion in site A for the 3′ → 5′ exonuclease interacts primarily with a water molecule (*24*), whereas the metal ion in site A for the polymerase interacts primarily with PP_i (or the PP_i moiety of ddCTP) (*65*).

In summary, the overall nucleotidyl transfer reaction, as catalyzed by pol β, probably proceeds as follows (Fig. 6):

i) As pol β binds to the template-primer, Lys^{234}, Tyr^{271}, and Arg^{283} break up the

water structure of the minor groove, causing the minor groove width to increase near the active site.

ii) An incoming nucleotide is positioned in the active site by base-pairing with the template, by a hydrogen bond to Asn^{279}, by a van der Waals contacts with Asp^{276}, by steric hindrance with the protein backbone between Tyr^{271} and Gly^{274}, by six hydrogen bonds between protein and ddCTP phosphates, and by two Mg^{2+} ions that are, in turn, positioned by Asp^{190}, Asp^{192}, and Asp^{256}.

iii) The Mg^{2+} ion in metal site B, acting as a Lewis acid, activates the 3′-OH of the primer terminus, while one of its ligands, Asp^{256}, probably acts as the proton acceptor for the 3′-OH.

iv) After attack on the α-phosphate by the activated 3′-OH, the reaction proceeds through the transition state in which the α phosphate is pentacoordinated, with the attacking 3′ oxygen of the primer terminus and the leaving oxygen of the PP_i group occupying the two apical positions. The pentacoordinated transition state is stabilized by the Mg^{2+} ion in site B, which coordinates both an apical and an equatorial oxygen of the α phosphate.

v) After PP_i (or Mg-PP_i) is released, pol β is ready for another cycle, but only after the enzyme either releases from the template-primer (distributive mode of synthesis) or pulls itself along the template (processive mode of synthesis); one or both of these activities may be facilitated by a conformational change of cis- to trans-peptide at Gly^{274}–Ser^{275}.

Metal ions play crucial roles in the mechanism of pol β and of the 3′ → 5′ exonuclease of pol I. This kind of independence from direct involvement of protein side chains during catalysis has led to proposals that perhaps hydrolysis reactions involving nonprotein catalysts such as self-splicing ribozymes (*66, 67*), where positioning of the two metal ions can be achieved just as easily by RNA backbone phosphates, proceed through a similar two-metal ion mechanism as that proposed for the 3′ → 5′ exonuclease of pol I (*23, 68*). In much the same way, perhaps the nucleotidyl transfer mechanism that we present also applies, to some extent, to those ribozymes that are capable of catalyzing the nucleotidyl transfer reaction (*69, 70*).

Comparisons with other polymerases. As discussed in the accompanying report (*14*), the most obvious structural overlap among all four polymerases, KF, RNAP, RT, and pol β, consists of a conserved pair of carboxylic acid side chains located in the palm subdomain. As revealed by the pol β active site, the primary function of these carboxylic acids is to position two catalytically critical divalent metal ions. The observation that even the most divergent polymerase structures retain this catalytic core is compelling evidence that all polymerases share a common catalytic mechanism (*11, 14*). We believe that the pol β ternary complex structures presented here are physiologically relevant and that the nucleotidyl transfer mechanism that we propose based on these structures represents a common catalytic mechanism found in all polymerases. In the absence of evidence that suggests otherwise, the next logical argument would be that all polymerases, because they share a common catalytic mechanism, should bind to the DNA (or RNA) template-primer in a fashion very similar—at least with respect to the highly conserved catalytic residues—to that in which pol β has attached itself to the DNA template-primer in our ternary complex (Fig. 7, A to C). All of the polymerase structures discussed have a DNA binding channel that can grasp a stationary rodlike DNA template-primer in one of two general directions, so the only other possibility for binding to the template-primer, provided that there are no gross conformational changes on the part of DNA or protein, is shown for the RT structure (Fig. 7D). In order to generate Fig. 7D, the template-primer in Fig. 7C was held stationary while the RT molecule was rotated by 180° about an axis normal to the plane of the page and passing through the polymerase active site. The mode of DNA binding depicted in Fig. 7D, which is opposite to that found in pol β (anti–pol β), is the one proposed for KF (*41*), for RNAP (*13*), and for RT (*11, 12*). We are thus faced with a dilemma.

Three main possibilities can be pursued. First, it could be argued that the other three polymerase-DNA models are basically correct, and that the pol β ternary complex structures presented here are not physiologically relevant. However, as stated above, there is strong evidence in favor of the physiological relevance of our structures, and we therefore bypass this possibility for the present. A second possibility could be that all four of the polymerase-DNA models are correct, including the pol β–DNA structures presented here, and that pol β uses an entirely different mode of template-primer binding from the other polymerases. However, that pol β has a palm subdomain that is structurally homologous with the other polymerases (*14*), and that this palm subdomain also contains the highly conserved catalytic residues, suggests otherwise. The two-metal ion mechanism that we propose for the nucleotidyl transfer reaction seems to depend heavily on the positioning of the metal ions by the conserved carboxylic acid residues. Furthermore, the catalytic site appears to be asymmetric in that the two metal sites possess different geometries and different binding affinities (*65*). This leads us to the third possibility, which is the one we choose to pursue in the following discussions. The other polymerases must bind to a template-primer in a manner similar to that of pol β so as to conserve the critical, asymmetric geometry of the active site. An inescapable inference, then, is that the other polymerase-DNA models are not correct with respect to the directionality of the template-primer in the binding channel. Because of the complexity of the existing data and interpretations, we address each of the

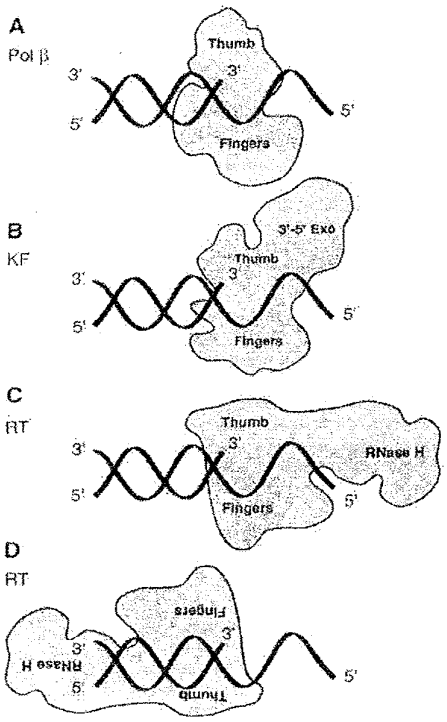

Fig. 7. (**A**) Schematic drawing showing the position of the template-primer as seen in the pol β ternary complex structure and (**B** and **C**) our proposed models for the KF-DNA and RT-DNA complexes, respectively. To generate (B and C) the palms of KF and RT were aligned with the palm subdomain of pol β as viewed in (A). In the text, (A to C) represent the pol β–like mode of binding to the template-primer. To generate (**D**), which represents the anti–pol β mode of binding to the template-primer, the template-primer in (C) was held stationary, and the RT molecule was rotated by 180° about an axis normal to the plane of the page and running through the RT active site. The template-primer was kept stationary in all four diagrams because that is a reasonable representation of in vivo polymerization, where the DNA molecule is usually very long and immovable in comparison to the polymerase molecule. The active site in all four diagrams is at the 3′ terminus of the primer strand, and the view looks down at the palm, which forms the bottom of the template-primer binding channel.

three other polymerases, KF, RNAP, and RT, separately.

DNA pol I of *E. coli* is a 103-kD monomer and can be cleaved by limited proteolysis into a large (68-kD) COOH-terminal fragment [commonly referred to as the Klenow fragment (KF)], and a smaller (35-kD) NH_2-terminal fragment (*1*). KF has both polymerase and 3′ → 5′ exonuclease (editing) activities, whereas the small NH_2-terminal fragment functions solely as a 5′ → 3′ exonuclease. The crystal structure of KF alone revealed two distinct domains (*10*), confirming previous proposals that the polymerase and 3′ → 5′ exonuclease functions of KF lie on separate, independent folding units. From its position in the pol I amino acid sequence, it was then inferred that the missing NH_2-terminal 5′ → 3′ exonuclease domain should be positioned somewhere to the right of the 3′ → 5′ exonuclease as viewed in Fig. 7B (*10*). During nick translation, a function intrinsic to pol I, it is proposed that the polymerase, starting at a nick in the DNA and polymerizing in a 5′ → 3′ direction, works in conjunction with the activities of the 5′ → 3′ exonuclease so that the net result is simply a translation of the nick along the DNA in a 5′ → 3′ direction (*1*). It was this property of pol I that led to proposals that the polymerase domain of pol I bound to the DNA template-primer in a manner not too different from that shown in Fig. 7B, so that the polymerase and the 5′ → 3′ exonuclease were positioned properly, relative to the DNA nick, to perform their separate activities in conjunction with one another (*10*). Subsequent KF-DNA models were proposed to show how the polymerase and the 3′ → 5′ exonuclease of KF could work in conjunction with one another during the DNA editing process (*23, 24, 71, 72*). Once again, these earlier models require that KF binds to the DNA template-primer in a manner similar to that shown in Fig. 7B. Therefore, all of the earlier pol I–DNA models are in agreement with a pol β–like mode of DNA binding.

The recent crystal structure determination of KF crystallized in the presence of a putative template-primer revealed an unexpected complex in which the KF had bound to the template-primer in neither of the two binding modes discussed above, but instead, in such a way that the DNA lay in a separate, less obvious channel running between the thumb subdomain of the polymerase and the 3′ → 5′ exonuclease (*41*). From this structure it was proposed that during polymerization, the KF bent the DNA by 80° so that the DNA entered the larger, more obvious DNA binding channel in a direction opposite to that of all the previously proposed KF-DNA models. We now find ourselves in the awkward position of presenting arguments in favor of the idea that the initial KF-DNA models were correct, at least with respect to the direction of DNA binding, and that the recent KF-DNA crystal structure (*41*) might not be physiologically relevant.

Possibly the most compelling reason to believe that the previously reported KF-DNA crystal structure (*41*) might not be physiologically relevant is that it is not in accord with the nick translation activities of pol I. Other reasons, which concern the nature of the KF-DNA crystals themselves, are (i) the complex crystals were grown under relatively high, nonphysiological salt concentrations; (ii) the template-primer in the crystallization experiments included an unnatural epoxy-ATP; (iii) the template-primer had been unexpectedly modified during crystallization in such a way that the primer strand was longer than the template strand in the crystal structure; and finally, (iv) the complex crystals were isomorphous with the apo KF crystals, which in itself is not a strong argument, but is made stronger by the observation that none of the pol β ternary complex crystals presented here is isomorphous with the apo crystals. Because the 3′ → 5′ exonuclease domain of KF was shown to have a high affinity for the primer terminus during other attempts at obtaining KF-DNA complex crystals (*23*), perhaps the recently reported KF-DNA structure (*41*) is solely an editing complex in which the primer terminus bound to the 3′ → 5′ exonuclease site, and the leftover "tethered" DNA, guided by crystal packing forces, bound wherever it could under the circumstances. This is similar to the argument presented above that the position of the tethered 8-kD domain in our complex crystals, though ordered in both structures, is probably not physiologically relevant.

Further support for the idea that the KF binds to the template-primer in a manner very similar to that of pol β comes from closer inspection of the DNA binding channels of both enzymes. KF possesses what appears to be the structural equivalent to helices M and N of the pol β thumb subdomain. As described above, helices M and N protrude into the pol β DNA binding channel and interact with the nucleotide substrate (Fig. 3). Though not structurally homologous with helices M and N, helices J and K of KF also protrude into the KF-DNA binding channel in an area near the catalytic carboxylic acids that overlaps quite well with helices M and N of pol β, suggesting a similar role for helices J and K in nucleotide specificity for KF.

RNA polymerase (RNAP) from bacteriophage T7 is a monomer of molecular weight 99-kD, and the crystal structure determination of RNAP revealed that it folds into two distinct domains (*13*). One domain possesses a polymerase fold that is highly homologous with the polymerase domain of KF, while a smaller, NH_2-terminal domain, though not an exonuclease, is located in the same relative position as the 3′ → 5′ exonuclease domain of KF. That the polymerase domains of RNAP and KF are so structurally homologous was one of the strongest arguments presented in favor of a model (*13*) wherein RNAP binds to a DNA-RNA duplex in a manner similar to that of the recent KF-DNA model (*41*). However, as pointed out above, we believe that the recent KF-DNA complex structure might not be physiologically relevant and that the KF, instead, probably utilizes a pol β–like mode of binding to the template-primer. Therefore, we propose that like KF, RNAP also utilizes a pol β–like mode of binding to an RNA-DNA duplex.

Other evidence besides homology with KF was also presented in favor of an anti–pol β mode of template-primer binding for RNAP (*13*)—in disagreement with our proposals for an RNAP-RNA-DNA complex structure. Results from mutagenesis studies (*73, 74*) as well as binding studies with proteolytically modified RNAP fragments (*75*) suggest that the NH_2-terminal domain of RNAP binds to the nascent RNA of the emerging RNA-DNA duplex (*73, 75*) and that Gln^{748} of RNAP recognizes the -10 and -11 bases of the DNA promoter upon complex formation (*74*). These observations suggested positioning the RNA-DNA duplex in the template-primer binding channel of RNAP in an anti–pol β fashion (*13*). Although more work will be required to settle this issue, the structural evidence presented by the pol β ternary complex seems very compelling and interpretations of results from the mutagenesis studies as well as binding studies of proteolytically modified RNAP fragments may require a more careful analysis. As an example of the difficulties involved in interpreting mutagenesis and binding studies in the absence of structural data on a polymerase-DNA complex, mutagenesis studies on pol β implicated Arg^{183} as taking part in primer strand recognition upon complex formation (*76*), and binding studies with pyridoxal 5′-phosphate suggested that the 8-kD domain of pol β formed a part of the nucleotide binding pocket (*77*). Neither of these conclusions is in agreement with our ternary complex structures, though the results from these studies, when re-evaluated, are not necessarily in disagreement with our structural work [reference 41 in (*14*)].

Retroviral RTs, responsible for making double-stranded DNA copies of single-stranded RNA viral genomes, had been well studied (*78*) prior to the discovery that the retrovirus HIV-1 was the cause of AIDS (*79–81*). This facilitated the study of the

HIV-1 RT because its mechanism of operation is similar to that of many previously studied RTs (*82*). HIV-1 RT functions as a heterodimer, and the crystal structure determination of RT showed that one monomer of the heterodimer, called p66 because it has a molecular mass of 66 kD, folds into two distinct domains: a typical polymerase domain (palm, fingers, and thumb) and a connected ribonuclease (RNase) H domain (*11, 12*). The second monomer of the RT heterodimer, p51, is simply a copy of p66 in which the 15-kD RNase H COOH-terminal segment has been proteolytically cleaved. In contrast to the fingers, palm, and thumb of the polymerase domain of p66, the fingers, palm, and thumb of p51 occupy relatively different positions in the crystal structure, resulting in something other than a typical polymerase fold for p51 in the p66-p51 heterodimer (*11, 12*). Only p66 is shown in Fig. 7C for clarity and to highlight how the RNase H domain lies in approximately the same direction with respect to the polymerase as the 3′ → 5′ exonuclease domain of KF. The function of the RNase H is to remove the viral RNA template from the RNA-DNA hybrid that results after RNA-directed DNA polymerization (reverse transcription) of the viral genome.

In keeping with our proposals that all polymerases share a common catalytic mechanism, and hence a common template-primer binding mode, we suggest that the polymerase domain of the p66 monomer of RT also binds to a template-primer in a manner similar to that of pol β (Fig. 7C). Some evidence of this exists in that, as with KF and RNAP, RT also seems to possess the structural equivalent to helices M and N of pol β. Though not quite as obvious and pronounced as in the other polymerases, beta strands 12 and 13 of RT do protrude into the template-primer binding channel near the catalytically important carboxylic acid residues in an area that overlaps quite well with helices M and N of pol β and helices J and K of KF, implicating beta strands 12 and 13 of RT as possibly playing a similar role in substrate specificity.

In disagreement with this proposal is the crystal structure of RT complexed with a template-primer, which shows that HIV-1 RT has bound to the template-primer in a manner opposite to that of pol β (*12*) (Fig. 7D may serve as an approximate representation of that structure). We can present some of the same reservations as to the physiological relevance of the RT-DNA complex as were presented above for the KF-DNA complex: (i) The RT-DNA complex crystals were grown at relatively high, nonphysiological salt concentrations; (ii) the template-primer in the crystallization experiments was odd in that it had only a single base template overhang; and (iii) the complex crystals were isomorphous with the apo structure crystals. However, unlike KF, there exists strong evidence in favor of the idea that the RT nevertheless binds to a template-primer in the manner depicted in Fig. 7D, regardless of the physiological relevance of the RT-DNA crystal structure. This evidence comes from kinetic studies that show a tight temporal coupling between the polymerase and RNase H activities of RT during the reverse transcription process (*83*). These results suggest that RT binds to the template-primer in such a way that the RNase domain comes in contact with the RNA template of the emerging RNA-DNA duplex during reverse transcription, which would be possible only if the anti–pol β mode of binding were employed, as shown in Fig. 7D.

Although we have presented strong structural evidence in favor of the idea that RT binds to the template-primer in a manner similar to that of pol β, KF, and RNAP, there seems to be equally strong kinetic evidence (*83*) in support of an anti–pol β mode of template-primer binding for RT during RNase H coupled polymerization activity. Thus it may be that RT can bind to the template-primer in two different catalytically competent ways; an RNase H independent, pol β–like mode of binding and an RNase H–coupled, anti–pol β mode of binding.

The method by which RT makes double-stranded DNA copies of the single-stranded RNA genome is quite complicated (*82*), so it is not too difficult to imagine that one enzyme performing so many different functions might employ two modes of binding to a template-primer (Fig. 7, C and D). Although this appears to be in disagreement with our arguments that the asymmetric geometry of the polymerase active site must be conserved, closer inspection of the RT active site reveals that our arguments may nevertheless hold true. As it turns out, the 180° rotation performed on the RT in Fig. 7C to produce Fig. 7D resulted in an active site where Asp^{185} and Asp^{186} of RT (the equivalent to Asp^{190} and Asp^{192} in pol β) have simply switched positions, and Asp^{110} (the equivalent to pol β's Asp^{256}) has been replaced by Tyr^{183}. Instead of Asp^{110}, Tyr^{183} is now near the primer 3′ terminus for RT in the anti–pol β mode of template-primer binding, suggesting that perhaps Tyr^{183} performs some of the same functions that Asp^{110} performs when RT is in a pol β–like binding mode. Thus the geometry of the active site seems to be conserved. In support of this idea, the YMDD sequence of RT (Tyr^{183}, Met^{184}, Asp^{185}, Asp^{186}) is the most highly conserved amino acid sequence in all known RTs, and mutations to the tyrosine residue of this segment have shown it to be highly critical for catalysis (*84*). None of the other polymerases discussed, neither pol β, KF, nor RNAP, has a structural equivalent to Tyr^{183} in RT.

Another aspect of the models in Fig. 7, C and D, is that a much smaller part of RT is in contact with duplex DNA in Fig. 7C, possibly suggesting a more distributive mode of synthesis when RT invokes a pol β–like mode of template-primer binding. A more distributive mode of polymerization has been observed for RT when a DNA template is utilized instead of an RNA template (*85, 86*). All this leads us to propose that RT might use a pol β–like mode of binding for a DNA template, and the reverse, anti–pol β mode of binding for an RNA template, thus making it a reverse transcriptase in another sense of the word. The anti–pol β mode of template-primer binding (Fig. 7D) could be operative during the highly processive, RNase H–coupled, RNA-directed DNA polymerization of the viral minus strand (*82*), whereas a pol β–like mode of template-primer binding (Fig. 7C) could be employed during the more distributive DNA-directed DNA polymerization of the viral plus strand (*82*), when RNase H coupled polymerization is no longer required. The most salient feature of this proposal is that RT may distinguish between the two types of DNA substrates involved: the A form of an RNA-DNA hybrid versus the B form of a DNA duplex. For instance, RT might possess the structural equivalents of pol β's Lys^{234}, Tyr^{271}, and Arg^{283} (which, as discussed above, break up the water structure of the minor groove near the active site) only when RT employs a pol β–like mode of binding. If all DNA-directed DNA polymerization steps require that the water structure in the minor groove of the B-DNA template-primer be disrupted, this would make it unfavorable for RT to bind to a B form substrate in any other way but in a pol β–like fashion.

Although the aforementioned proposals restrict RT from utilizing an anti-pol β mode of binding on B form substrates, they do not necessarily restrict RT from utilizing a pol β–like mode of binding on A-form substrates, which already possess a broad minor groove. It is therefore feasible that other, possibly non–RNase H coupled steps during the replication of the viral genome, such as tRNA primed synthesis of the minus strand strong stop DNA or synthesis of the RNase H insensitive polypurine tract (ppt) (*82*), might employ a pol β–like mode of template-primer binding for RT as well, despite the fact that an RNA template is utilized during these steps. That it might be possible for RT, under certain circumstances, to employ the same mode of bind-

ing for an RNA template as for a DNA template is supported by kinetic studies showing that pol I, under the appropriate conditions, can utilize an RNA template almost as efficiently as its natural DNA template (*87*). In the case of the synthesis of minus stand strong stop DNA, the length of the tRNA primer (18 nt) is the same as the distance between the polymerase active site and the RNase H active site (about 18 bp) (*83*). Accordingly we suggest that perhaps the bulky tRNA molecule attached to the primer strand functions as a steric hindrance to binding at the RNase H active site, forcing RT to use the pol β–like mode of template-primer binding during this step of the cycle. In favor of this argument is the observation that RT polymerization during minus strand strong stop synthesis appears to be more distributive than during reverse transcription of the viral genome (*88*, *89*). Also supporting this idea are results from primer utilization studies showing that tRNA primed synthesis of minus strand strong stop DNA, at least in vitro, does not require a specific tRNA such as human $tRNA^3_{Lys}$, which is utilized by RT in vivo (*90*).

Further indication of a possible dual mode of template-primer binding by RT comes from many independent studies. Active site titration studies showed that the RT heterodimer possesses a possible second template-primer binding site, but what is most intriguing about these results is that this second binding site only reveals itself after the template strand of the template-primer has been shortened to 16 nt (*91*). Along the same lines, although a model proposing an RNA-DNA-RNA intermediate for a RT strand transfer mechanism does not suggest a second mode of template-primer binding (*92*), perhaps some of the kinetic and crosslinking data leading up to that model can also be interpreted as evidence in favor of our arguments. Furthermore, kinetic studies on the individual p66 and p51 monomers of RT showed that both of these monomers are fully capable of catalyzing the nucleotidyl transfer reaction, but only under optimal conditions for each monomer (*93*). That the optimal template for p66 was RNA and the optimal template for p51 was DNA strengthened the proposal that the RT heterodimer "may be functionally asymmetric with distinct plus and minus strand polymerases" (*93*). It was further suggested that the p66 monomer of the p66-p51 RT heterodimer was responsible for RNA-directed DNA polymerase functions (reverse transcription), whereas the p51 monomer of the heterodimer was responsible for the DNA-directed DNA polymerization activities of RT (*93*). However, as discussed above, the crystal structure of the p66-p51 RT heterodimer clearly shows that the p51 monomer does not possess a polymerase fold, at least while p51 RT is a part of the p66-p51 heterodimer (*11*, *12*). Therefore, our hypothesis that the p66 monomer of the p66-p51 RT heterodimer may act as two different polymerases that happen to share a common active site is not only in agreement with the idea that the RT heterodimer is functionally asymmetric with distinct plus and minus strand polymerases (*93*), but is in accord with the structural work (*11*, *12*) as well.

Finally, a dual mode of template binding by RT might go a long way toward explaining why drugs such as AZT can specifically target HIV-1 RT in preference to host cell polymerases. Pol β, KF, RNAP, and even RT (when in a pol β–like mode of template-primer binding) all seem to possess structural features near the active site, such as helices M and N of pol β, that may be responsible for nucleotide selectivity. However, there appears to be no structural equivalent to helices M and N of pol β when RT is in an anti–pol β mode of template-primer binding, which might explain why RT is so error prone and more sensitive to AZT-TP inhibition. In support of this idea are kinetic studies that show decreasing K_i values for AZT-TP in its binding to pol β (K_i = 73 μM) and to RT (K_i = 0.3 μM) when calf thymus DNA is utilized as a template in both cases, as compared to RT when the native RNA template is utilized (K_i = 0.01 μM) (*55*). The decreasing order of K_i values (tighter binding) might reflect the decreasing number of structural elements in the active site that can, through steric hindrance, induce nucleotide specificity, the order of structural hindrance being: the pol β's helices M and N ≫ RT's β strand 12 and 13 (when employing a pol β–like mode of template-primer binding on a DNA template) > RT when employing an anti–pol β mode of binding on an RNA template. With this in mind, one could imagine how AZT, ddC, and ddI resistant RT mutants might arise in AIDS patients after prolonged treatment with these drugs (*94*–*97*). As discussed above, much of the nucleotide binding pocket in pol β is determined by the exact position of the template-primer, so a single mutation that affects how RT binds to the template-primer could in turn affect the nucleotide binding site. Indeed, it has been proposed that many of the drug resistant mutations in RT, because they are so distant from the active site, interact primarily with the template strand (*11*). Perhaps these RT mutants affect template-primer binding in such a way as to introduce structural elements, similar to helices M and N of pol β, into the nucleotide binding pocket of RT, possibly causing greater selectivity and more resistance to these anti-HIV drugs.

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that had a slight yellow hue and were difficult to dissolve in the buffer solution inevitably failed to produce complex crystals. We therefore recommend that a reversed-phase cartridge, or other DNA purification techniques, be employed before the crystallization experiments.
28. A more common method for annealing the DNA template-primer, in which samples are heated to 90° for 3 minutes then allowed to cool slowly to room temperature, was attempted in order to obtain better crystals, but this method resulted in crystals with no noticeable improvements in diffraction power.
29. The ddCTP was purchased (Sigma) as 4 μmol samples and stored at −20°C. The H_2O used throughout all crystallization procedures had been deionized, then distilled, prior to use.
30. Crystals were grown at room temperature in MVD-24 sitting drop trays (Charles Supper Co.), which were subsequently sealed with clear packaging tape (Manco, purchased from Sears) to allow vapor diffusion between the reservoir solution [7 to 9 percent (w/v) PEG 3350, 0.1 M MES, 75 mM lithium sulfate] and the crystallization medium, which was made by mixing 20 μl of the reservoir solution with 20 μl of the protein-DNA-ddCTP sample.
31. AZT-TP was provided by R. F. Schinazi, Emory University, Georgia.
32. A partial (31-kD) structure was determined by molecular replacement techniques, then refined, in a manner very similar to that described in the text for the ternary complex structures. It was difficult to interpret $F_o - F_c$, α_c, difference maps at this resolution (4 Å), but what little positive electron density that was observed in the maps was located adjacent to the fingers subdomain of pol β and was attributed to the missing 8-kD domain. No further work has been done on this structure.
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101. Supported in part by NIH grants GM10928 and CA17374 (J.K.), NIH grant ES06839, R. A. Welch Foundation grant H-1265 (S.H.W.), and a grant of computing time from the San Diego Supercomputer Center. We thank the personnel of the N. H. Xuong Laboratory for aid in data collection; C. Nielsen, N. Nguyen, D. Sullivan, V. Ashford, and W. Wolfle for technical assistance in protein preparation. Full coordinates for both ternary complex structures are available from the Brookhaven Protein Data Bank and are designated 1bpf and 1bpg for the $P6_1$ and $P2_1$ structures, respectively.

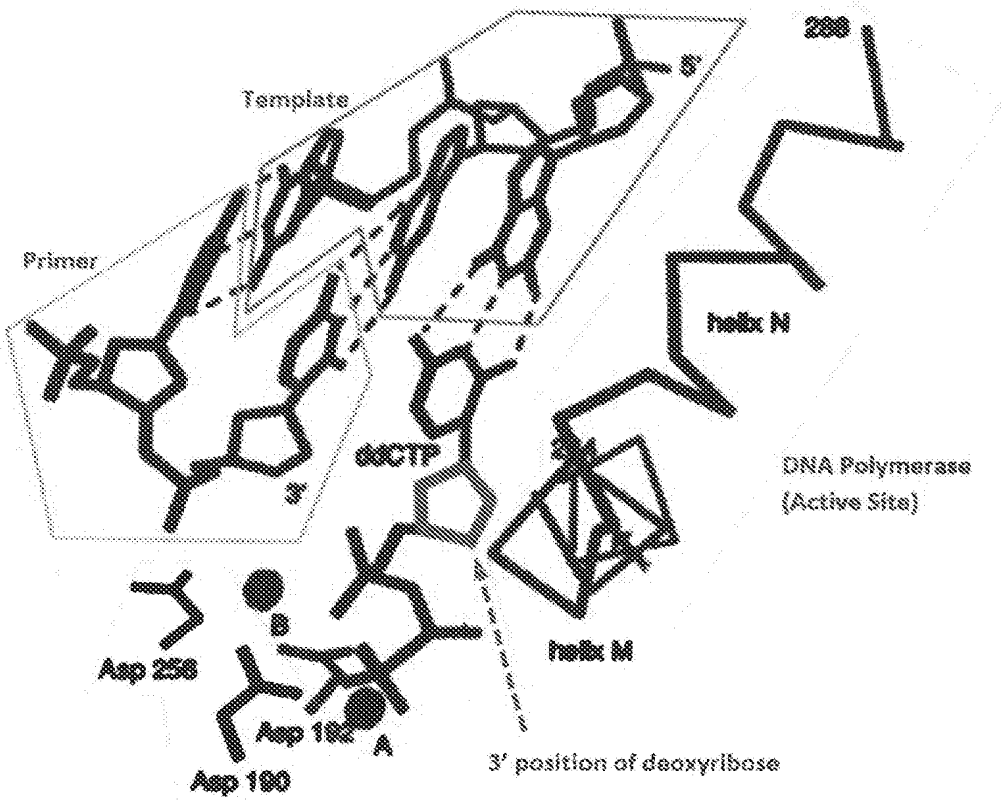
28 February 1994; accepted 10 May 1994

Dkt. 62239-BZA7

Analysis of Space Available Within the Active Site of a DNA Polymerase Ternary Complex (*polymerase, DNA template/primer, nucleotide*) to Accommodate a 3'-Capped dNTP

Based on the 3-dimensional structure of the ternary complex (*polymerase, DNA template/primer, nucleotide*) determined by Pelletier et al. (Pelletier et al. "*Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP*." *Science* 1994, 264, 1891-1903), which is cited in U.S. Serial No. 15/167,917 (Ju et al. *Massive parallel method for decoding DNA and RNA*), an analysis was performed to determine the space available for a 3'-O capping group on the 3' carbon of the deoxyribose of the nucleotide. The results indicate that there is only a small space available between amino acids in the active site of the polymerase and the 3' carbon of the deoxyribose of the nucleotide, as shown in the Figure below (corresponding to Fig. 1 of U.S. Serial No. 15/167,917 and to Fig. 6 of Pelletier et al.; color and labels added for clarity). This space can only accommodate a capping group of limited diameter on the 3' position of the deoxyribose of the nucleotide. Pelletier et al. (1994) determined that three amino acids of the polymerase, Tyr 271, Phe272, and Gly274, are in close proximity to the 3' carbon of the deoxyribose of the nucleotide. (Pelletier et al. 1994, Table 3). In Table 3 Pelletier et al. highlight the distances from the nucleotide to these amino acids in the polymerase ternary complex as follows: 3.2 Å between the 3' carbon of the deoxyribose ring and Phe272; 3.2 Å between the 2' carbon of the deoxyribose ring and Gly274; and 3.5 Å between the 2' carbon and Tyr271.

The distances given in Pelletier et al. were used to calculate the available space around the 3' carbon of the deoxyribose ring of the nucleotide. It was determined that the diameter of the available space in the active site of the polymerase ternary complex is approximately 3.7 Å.

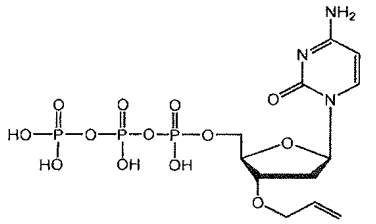


The Figure above from Pelletier et al. (1994) shows the 3-dimensional structure of the ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate. The active site of the polymerase

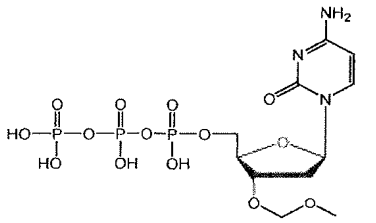
Applicant: The Trustees of Columbia University in the City of New York
U.S. Serial No.: 15/167,917
Filed: May 27, 2016
Exhibit C

is highlighted. Note that the 3' position of the dideoxyribose ring (blue) is very crowded. (See also Brief Description of the Figures of U.S. Serial No. 15/167,917 for FIG. 1).

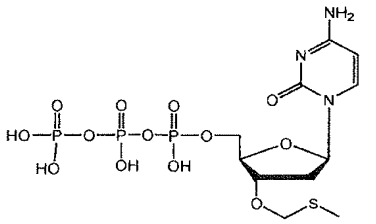
The diameters of five groups possibly useful as 3'-oxygen capping groups for a nucleotide analogue (allyl, methoxymethyl (MOM), methylthiomethyl, azidomethyl, and 2-nitrobenzyl, whose structures are shown below) were calculated. The calculations were based on the lengths and angles of the bonds beyond the 3' carbon of the deoxyribose ring of the nucleotide, and were determined using *Chem3D Pro* software (MC Squared, *"Software Review: CS Chem3D Pro 3.5 and CS MOPAC Pro" Electronic Journal of Theoretical Chemistry*, 1997, 2, 215–217).



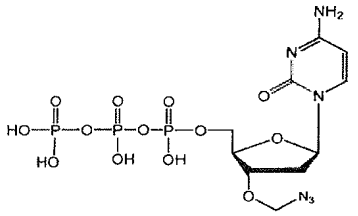
3'-*O*-Allyl-dCTP



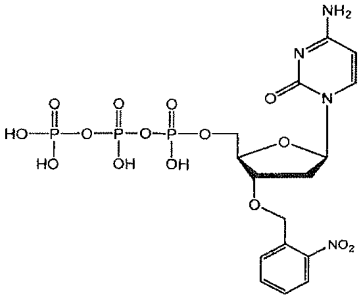
3'-*O*-Methoxymethyl-dCTP



3'-*O*-Methylthiomethyl-dCTP



3'-*O*-Azidomethyl-dCTP



3'-*O*-2-Nitrobenzyl-dCTP

The calculated diameter (D) for each group is as follows:

1. Allyl ($-CH_2-CH=CH_2$): D = 3.0 Å

2. Methoxymethyl (MOM; $-CH_2-OCH_3$): D= 2.1 Å

3. Methylthiomethyl ($-CH_2-SCH_3$): D= 2.4 Å

4. Azidomethyl ($-CH_2-N_3$): D= 2.1 Å

5. 2-Nitrobenzyl ($-C_7H_6O_2N$): D = 5.0 Å

Conclusion:

The available space in the active site of the polymerase around the 3' position of the deoxyribose ring of the nucleotide in the polymerase ternary complex has a diameter of approximately 3.7 Å. The allyl, MOM, methylthiomethyl and azidomethyl groups (diameters ranging from 2.1 Å to 3.0 Å) will fit into the available space, but the rigid aromatic 2-nitrobenzyl group (diameter 5.0 Å) will not fit into the active site of the polymerase due to its larger diameter.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/149,098	09/10/2019	10407458	62239-BZA6AA/JPW/BI	3820

23432 7590 08/21/2019

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ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Jingyue Ju, Englewood Cliffs, NJ;
The Trustees of Columbia University in the City of New York, New York, NY;
Zengmin Li, Flushing, NY;
John Robert Edwards, St. Louis, MO;
Yasuhiro Itagaki, New York, NY;

Paper No. 13
Filed: May 4, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK
Patent Owner.

Case IPR2018-00385
Patent 9,725,480

PATENT OWNER'S PRELIMINARY RESPONSE

A. Introduction

In 2000, Columbia Professor Dr. Jingyue Ju and Zengmin Li, John Robert Edwards, and Yasuhiro Itagaki ("the Columbia inventors") solved a decade-old problem that Petitioner, Illumina, and its expert admitted was a "major challenge" and a "formidable obstacle." Ex-2007 ¶60; Ex-2008 at 4-5; Ex-2042 at 6. They invented nucleotide analogues that permitted Sequencing by Synthesis (SBS) to become a practical reality. Others described a wide variety of features for a deoxyribonucleotide analogue for use in SBS, but no one defined the combination of features necessary for success. The Columbia inventors conceived nucleotide analogues comprising, *inter alia*, (i) on the deoxyribose 3'-oxygen, a chemically cleavable, chemical capping group having a "small" diameter (i.e., less than 3.7Å), which does not contain a ketone and does not form an ester or a methoxy group with the 3'-oxygen and (ii) on the 7-position of a deaza-guanine base, a chemically cleavable, chemical linker attached to a detectable fluorescent moiety.

Contrary to Illumina's arguments, the '480 patent claim ("the challenged claim") differs from the claims found unpatentable in prior IPR proceedings. Specifically, the challenged claim is substantially narrower than, and patentably distinct from, those earlier claims. Before allowing the challenged claim, the Examiner considered the prior art and the Board's earlier rulings and correctly

challenged claim. Mischaracterizing a reference is not presenting the reference in a new light and the Board should exercise its discretion under 35 U.S.C. §325(d) and deny institution of Ground 1.

G. Illumina's Ground 2 Challenge For Obviousness Over Dower In View Of Prober And Metzker Is Flawed

Illumina's Ground 2 challenge fails for many of the same reasons as its Ground 1 challenge, but also because none of its references (Dower, Prober, or Metzker) disclose a "chemically cleavable, chemical linker" as required by the challenged claim.

1. Missing Claim Features In Illumina's Challenge

a. Dower Does Not Disclose A Chemically Cleavable, Chemical Linker

Illumina asserts that "Dower discloses cleavably linking the label to the base." Petition at 56; *see also* Petition at 71 ("Dower discloses a nucleotide analogue having a cleavably linked fluorescent label"). The challenged claim, however, does not simply require that the label be linked to the base in a manner that is cleavable, it requires the presence of "Y," a "chemically cleavable, chemical linker" between the label and the base. The Board rejected the same argument by Illumina in IPR2012-00007. *See* Ex-2036 at 14-15. There, as here, Illumina cited portions of Dower that describe attaching a label ***directly*** to the nucleotide base

(i.e., with no cleavable linker) and characterized those portions of Dower as disclosing a cleavable linker. *Id*. The Board disagreed:

> The cited disclosures in Dower ***do not expressly identify a "cleavable linker" as joining the "base" to the "detectable label."*** Removal of a detectable label from a nucleotide is described, but such disclosure does not specify that removal occurs at cleavable linker which joins the label to the base. In the absence of such description, there is not a reasonable likelihood that Illumina would prevail in demonstrating unpatentability based on Dower, alone.

Id. at 15 (emphasis added). As the Board found, Dower does not disclose a label attached to a nucleotide base by a cleavable linker, let alone a chemically cleavable, chemical linker as required by the challenged claim. The portions of Dower cited in Illumina's Petition show the label directly attached to the base. *E.g.*, Petition at 58 (citing Ex-1015 at Fig. 9).[14] Illumina has not established that Dower discloses the claimed feature Y, a "chemically cleavable, chemical linker" attaching the label to the base.

[14] The portions of Dower cited by Illumina in its Petition for this limitation (*see* Petition at 65-66) are the same, or substantively the same, as the portions of Dower the Board previously considered.

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Paper 66
Entered: June 21, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK,
Patent Owner.

Case IPR2018-00291 (Patent 9,718,852 B2)
Case IPR2018-00318 (Patent 9,719,139 B2)
Case IPR2018-00322 (Patent 9,708,358 B2)
Case IPR2018-00385 (Patent 9,725,480 B2)[1]

Before JAMES A. WORTH, MICHELLE N. ANKENBRAND, and BRIAN D. RANGE, *Administrative Patent Judges.*

Opinion for the Board *per curiam*.
Opinion Dissenting filed by Administrative Patent Judge WORTH.

Per curiam

---

[1] The proceedings have not been consolidated. The parties are not authorized to use a combined caption unless an identical paper is being entered into each proceeding and the paper contains a footnote indicating the same.

FINAL WRITTEN DECISION
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

I. INTRODUCTION

This is a Final Written Decision addressing four *inter partes* reviews challenging each claim of U.S. Patent Nos. 9,718,852 B2 ("the '852 patent"), 9,719,139 B2 ("the '139 patent"), 9,708,358 B2 ("the '358 patent"), and 9,725,480 B2 ("the '480 patent"). We have jurisdiction under 35 U.S.C. § 6. For the reasons that follow, we determine that Illumina, Inc. ("Petitioner" or "Illumina") demonstrates, by a preponderance of the evidence, that the challenged claims are unpatentable.

A. Procedural History

Petitioner filed four Petitions (Paper 1,[2] "Pet.") requesting an *inter partes* review of the '852 patent, the '139 patent, the '358 patent, and the '480 patent. We instituted trial on the following grounds:[3]

Patent	References	Basis	Claim Challenged
'852	Tsien,[4] Prober[5]	§ 103(a)	1

[2] Unless this opinion otherwise indicates, all citations are to IPR2018-00291 ("the '291 IPR").

[3] *See* IPR2018-00291, Paper 16 (June 25, 2018); IPR2018-00318, Paper 16 (July 2, 2018); IPR2018-00322, Paper 16 (July 2, 2018); IPR2018-00385, Paper 20 (July 26, 2018).

[4] Tsien et al., WO 91/06678, May 16, 1991 ("Tsien") (Ex. 1013).

[5] James M. Prober et al., *A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides*, 238 SCIENCE 336–341 (Oct. 16, 1987) ("Prober") (Ex. 1014).

application. The parties agree that the priority date for the patent at issue is October 6, 2000. Tr. at 24:21–24.

C. Level of Ordinary Skill in the Art

We consider each asserted ground of unpatentability in view of the understanding of a person of ordinary skill in the art. Petitioner proposes a definition of the level of skill in the art (Pet. 7–8), and Patent Owner does not dispute this definition (Resp. 3). Petitioner's proposal is consistent with the evidence before us. *See* Findings of Fact, *infra*. We, therefore, adopt Petitioner's proposal and find that a person of ordinary skill in the art would have been a member of a team of scientists developing nucleotide analogues, researching DNA polymerases, and/or addressing DNA techniques. A person of ordinary skill in the art would have held a doctoral degree in chemistry, molecular biology, or a closely related discipline, and would have had at least five years of practical academic or industrial laboratory experience.

D. Claim Construction

The Board interprets claims in an unexpired patent using the "broadest reasonable construction in light of the specification of the patent." 37 C.F.R. § 42.100(b) (2017)[10]; *Cuozzo Speed Techs., LLC v. Lee*, 136 S. Ct.

[10] The Office recently changed the claim construction standard applicable to an *inter partes* review. *See* Changes to the Claim Construction Standard for Interpreting Claims in Trial Proceedings Before the Patent Trial and Appeal Board, 83 Fed. Reg. 51,340 (Oct. 11, 2018). The rule changing the claim construction standard, however, does not apply to this proceeding because Petitioner filed its Petition before the effective date of the final rule, i.e., November 13, 2018. *Id.* at 51,340 (rule effective date and applicability date), 51,344 (explaining how the Office will implement the rule).

Motor Co. Ltd., 868 F.3d 1013, 1017 (Fed. Cir. 2017) (quoting *Vivid Techs* when addressing an *inter partes* review proceeding on appeal).

"*chemical linker*"

Each challenged claim recites that the Y on the nucleotide structure "represents a chemically cleavable, chemical linker." *See, e.g.*, Ex. 1001, 34:30–31. Patent Owner argues that "'chemical linker' means a chemical moiety attached by covalent bonds at one end to a specified position on the base of a nucleotide and at the other end to a tag (detectable fluorescent moiety)." Resp. 10. Based on our review of the record, we determine that this term does not require express construction in order to resolve the parties' controversy. *Vivid Techs.*, 200 F.3d at 803.

E. Fact Findings

The fact findings below focus on issues that must be resolved in order to assess Petitioner's obviousness challenges. *Graham* 383 U.S. at 17–18. (1966). Each finding is based upon consideration of the record as a whole and is supported by the preponderance of the evidence.

1. Technology Overview

Deoxyribonucleotides make up the building blocks of DNA, and the chemical formula, nomenclature, and uses of deoxyribonucleotides were generally known before October 6, 2000. Ex. 1011, 46, 47, 58–60, 98–103. A strand of DNA consists of deoxyribonucleotides where the 5'-phosphate of one nucleotide is attached to the 3′-oxygen of the adjacent nucleotide. Ex. 1078 ¶¶ 33–36; Pet. 12–13.

Before October 6, 2000, persons having ordinary skill in the art would have been aware of several methods for determining the sequence of DNA, including Sanger sequencing and sequencing-by-synthesis ("SBS").

Paper No. _
Filed: February 5, 2019

UNITED STATES PATENT AND TRADEMARK OFFICE

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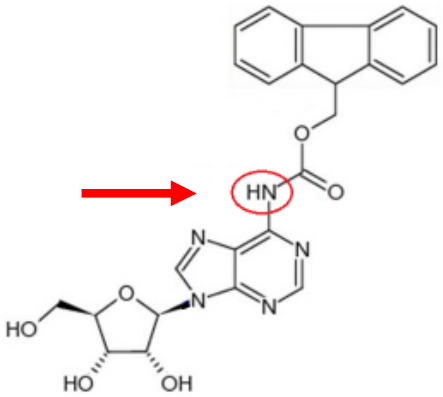
IN THE CITY OF NEW YORK
Patent Owner.

IPR2018-00291 (Patent 9,718,852)
IPR2018-00318 (Patent 9,719,139)
IPR2018-00322 (Patent 9,708,358)
IPR2018-00385 (Patent 9,725,480)[1]

PATENT OWNER'S SUR-REPLY

[1] An identical Paper is being entered into each listed proceeding.



Reply, 26 (relabeled); Ex. 2140, 198:10-16. Dower's FMOC is not a cleavable linker (Y) attached to a ***carbon***, and Dr. Romesberg admitted he is not aware of evidence showing FMOC could be so attached. Ex. 2140, 201:8-23. Instead, Dr. Romesberg now says that FMOC's cleavable linker could be attached to the carbon in the challenged claim via "***an additional linker***[.]" Ex. 2140, 197:16-198:8. No such theory exists in Illumina's Petition. And, Illumina's double-linker is excluded from the claim, which requires one linker (Y), not two linkers (Y Y). Moreover, the claim mandates non-interference and stability properties, and there is no evidence Illumina's double-linker satisfies those properties. Further, Dr. Romesberg provided only conclusory testimony that a POSA knew the chemistry to accomplish this double-linker attachment (Ex. 2140, 217:2-218:3). Thus, Ground 2 fails.

While irrelevant, Columbia's patent does not "merely say[] that the linker can be chemically cleaved" without providing an example. Reply, 26. It discloses

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Paper 65
Entered: September 9, 2019

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK,
Patent Owner.

Case IPR2018-00797
Patent 9,868,985 B2

Before MICHELLE N. ANKENBRAND, *Acting Vice Chief Administrative Patent Judge*, JAMES A. WORTH and BRIAN D. RANGE, *Administrative Patent Judges.*

Opinion for the Board *per curiam*.
Opinion Dissenting filed by *Administrative Patent Judge*, WORTH.

Per curiam

FINAL WRITTEN DECISION
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

D. Claim Construction

The Board interprets claims in an unexpired patent using the "broadest reasonable construction in light of the specification of the patent." 37 C.F.R. § 42.100(b) (2017)[9]; *Cuozzo Speed Techs., LLC v. Lee*, 136 S. Ct. 2131, 2144–46 (2016). Under that standard, claim terms are given their ordinary and customary meaning in view of the specification, as would have been understood by one of ordinary skill in the art at the time of the invention. *In re Translogic Tech., Inc.*, 504 F.3d 1249, 1257 (Fed. Cir. 2007). Any special definitions for claim terms must be set forth in the specification with reasonable clarity, deliberateness, and precision. *In re Paulsen*, 30 F.3d 1475, 1480 (Fed. Cir. 1994).

Here, Petitioner addresses claim 1's recitation of "or" between the claim's recitation of the four nucleotide analogues. Pet. 11. Patent Owner requests construction of "small" and "chemical linker." Resp. 9–10. Additionally, the parties address claim 1's recitation of "[a] method for sequencing a nucleic acid." *Id.* at 11; Reply 3–5. We address these four issues below.

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[9] The Office recently changed the claim construction standard applicable to an *inter partes* review. *See* Changes to the Claim Construction Standard for Interpreting Claims in Trial Proceedings Before the Patent Trial and Appeal Board, 83 Fed. Reg. 51,340 (Oct. 11, 2018). The rule changing the claim construction standard, however, does not apply to this proceeding because Petitioner filed its Petition before the effective date of the final rule, i.e., November 13, 2018. *Id.* at 51,340 (rule effective date and applicability date), 51,344 (explaining how the Office will implement the rule).

APPENDIX A: AGREED-TO CLAIM CONSTRUCTIONS

Terms (Patent/Claims)	Joint Proposed Construction
"R" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2 '984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	**Proposed Construction:** "A chemical group used to cap the 3' OH group, as depicted in the illustration of the nucleotide analogue in the claim"
"does not interfere with recognition of the analogue as a substrate by a DNA polymerase" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2	**Proposed Construction:** "Does not interfere with the use of the analogue as a DNA polymerase substrate"

Terms (Patent/Claims)	Joint Proposed Construction
'984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	
"Y . . . is stable during a DNA polymerase reaction" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2 '984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	**Proposed Construction:** "Y remains bonded to base and tag during a DNA polymerase reaction"
"chemically cleavable" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2	**Proposed Construction:** Plain and ordinary meaning, namely "cleavable by chemical means"

Terms (Patent/Claims)	Joint Proposed Construction
’984 Patent: Claim 1, 2 ’380 Patent: Claim 1, 3	

APPENDIX B: DISPUTED CLAIM TERMS AND PROPOSED CONSTRUCTIONS

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
"Y" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2 '984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	**Proposed Construction:** "Represents a part of the nucleotide analogue, attaching the base of the nucleotide analogue to a tag, as depicted in the illustration of the nucleotide analogue in the claim" **Intrinsic Evidence:** The claims, specification, and prosecution history of the: • '458 Patent • '459 Patent	**Proposed Construction:** "A single linker that directly connects the base to the label" **Intrinsic Evidence:** - '458 Patent Figs. 2A, 2B; 3:18-54, 4:45-5:48, 8:33-9:11, 10:64-11:18, and 13:6-7 - Claims 1 and 2 of the Nucleotide Patents-in-Suit - Claims 1 and 3 of the '380 Patent - '380 Patent PH: March 12, 2019 First Action Interview Pilot Program Pre-

[1] The "'458 Patent" citations are to the specification for the '458 Patent. The other patents-in-suit, the '459 Patent, '742 Patent, '984 Patent (with the '458 Patent, the "Nucleotide Patents-in-Suit"); and '380 Patent (with the Nucleotide Patents-in-Suit, the "Patents-in-Suit"), have identical specifications as the '458 Patent (sans minor stylistic differences) and, thus, any citations to the '458 Patent are found in each of the specifications for and apply to the Patents-in-Suit respectively.

[2] The prosecution history for the '380 Patent ("'380 Patent PH") is a "superset" of the prosecution histories for each of the Nucleotide Patents-in-Suit and, thus, any citations to '380 Patent PH are substantively found in each of the prosecution histories for and apply to the Nucleotide Patents-in-Suit respectively.

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• '742 Patent • '984 Patent • '380 Patent *See e.g.*, '458 Patent[3] (Ex. A) at Abstract; 3:4–11; 4:58–63; 5:33–41; 5:60–67; 6:15–18; 6:24–25; 6:37–38; 6:57–58; 6:64–67; 7:10–16; 7:20–27; 8:21–24; 8:46–51; 10:64–11:4; 12:66–13:7; 14:8–14:15; 14:29–19:26; 24:4–6; 26:64–27:3; FIGS. 2A–B, 7, 8, 10, 15A, 16, 19, 21, 22 '458 Patent (Appl. No. 16/149,098) Prosecution History[4]: *See e.g.*,	Interview Communication; April 12, 2019 Communication in Response to March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication and all exhibits attached thereto; May 8, 2019 Summary of May 1, 2019 Examiner Interview - All the corresponding prosecution history communications and exhibits as those cited from the '380 Patent PH above in the Nucleotide Patents-in-Suit prosecution histories

[3] Because each of the Patents-in-Suit shares a common specification, for the Court's convenience, Plaintiffs cite to the specification of the '458 Patent as representative of the specifications of each of the Patents-in-Suit. Plaintiffs' citations to the '458 Patent's specification incorporate by reference the corresponding passages from the specification of the '459 Patent, the '742 Patent, the '984 Patent, and the '380 Patent. Citations to the patent incorporate by reference the corresponding passages in the patent application (as filed and as published).

[4] Because of the similarity in the prosecution histories of the Patents-in-Suit, for the Court's convenience, Plaintiffs cite to the prosecution file history of the '458 Patent as representative of the prosecution file histories of each of the Patents-in-Suit. Plaintiffs' citations to the '458 Patent's prosecution file history incorporate by reference the corresponding passages from the prosecution file histories of the '459 Patent, the '742 Patent, the '984 Patent, and

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• May 9, 2019 Reply to Pre-Interview Communication, pp 6–7 • Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to May 9, 2019 Reply to Pre-Interview Communication, ¶ 20 • Ex. 2 (Claim Support Table) to May 9, 2019 Reply to Pre-Interview Communication '380 Patent (Appl. No. 16/150,191) Prosecution History: *See e.g.*, • March 12, 2019 Pre-Interview First Office Action • June 12, 2019 Applicant Summary of June 5, 2019 Examiner Interview **Related Patents:**[5]	- The October 2, 2017 Non-Final Rejection; March 2, 2018 Amendment In Response to the October 2, 2017 Non-Final Office Action and Petition for a Two-Month Extension of Time; November 21, 2018 Non-Final Rejection; May 5, 2019 Communication in Reponses to November 21, 2018 Office Action and Petition for A Three-Month Extension of Time; July 31, 2019 Final Rejection; October 28, 2019 Amendment as a Submission Accompanying a Request for Continued Examination under 37 C.F.R. § 1.114 in Response to July 31, 2019 Final Office Action; and all exhibits attached thereto in the prosecution history of U.S. Patent

the '380 Patent. To the extent the prosecution file history of the '380 Patent contains *additional* relevant information, Plaintiffs separately cite to the relevant passages of the '380 Patent's prosecution file history.

[5] The Patents-in-Suit claim priority to the same patent application (No. 09/684,670) as, inter alia, U.S. Patent Nos. 9,718,852 ("the '852 Patent"), 9,719,139 ("the '139 Patent"), 9,708,358 ("the '358 Patent"), 9,725,480 ("the '480 Patent"), and 9,868,985 ("the '985 Patent").

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	'480 Patent (Appl. No. 15/167,917) Prosecution History: *See e.g.*, • March 30, 2017 Office Action, p 5–6, 10–12 • May 26, 2017 Response to March 30, 2017 Office Action, pp 5–6 • Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to Response to March 30, 2017 Office Action, ¶ 20 • Ex. 2 (Claim Support Table) to Response to March 30, 2017 Office Action • June 23, 2017 Notice of Allowance at Reasons of Allowance, p 2 IPR2018-00291:[6] *See e.g.*,	Application No. 14/670,748, to which the Patents-in-Suit claim priority - *Illumina, Inc. v. The Trustees Of Columbia University In The City Of New York*, IPR2018-00291, Paper 49 (Feb. 5, 2019) at 24; Ex. 1139 at slides 46-51; Ex. 2140; Ex. 2141 at slide 59 - All corresponding documents and exhibits as those identified-above from *Illumina, Inc. v. The Trustees Of Columbia University In The City Of New York*, IPR2018-00291 that were filed in IPR2018-00318, -00322, -00385, and -00797

[6] Defendant filed petitions for *inter partes review* of each of the '852, '139, '358, '480, and '985 Patents. The Patent Trial and Appeal Board instituted IPR proceedings for each of these five patents (IPR2018-00291, IPR2018-00318, IPR2018-00322, IPR2018-00385, and IPR2018-00797, respectively). For the Court's convenience, Plaintiffs primarily cite to the filings in IPR2018-00291 as representative of the filings in IPR2018-00318, IPR2018-00322, IPR2018-00385, and IPR2018-00797. Accordingly, Plaintiffs' citations to the filings in IPR2018-00291 incorporate by reference the corresponding passages of the corresponding filings in IPR2018-00318, IPR2018-00322, IPR2018-00385, and IPR2018-00797.

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Petition (December 8, 2017) at pp 13, 17–18, 20–21, 27–29, 36–37, 42, 46–47, 48–49 • Exhibit 1012 Romesberg Declaration (December 8, 2017) at pp 25, 28–29, 37–39, 43, 49, 52–56, 62–68 • Preliminary Response (March 27, 2018) at pp 10–11 • Patent Owner's Response (October 26, 2018) at pp 10–11 • Exhibit 2116 Menchen Declaration (October 25, 2018) at pp 9–11 • Final Written Decision (June 21, 2018) at pp 17, 53 (n.33), 54–55 IPR2018-00797: *See e.g.*, • IPR2018-00797 Petition (March 16, 2018) at pp 24–25, 47–50 • Exhibit 1078 Romesberg Declaration (March 16, 2018) at pp 42–43, 55–56, 73–75, 89–98	
"small"	**Proposed Construction:**	**Proposed Construction:**

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2 '984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	"A chemical group that has a diameter, *i.e.*, width, that is less than 3.7Å" **Intrinsic Evidence:** The claims, specification, and prosecution history of the: • '458 Patent • '459 Patent • '742 Patent • '984 Patent • '380 Patent *See e.g.*, '458 Patent (Ex. A) at Abstract; 2:52–3:54; 5:52–59; 8:24–27; 20:46–51; 21:23–34; 25:54–64; FIG. 1 '458 Patent (Appl. No. 16/149,098) Prosecution History: *See e.g.*, • May 9, 2019 Reply to Pre-Interview Communication, pp 3–5, 8–9 • Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to May 09, 2019 Reply to Pre-Interview Communication, ¶¶ 10–18, 22, 24–27	"A chemical group that fits within the rat DNA polymerase active site shown in Fig. 1 of the patent, *i.e.* has a longest dimension less than 3.7Å, including the 3' oxygen" **Intrinsic Evidence:** - '458 Patent: Figs. 1, 2A, 2B, 13, 14, 19; 2:47-3:3, 3:4-17, 3:18-54, 8:6-28, 13:12-15, 15:20-17:19, 18:42-19:26, 21:33-64, 23:25-44, 25:36-53 - Claims 1 and 2 of the Nucleotide Patents-in-Suit - Claims 1 and 3 of the '380 Patent - '380 Patent PH: March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication; April 12, 2019 Communication in Response to March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication and all exhibits attached thereto; May 8, 2019 Summary of May 1, 2019 Examiner Interview

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Ex. C to Decl. of Jingyue Ju, Ph.D. • Ex. 2 (Claim Support Table) to May 9, 2019 Reply to Pre-Interview Communication '380 Patent (Appl. No. 16/150,191) Prosecution History: *See e.g.*, • March 12, 2019 Pre-Interview First Office Action • June 12, 2019 Applicant Summary of June 5, 2019 Examiner Interview **Related Patents:** '480 Patent (Appl. No. 15/167,917) Prosecution History: *See e.g.*, • March 30, 2017 Office Action, p 5 • May 26, 2017 Response to March 30, 2017 Office Action, pp 2–5, 8–11 • Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to Response to March 30, 2017 Office Action, ¶¶ 10–18, 22, 24–27 • Ex. C to Decl. of Jingyue Ju, Ph.D.	- All the corresponding prosecution history communications and exhibits as those cited from the '380 Patent PH above in the Nucleotide Patents-in-Suit prosecution histories - The October 2, 2017 Non-Final Rejection; March 2, 2018 Amendment In Response to the October 2, 2017 Non-Final Office Action and Petition for a Two-Month Extension of Time; November 21, 2018 Non-Final Rejection; May 5, 2019 Communication in Reponses to November 21, 2018 Office Action and Petition for A Three-Month Extension of Time; July 31, 2019 Final Rejection; October 28, 2019 Amendment as a Submission Accompanying a Request for Continued Examination under 37 C.F.R. § 1.114 in Response to July 31, 2019 Final Office Action; and all exhibits attached thereto in the prosecution history of U.S. Patent Application No. 14/670,748, to which the Patents-in-Suit claim priority

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Ex. 2 (Claim Support Table) to Response to March 30, 2017 Office Action • June 23, 2017 Notice of Allowance at Reasons of Allowance, p 2 IPR2018-00291: *See e.g.*, • Petition (December 8, 2017) at pp 6, 11–12, 22, 59–60 • Exhibit 1012 Romesberg Declaration (December 8, 2017) at pp 17–18, 30, 99 • Preliminary Response (March 27, 2018) at pp 1, 9–10, 12, 15–16, 31, 40–42, 55, 58–59 • Institution Decision (June 25, 2018) at p 21 • Patent Owner's Response (October 26, 2018) at pp 9–10, 46–48 • Petitioner's Reply (January 22, 2019) at p 22 • Exhibit 1119 Romesberg Second Declaration (January 22, 2019) at p 21–27	

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Patent Owner's Sur-reply (February 5, 2019) at p 3	
"R . . . is stable during a DNA polymerase reaction" ***** '458 Patent: Claim 1, 2 '459 Patent: Claim 1, 2 '742 Patent: Claim 1, 2 '984 Patent: Claim 1, 2 '380 Patent: Claim 1, 3	**Proposed Construction:** "R remains bonded to 3′ oxygen during a DNA polymerase reaction" **Intrinsic Evidence:** The claims, specification, and prosecution history of the: • '458 Patent • '459 Patent • '742 Patent • '984 Patent • '380 Patent *See e.g.*, '458 Patent (Ex. A) at 3:4–17; 3:44–51; 8:41–53; 8:56–59; 8:65–67; 10:13–17; 10:66–11:4; 14:8–15; 21:41–64 '458 Patent (Appl. No. 16/149,098) Prosecution History: *See e.g.*, • May 9, 2019 Reply to Pre-Interview Communication, pp 5–7	**Proposed Construction:** "R has at least the stability of a MOM ether ($-CH_2OCH_3$) or allyl ($-CH_2CH=CH_2$) group" **Intrinsic Evidence:** - '458 Patent: Figs. 7, 13A, 13B, 14, 15A, 16; 4:25-41, 10:13-19, 10:64-11:5, 14:8-16 - Claims 1 and 2 of the Nucleotide Patents-in-Suit - Claims 1 and 3 of the '380 Patent - '380 Patent PH: March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication; April 12, 2019 Communication in Response to March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication and all exhibits attached thereto; May 8, 2019 Summary of May 1, 2019 Examiner Interview

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to May 09, 2019 Reply to Pre-Interview Communication, ¶ 21 • Ex. 2 (Claim Support Table) to May 9, 2019 Reply to Pre-Interview Communication **Related Patents:** '480 Patent (Appl. No. 15/167,917) Prosecution History: *See e.g.*, • March 30, 2017 Office Action, pp 5–6, 9 • May 26, 2017 Response to March 30, 2017 Office Action, pp 4–6 • Ex. 1 (Decl. of Jingyue Ju, Ph.D.) to Response to March 30, 2017 Office Action, ¶ 21 • Ex. 2 (Claim Support Table) to Response to March 30, 2017 Office Action IPR2018-00291: *See e.g.*,	- All the corresponding prosecution history communications and exhibits as those cited from the '380 Patent PH above in the Nucleotide Patents-in-Suit prosecution histories - The October 2, 2017 Non-Final Rejection; March 2, 2018 Amendment In Response to the October 2, 2017 Non-Final Office Action and Petition for a Two-Month Extension of Time; November 21, 2018 Non-Final Rejection; May 5, 2019 Communication in Reponses to November 21, 2018 Office Action and Petition for A Three-Month Extension of Time; July 31, 2019 Final Rejection; October 28, 2019 Amendment as a Submission Accompanying a Request for Continued Examination under 37 C.F.R. § 1.114 in Response to July 31, 2019 Final Office Action; and all exhibits attached thereto in the prosecution history of U.S. Patent Application No. 14/670,748, to which the Patents-in-Suit claim priority

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	• Petition (December 8, 2017) at pp 25, 29, 61–62, 64 • Exhibit 1012 Romesberg Declaration (December 8, 2017) at pp 34–35, 41–42, 81–83, 86–89 • Institution Decision (June 25, 2018) at pp 22–23, 30–32	
"A method for sequencing a nucleic acid" ***** '380 Patent: Claim 1, 3	**Proposed Construction:** "A method for detecting the identity and sequence of a strand of nucleotides" **Intrinsic Evidence:** The claims, specification, and prosecution history of the: • '380 Patent *See e.g.*, '380 Patent (Ex. E) at Abstract; 3:4–17; 4:25–31; 4:45–48; 5:17–19; 5:60–67; 8:33–36; 9:5–7; 12:29–35; 21:52–67; 21:33–64; FIGS. 2A–B	**Proposed Construction:** Preamble is not limiting **Intrinsic Evidence:** - Claims 1 and 3 of the '380 Patent - '380 Patent PH: March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication; April 12, 2019 Communication in Response to March 12, 2019 First Action Interview Pilot Program Pre-Interview Communication and all exhibits attached thereto; May 8, 2019 Summary of May 1, 2019 Examiner Interview - All the corresponding prosecution history communications and exhibits as

Terms (Patent/Claims)	Plaintiffs' Proposed Construction and Supporting Intrinsic Evidence	Defendant's Proposed Construction and Supporting Intrinsic Evidence[1, 2]
	Related Patents: IPR2018-00797: *See e.g.*, • Patent Owner's Response (October 26, 2018) at pp 11, 48–49 • Exhibit 2114 Menchen Declaration (October 25, 2018) at pp 10–11, 45 • Patent Owner's Sur-reply (February 5, 2019) at p 5 • Paper 60 Oral Hearing Transcript at pp 35-36.	those cited from the '380 Patent PH above in the Nucleotide Patents-in-Suit prosecution histories - The October 2, 2017 Non-Final Rejection; March 2, 2018 Amendment In Response to the October 2, 2017 Non-Final Office Action and Petition for a Two-Month Extension of Time; November 21, 2018 Non-Final Rejection; May 5, 2019 Communication in Reponses to November 21, 2018 Office Action and Petition for A Three-Month Extension of Time; July 31, 2019 Final Rejection; October 28, 2019 Amendment as a Submission Accompanying a Request for Continued Examination under 37 C.F.R. § 1.114 in Response to July 31, 2019 Final Office Action; and all exhibits attached thereto in the prosecution history of U.S. Patent Application No. 14/670,748, to which the Patents-in-Suit claim priority

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: Patent of Shankar Balasubramanian *et al.*
Patent No.: 8,158,346
Appl. No.: 12/804,025
Issue Date: April 17, 2012
For: LABELLED NUCLEOTIDES

Mail Stop PATENT BOARD
Patent Trial and Appeal Board
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Dr. Bruce P. Branchaud in Support of Petition for *Inter Partes* Review of U.S. Patent No. 8,158,346

I, Bruce P. Branchaud, Ph.D., declare as follows:

1. I have been retained by the firm of Ballard Spahr LLP, who represents Intelligent Bio-Systems, Inc. ("IBS"), to provide expert testimony in support of IBS's Petition for *Inter Partes* Review of U.S. Patent No. 8,158,346 (the "Petition").

2. I understand that this proceeding involves U.S. Patent No. 8,158,346 ("the '346 Patent") entitled "Labelled Nucleotides." I also understand that the '346 Patent issued from U.S. Patent App. No. 12/804,025 filed on July

50. Moreover, *Ju* also provides specific examples of nucleotide analogs wherein the label and the protecting group can be removed under a single set of conditions. While the examples of *Ju* specifically teach cleaving the linker with light at Cleavage Site #2 shown in Figure 7 below, one of ordinary skill in the art would recognize that the chemical cleavage conditions taught by *Ju* to remove the allyl protecting group would necessarily also cleave the linkers (and thus remove the labels) disclosed by *Ju*.

51. *Ju* teaches that the cleavable chemical group may be an allyl group ($-CH_2CH=CH_2$). *See Ju* Figure 7 and col. 10, ll. 1-7.

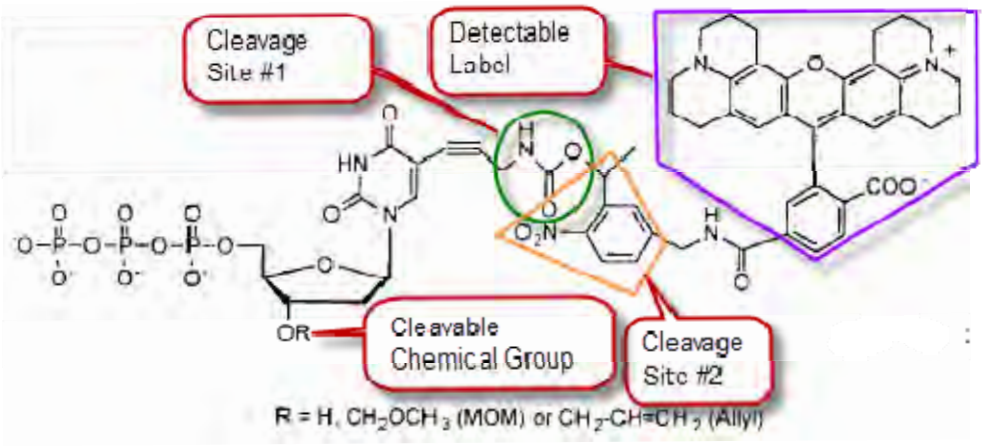
52. *Ju* also teaches specific conditions to cleave the allyl group of the nucleotide analog to yield an –OH group at the 3' position after cleavage. Specifically, *Ju* cites the methods of Kamal *et al.*, A Mild and Rapid Regeneration of Alcohols from their Allylic Ethers by Chlorotrimethylsilane/ Sodium Iodide, Tetrahedron Letters 40(2):371-372, 1999 ("*Kamal*") for removing an allyl group used to cap an –OH group at the 3' position of a nucleotide analog. *See Ju*, col. 26, ll. 19-24; col. 28, ll. 16-17; and Figure 14.

53. *Kamal* teaches using chlorotrimethlysilane (ClTMS) and sodium iodide (NaI) in combination to form Trimethylsilyl iodide (TMSi-I). TMSi-I is

then used to cleave an allyl group used to cap an –OH group at the 3' position of the nucleotide analog. *See Kamal* pp. 371-72.

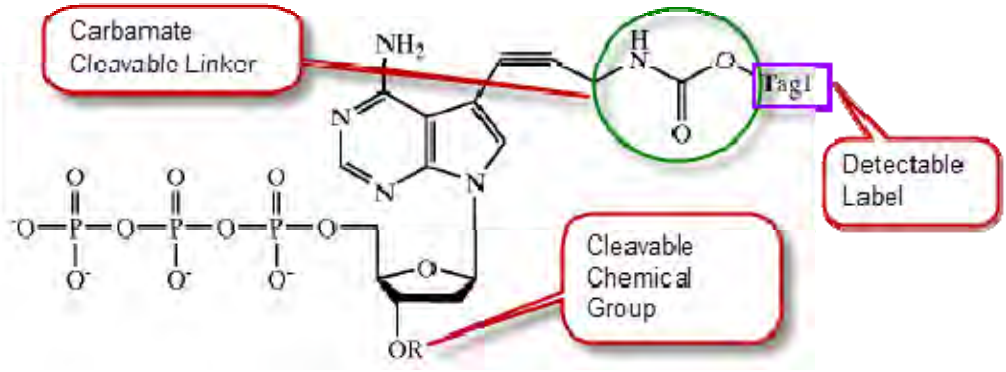
54. With regard to the nucleotide analog of Figure 7, a person of ordinary skill in the art would recognize that among the locations at which the linker could be cleaved are (1) the carbamate (urethane) group at Cleavage Site #1, and (2) the 2-nitrobenzyl group at Cleavage Site #2.

FIG. 7



55. With regard to the nucleotide structure shown at the bottom of columns 17 and 18, a person of ordinary skill in the art would recognize that the linker could be cleaved at the carbamate (urethane) group.

Structure Shown at the Bottom of Column 17 of *Ju*



56. Notably, the conditions taught by *Ju* to cleave the allyl group will also necessarily cleave the attached labels shown in the annotated figures above via the reaction of TMSi-I with the carbamate (urethane) groups of the linkers. This is based on well-established chemistry exemplified in Jung *et al.*, *Conversion of Alkyl Carbamates into Amines vie Treatment with Trimethylsilyl Iodide*, 7 J.C.S. CHEM. COMM. 315 (1978) ("*Jung*"; Ex. 1005), which teaches cleaving carbamate (urethane) with TMSi-I. These principles were well known in the art prior to the Earliest Filing date.

57. Thus, *Ju* teaches sequencing by synthesis methods utilizing nucleotide analogs wherein the attached label and the 3'-OH capping group is removed under a single set of conditions, that is, they are removed concurrently in one step, via a single treatment.

83. Accordingly, in order to improve the efficiency, reliability, and robustness of a sequencing by synthesis method taught in one of the references, one of ordinary skill in the art would be motivated to look to and to use aspects of the methods taught by one of the other references.

I declare under penalty of perjury that the foregoing is true and correct.

This 3rd day of May, 2013.

Bruce P. Branchaud

Bruce P. Branchaud, Ph.D.

US007566537B2

(12) United States Patent

Balasubramanian et al.

(10) Patent No.: US 7,566,537 B2
(45) Date of Patent: Jul. 28, 2009

(54) **LABELLED NUCLEOTIDES**

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(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**

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C12P 19/34	(2006.01)
C12M 1/34	(2006.01)
C07H 21/00	(2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/287.2; 536/23.1

(58) **Field of Classification Search** 536/23.1; 435/6, 91.1, 287.2

See application file for complete search history.

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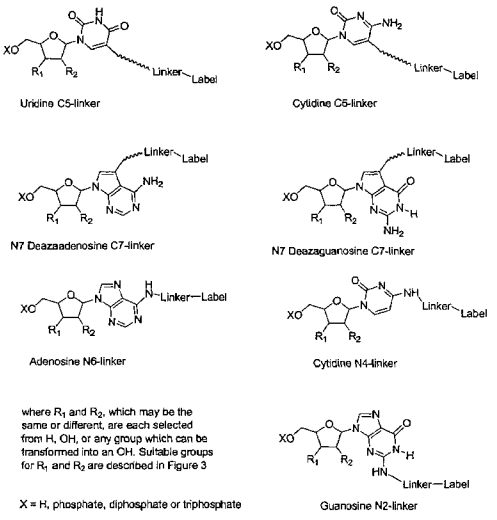
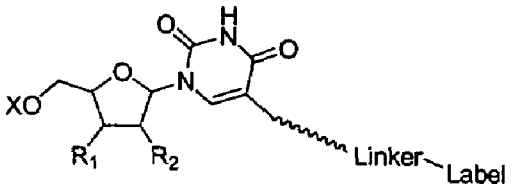
(57) **ABSTRACT**

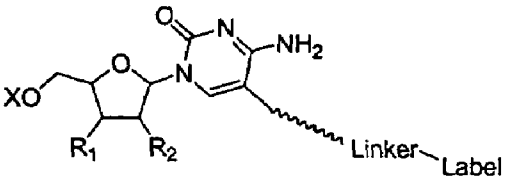
Nucleosides and nucleotides are disclosed that are linked to detectable labels via a cleavable linker group.

15 Claims, 6 Drawing Sheets

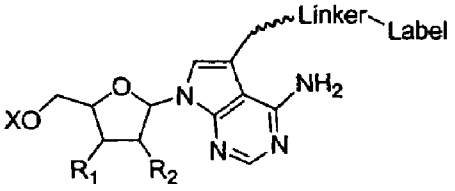




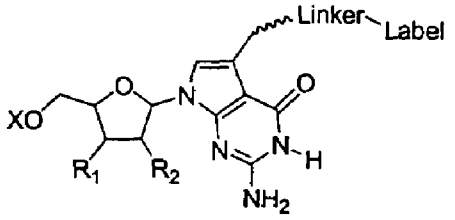
Uridine C5-linker



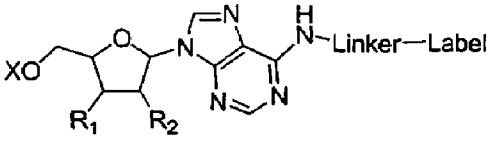
Cytidine C5-linker



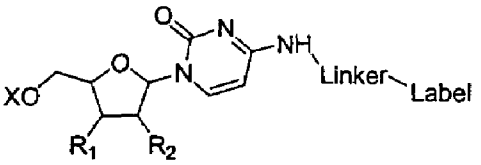
N7 Deazaadenosine C7-linker



N7 Deazaguanosine C7-linker



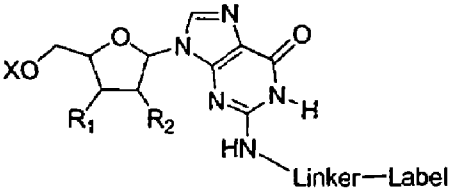
Adenosine N6-linker



Cytidine N4-linker

where R_1 and R_2, which may be the same or different, are each selected from H, OH, or any group which can be transformed into an OH. Suitable groups for R_1 and R_2 are described in Figure 3

X = H, phosphate, diphosphate or triphosphate



Guanosine N2-linker

Fig. 1

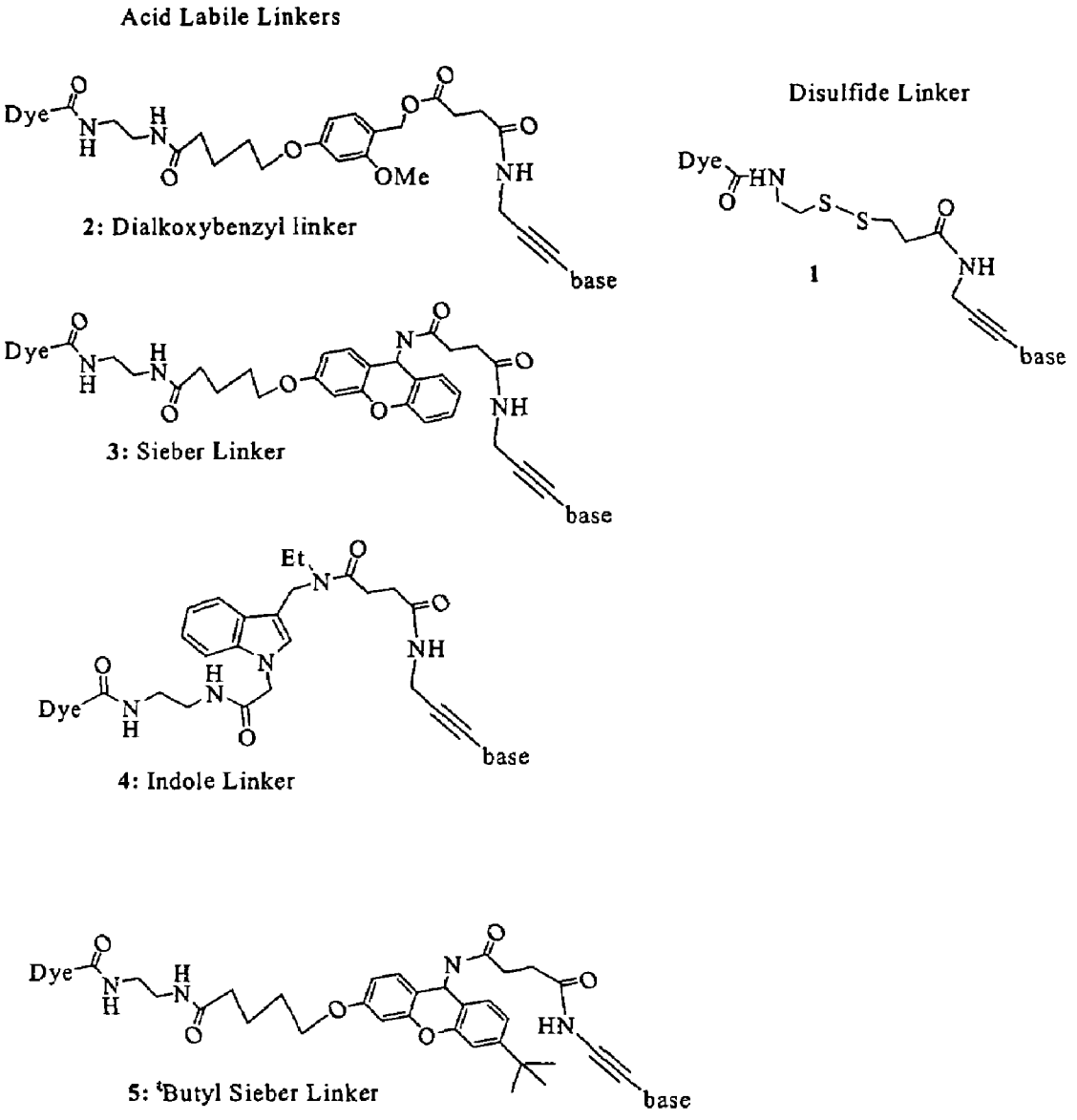
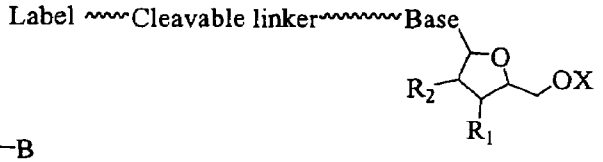
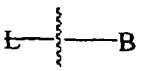
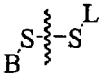

Acid Labile Linkers
Disulfide Linker
2: Dialkoxybenzyl linker
1
3: Sieber Linker
4: Indole Linker
5: tButyl Sieber Linker
Dye
base
OMe
NH
HN
Et

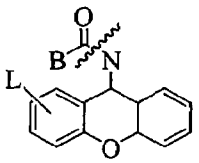
Fig. 2

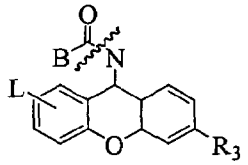


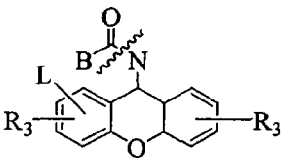


Cleavable linkers may include:







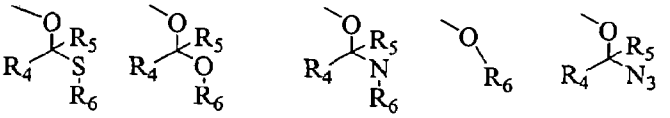


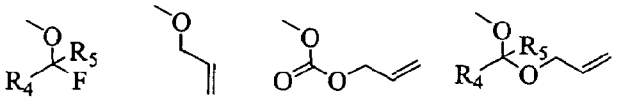
R3 represents one or more substituents independently selected from alkyl, alkoxy, amino or halogen

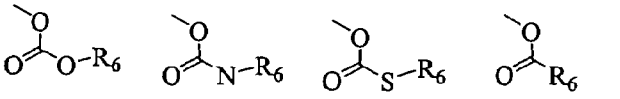
Alternatively, cleavable linkers may be constructed from any labile functionality used on the 3'-block

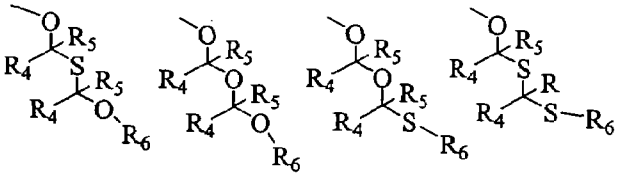
where R_1 and R_2, which may be the same or different, are each selected from H, OH, or any group which can be transformed into an OH, including a carbonyl

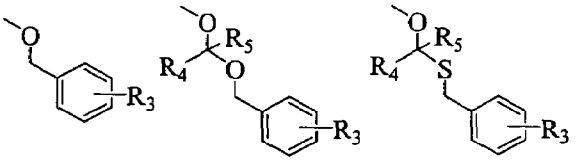
R_1 and R_2 groups may include











where R_4 is H or alkyl, R_5 is H or alkyl and R_6 is alkyl, cycloalkyl, alkenyl, cycloalkenyl or benzyl

and X is H, phosphate, diphosphate or triphosphate

Fig. 3

LABELLED NUCLEOTIDES

RELATED APPLICATIONS

This application is a Divisional Application of U.S. application Ser. No. 10/227,131, now U.S. Pat. No. 7,057,026 filed Aug. 23, 2002 which in turn claims benefit of United Kingdom Application No. GB0129012.1, filed Dec. 4, 2001. The entire teachings of the above applications are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to labelled nucleotides. In particular, this invention discloses nucleotides having a removable label and their use in polynucleotide sequencing methods.

BACKGROUND

Advances in the study of molecules have been led, in part, by improvement in technologies used to characterise the molecules or their biological reactions. In particular, the study of the nucleic acids DNA and RNA has benefited from developing technologies used for sequence analysis and the study of hybridisation events.

An example of the technologies that have improved the study of nucleic acids, is the development of fabricated arrays of immobilised nucleic acids. These arrays consist typically of a high-density matrix of polynucleotides immobilised onto a solid support material. See, e.g., Fodor et al., *Trends Biotech.* 12:19-26, 1994, which describes ways of assembling the nucleic acids using a chemically sensitized glass surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotide phosphoramidites. Fabricated arrays can also be manufactured by the technique of "spotting" known polynucleotides onto a solid support at predetermined positions (e.g., Stimpson et al., *Proc. Natl. Acad. Sci. USA* 92:6379-6383, 1995).

A further development in array technology is the attachment of the polynucleotides to the solid support material to form single molecule arrays. Arrays of this type are disclosed in International Patent App. WO 00/06770. The advantage of these arrays is that reactions can be monitored at the single molecule level and information on large numbers of single molecules can be collated from a single reaction.

For DNA arrays to be useful, the sequences of the molecules must be determined. U.S. Pat. No. 5,302,509 discloses a method to sequence polynucleotides immobilised on a solid support. The method relies on the incorporation of 3'-blocked bases A, G, C and T having a different fluorescent label to the immobilised polynucleotide, in the presence of DNA polymerase. The polymerase incorporates a base complementary to the target polynucleotide, but is prevented from further addition by the 3'-blocking group. The label of the incorporated base can then be determined and the blocking group removed by chemical cleavage to allow further polymerisation to occur.

Welch et al. (*Chem. Eur. J.* 5(3):951-960, 1999) describes the synthesis of nucleotide triphosphates modified with a 3'-O-blocking group that is photolabile and fluorescent. The modified nucleotides are intended for use in DNA sequencing experiments. However, these nucleotides proved to be difficult to incorporate onto an existing polynucleotide, due to an inability to fit into the polymerase enzyme active site.

Zhu et al. (*Cytometry* 28:206-211, 1997) also discloses the use of fluorescent labels attached to a nucleotide via the base group. The labelled nucleotides are intended for use in fluorescence in situ hybridisation (FISH) experiments, where a series of incorporated labelled nucleotides is required to produce a fluorescent "bar code".

SUMMARY OF THE INVENTION

In the present invention, a nucleoside or nucleotide molecule is linked to a detectable label via a cleavable linker group attached to the base, rendering the molecule useful in techniques using Labelled nucleosides or nucleotides, e.g., sequencing reactions, polynucleotide synthesis, nucleic acid amplification, nucleic acid hybridization assays, single nucleotide polymorphism studies, and other techniques using enzymes such as polymerases, reverse transcriptases, terminal transferases, or other DNA modifying enzymes. The invention is especially useful in techniques that use Labelled dNTPs, such as nick translation, random primer labeling, end-labeling (e.g., with terminal deoxynucleotidyltransferase), reverse transcription, or nucleic acid amplification. The molecules of the present invention are in contrast to the prior art, where the label is attached to the ribose or deoxyribose sugar, or where the label is attached via a non-cleavable linker.

According to a first aspect of the invention, a nucleotide or nucleoside molecule, or an analog thereof, has a base that is linked to a detectable label via a cleavable linker.

The invention features a nucleotide or nucleoside molecule, having a base that is linked to a detectable label via a cleavable linker. The base can be a purine, or a pyrimidine. The base can be a deazapurine. The molecule can have a ribose or deoxyribose sugar moiety. The ribose or deoxyribose sugar can include a protecting group attached via the 2' or 3' oxygen atom. The protecting group can be removed to expose a 3'-OH. The molecule can be a deoxyribonucleotide triphosphate. The detectable label can be a fluorophore. The linker can be an acid labile linker, a photolabile linker, or can contain a disulphide linkage.

The invention also features a method of labeling a nucleic acid molecule, where the method includes incorporating into the nucleic acid molecule a nucleotide or nucleoside molecule, where the nucleotide or nucleoside molecule has a base that is linked to a detectable label via a cleavable linker. The incorporating step can be accomplished via a terminal transferase, a polymerase or a reverse transcriptase. The base can be a purine, or a pyrimidine. The base can be a deazapurine. The nucleotide or nucleoside molecule can have a ribose or deoxyribose sugar moiety. The ribose or deoxyribose sugar can include a protecting group attached via the 2' or 3' oxygen atom. The protecting group can be removed to expose a 3'-OH group. The molecule can be a deoxyribonucleotide triphosphate. The detectable label can be a fluorophore. The linker can be an acid labile linker, a photolabile linker, or can contain a disulphide linkage. The detectable label and/or the cleavable linker can be of a size sufficient to prevent the incorporation of a second nucleotide or nucleoside into the nucleic acid molecule.

In another aspect, the invention features a method for determining the sequence of a target single-stranded polynucleotide, where the method includes monitoring the sequential incorporation of complementary nucleotides, where the nucleotides each have a base that is linked to a detectable label via a cleavable linker, and where the identity of each nucleotide incorporated is determined by detection of the label linked to the base, and subsequent removal of the label.

The invention also features a method for determining the sequence of a target single-stranded polynucleotide, where the method includes: (a) providing nucleotides, where the nucleotides have a base that is linked to a detectable label via a cleavable linker, and where the detectable label linked to each type of nucleotide can be distinguished upon detection from the detectable label used for other types of nucleotides; (b) incorporating a nucleotide into the complement of the target single stranded polynucleotide; (c)detecting the label of the nucleotide of (b), thereby determining the type of nucleotide incorporated; (d) removing the label of the nucle-

otide of (b); and (e) optionally repeating steps (b)-(d) one or more times; thereby determining the sequence of a target single-stranded polynucleotide.

In the methods described herein, each of the nucleotides can be brought into contact with the target sequentially, with removal of non-incorporated nucleotides prior to addition of the next nucleotide, where detection and removal of the label is carried out either after addition of each nucleotide, or after addition of all four nucleotides.

In the methods, all of the nucleotides can be brought into contact with the target simultaneously, i.e., a composition comprising all of the different nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label(s).

The methods can comprise a first step and a second step, where in the first step, a first composition comprising two of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where in the second step, a second composition comprising the two nucleotides not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where the first steps and the second step can be optionally repeated one or more times.

The methods described herein can also comprise a first step and a second step, where in the first step, a composition comprising one of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where in the second step, a second composition. comprising the three nucleotides not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where the first steps and the second step can be optionally repeated one or more times.

The methods described herein can also comprise a first step and a second step, where in the first step, a first composition comprising three of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where in the second step, a composition comprising the nucleotide not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and where the first steps and the second step can be optionally repeated one or more times.

In a further aspect, the invention features a kit, where the kit includes: (a) individual the nucleotides, where each nucleotide has a base that is linked to a detectable label via a cleavable linker, and where the detectable label linked to each nucleotide can be distinguished upon detection from the detectable label used for other three nucleotides; and (b) packaging materials therefor. The kit can further include an enzyme and buffers appropriate for the action of the enzyme.

The nucleotides/nucleosides are suitable for use in many different DNA-based methodologies, including DNA synthesis and DNA sequencing protocols.

According to another aspect of the invention, a method for determining the sequence of a target polynucleotide comprises monitoring the sequential incorporation of complementary nucleotides, wherein the nucleotides comprise a detectable label linked to the base portion of the nucleotide via a cleavable linker, incorporation is detected by monitoring the label, and the label is removed to permit further nucleotide incorporation to occur.

DESCRIPTION OF THE DRAWINGS

FIG. **1** shows examplary nucleotide structures useful in the invention. For each structure, X can be H, phosphate, diphosphate or triphosphate. R_1 and R_2 can be the same or different, and can be selected from H, OH, or any group which can be transformed into an OH, including, but not limited to, a carbonyl. Some suitable functional groups for R_1 and R_2 include the structures shown in FIG. **3**.

FIG. **2** shows structures of linkers useful in the invention, including (1) disulfide linkers and acid labile linkers, (2) dialkoxybenzyl linkers, (3) Sieber linkers, (4) indole linkers and (5) t-butyl Sieber linkers.

FIG. **3** shows some functional molecules useful in the invention, including some cleavable linkers and some suitable hydroxyl protecting groups. In these structures, R_1 and R_2 may be the same of different, and can be H, OH, or any group which can be transformed into an OH group, including a carbonyl. R_3 represents one or more substituents independently selected from alkyl, alkoxyl, amino or halogen groups. Alternatively, cleavable linkers may be constructed from any labile functionality used on the 3'-block. R_4 and R_5 can be H or alkyl, and R_6 can be alkyl, cycloalkyl, alkenyl, cycloalkenyl or benzyl. X can be H, phosphate, diphosphate or triphosphate.

FIG. **4** shows a denaturing gel showing the incorporation of the triphosphate of Example 1 using Klenow polymerase.

FIG. **5** shows a denaturing gel showing the incorporation of the triphosphate of Example 3 using Klenow polymerase.

FIG. **6** shows a denaturing gel showing the incorporation of the triphosphate of Example 4 using Klenow polymerase.

DETAILED DESCRIPTION

The present invention relates to nucleotides and nucleosides that are modified by attachment of a label via a cleavable linker, thereby rendering the molecule useful in techniques where the labelled molecule is to interact with an enzyme, such as sequencing reactions, polynucleotide synthesis, nucleic acid amplification, nucleic acid hybridization assays, single nucleotide polymorphism studies, techniques using enzymes such as polymerase, reverse transcriptase, terminal transferase, techniques that use Labelled dNTPs (e.g., nick translation, random primer labeling, end-labeling (e.g., with terminal deoxynucleotidyltransferase), reverse transcription, or nucleic acid amplification).

As is known in the art, a "nucleotide" consists of a nitrogenous base, a sugar, and one or more phosphate groups. In RNA, the sugar is a ribose, and in DNA is a deoxyribose, i. e., a sugar lacking a hydroxyl group that is present in ribose. The nitrogenous base is a derivative of purine or pyrimidine. The purines are adenosine (A) and guanidine (G), and the pyrimidines are cytidine (C) and thymidine (T) (or in the context of RNA, uracil (U)). The C-1 atom of deoxyribose is bonded to N-1 of a pyrimidine or N-9 of a purine. A nucleotide is also a phosphate ester of a nucleoside, with esterification occurring on the hydroxyl group attached to C-5 of the sugar. Nucleotides are usually mono, di- or triphosphates.

A "nucleoside" is structurally similar to a nucleotide, but are missing the phosphate moieties. An example of a nucleoside analog would be one in which the label is linked to the base and there is no phosphate group attached to the sugar molecule.

Although the base is usually referred to as a purine or pyrimidine, the skilled person will appreciate that derivatives and analogs are available which do not alter the capability of the nucleotide or nucleoside to undergo Watson-Crick base

pairing. "Derivative" or "analog" means a compound or molecule whose core structure is the same as, or closely resembles that of, a parent compound, but which has a chemical or physical modification, such as a different or additional side groups, which allows the derivative nucleotide or nucleoside to be linked to another molecule. For example, the base can be a deazapurine. The derivatives should be capable of undergoing Watson-Crick pairing. "Deivative" and "analog" also mean a synthetic nucleotide or nucleoside derivative having modified base moieties and/or modified sugar moieties. Such derivatives and analogs are discussed in, e.g., Scheit, *Nucleotide Analogs* (John Wiley & Son, 1980) and Uhlman et al., *Chemical Reviews* 90:543-584, 1990. Nucleotide analogs can also comprise modified phosphodiester linkages, including phosphorothioate, phosphorodithioate, alkylphosphonate, phosphoranilidate and phosphoramidate linkages. The analogs should be capable of undergoing Watson-Crick base pairing. "Derivative" and "analog", as used herein, may be used interchangeably, and are encompassed by the terms "nucleotide" and "nucleoside" as defined herein.

The present invention can make use of conventional detectable labels. Detection can be carried out by any suitable method, including fluorescence spectroscopy or by other optical means. The preferred label is a fluorophore, which, after absorption of energy, emits radiation at a defined wavelength. Many suitable fluorescent labels are known. For example, Welch et al. (*Chem. Eur. J.* 5(3):951-960, 1999) discloses dansyl-functionalised fluorescent moieties that can be used in the present invention. Zhu et al. (*Cytometry* 28:206-211, 1997) describes the use of the fluorescent labels Cy3 and Cy5, which can also be used in the present invention. Labels suitable for use are also disclosed in Prober et al. (*Science* 238:336-341, 1987); Connell et al. (*BioTechniques* 5(4):342-384, 1987), Ansorge et al. (*Nucl. Acids Res.* 15(11): 4593-4602, 1987) and Smith et al. (*Nature* 321:674, 1986). Other commercially available fluorescent labels include, but are not limited to, fluorescein, rhodamine (including TMR, texas red and Rox), alexa, bodipy, acridine, coumarin, pyrene, benzanthracene and the cyanins.

Multiple labels can also be used in the invention. For example, bi-fluorophore FRET cassettes (*Tet. Letts.* 46:8867-8871, 2000) are well known in the art and can be utilised in the present invention. Multi-fluor dendrimeric systems (*J. Amer. Chem. Soc.* 123:8101-8108, 2001) can also be used.

Although fluorescent labels are preferred, other forms of detectable labels will be apparent as useful to those of ordinary skill. For example, microparticles, including quantum dots (Empodocles, et al., *Nature* 399:126-130, 1999), gold nanoparticles (Reichert et al., *Anal. Chem.* 72:6025-6029, 2000), microbeads (Lacoste et al., *Proc. Natl. Acad. Sci USA* 97(17):9461-9466, 2000), and tags detectable by mass spectrometry can all be used.

Multi-component labels can also be used in the invention. A multi-component label is one which is dependent on the interaction with a further compound for detection. The most common multi-component label used in biology is the biotin-streptavidin system. Biotin is used as the label attached to the nucleotide base. Streptavidin is then added separately to enable detection to occur. Other multi-component systems are available. For example, dinitrophenol has a commercially available fluorescent antibody that can be used for detection.

The label (or label and linker construct) can be of a size or structure sufficient to act as a block to the incorporation of a further nucleotide onto the nucleotide of the invention. This permits controlled polymerization to be carried out. The block can be due to steric hindrance, or can be due to a combination of size, charge and structure.

The invention will be further described with reference to nucleotides. However, unless indicated otherwise, the reference to nucleotides is also intended to be applicable to nucleosides. The invention will also be further described with reference to DNA, although the description will also be applicable to RNA, PNA, and other nucleic acids, unless otherwise indicated.

The modified nucleotides of the invention use a cleavable linker to attach the label to the nucleotide. The use of a cleavable linker ensures that the label can, if required, be removed after detection, avoiding any interfering signal with any labelled nucleotide incorporated subsequently.

Cleavable linkers are known in the art, and conventional chemistry can be applied to attach a linker to a nucleotide base and a label. The linker can be cleaved by any suitable method, including exposure to acids, bases, nucleophiles, electrophiles, radicals, metals, reducing or oxidising agents, light, temperature, enzymes etc. Suitable linkers can be adapted from standard chemical blocking groups, as disclosed in Greene & Wuts, *Protective Groups in Organic Synthesis*, John Wiley & Sons. Further suitable cleavable linkers used in solid-phase synthesis are disclosed in Guillier et al. (*Chem. Rev.* 100:2092-2157, 2000).

The use of the term "cleavable linker" is not meant to imply that the whole linker is required to be removed from the nucleotide base. The cleavage site can be located at a position on the linker that ensures that part of the linker remains attached to the nucleotide base after cleavage.

The linker can be attached at any position on the nucleotide base provided that Watson-Crick base pairing can still be carried out. In the context of purine bases, it is preferred if the linker is attached via the 7 position of the purine or the preferred deazapurine analogue, via an 8-modified purine, via an N-6 modified adenosine or an N-2 modified guanine. For pyrimidines, attachment is preferably via the 5 position on cytidine, thymidine or uracil and the N-4 position on cytosine. Suitable nucleotide structures are shown in FIG. **1**. For each structure in FIG. **1**, X can be H, phosphate, diphosphate or triphosphate. R_1 and R_2 can be the same or different, and can be selected from H, OH, or any group which can be transformed into an OH, including, but not limited to, a carbonyl. Some suitable functional groups for R_1 and R_2 include the structures shown in FIG. **3**.

Suitable linkers are shown in FIG. **2** and include, but are not limited to, disulfide linkers (1), acid labile linkers (2, 3, 4 and 5; including dialkoxybenzyl linkers (e.g., 2), Sieber linkers (e.g., 3), indole linkers (e.g., 4), t-butyl Sieber linkers (e.g., 5)), electrophilically cleavable linkers, nucleophilically cleavable linkers, photocleavable linkers, cleavage under reductive conditions, oxidative conditions, cleavage via use of safety-catch linkers, and cleavage by elimination mechanisms.

A. Electrophilically Cleaved Linkers.

Electrophilically cleaved linkers are typically cleaved by protons and include cleavages sensitive to acids. Suitable linkers include the modified benzylic systems such as trityl, p-alkoxybenzyl esters and p-alkoxybenzyl amides. Other suitable linkers include tert-butyloxycarbonyl (Boc) groups and the acetal system (e.g., as is shown in FIG. **3** as O—C(R_4)(R_5)—O—R_6.

The use of thiophilic metals, such as nickel, silver or mercury, in the cleavage of thioacetal or other sulphur-containing protecting groups can also be considered for the preparation of suitable linker molecules.

B. Nucleophilically Cleaved Linkers.

Nucleophilic cleavage is also a well recognised method in the preparation of linker molecules. Groups such as esters that are labile in water (i.e., can be cleaved simply at basic pH) and groups that are labile to non-aqueous nucleophiles, can be used. Fluoride ions can be used to cleave silicon-oxygen bonds in groups such as triisopropyl silane (TIPS) or t-butyldimethyl silane (TBDMS).

C. Photocleavable Linkers.

Photocleavable linkers have been used widely in carbohydrate chemistry. It is preferable that the light required to activate cleavage does not affect the other components of the modified nucleotides. For example, if a fluorophore is used as the label, it is preferable if this absorbs light of a different wavelength to that required to cleave the linker molecule. Suitable linkers include those based on O-nitrobenyl compounds and nitroveratryl compounds. Linkers based on benzoin chemistry can also be used (Lee et al., *J. Org. Chem.* 64:3454-3460, 1999).

D. Cleavage Under Reductive Conditions

There are many linkers known that are susceptible to reductive cleavage. Catalytic hydrogenation using palladium-based catalysts has been used to cleave benzyl and benzyloxycarbonyl groups. Disulphide bond reduction is also known in the art.

E. Cleavage Under Oxidative Conditions

Oxidation-based approaches are well known in the art. These include oxidation of p-alkoxybenzyl groups and the oxidation of sulphur and selenium linkers. The use of aqueous iodine to cleave disulphides and other sulphur or selenium-based linkers is also within the scope of the invention.

F. Safety-Catch Linkers

Safety-catch linkers are those that cleave in two steps. In a preferred system the first step is the generation of a reactive nucleophilic center followed by a second step involving an intra-molecular cyclization that results in cleavage. For example, levulinic ester linkages can be treated with hydrazine or photochemistry to release an active amine, which can then be cyclised to cleave an ester elsewhere in the molecule (Burgess et al., *J. Org. Chem.* 62:5165-5168, 1997).

G. Cleavage by Elimination Mechanisms

Elimination reactions can also be used. For example, the base-catalysed elimination of groups such as Fmoc and cyanoethyl, and palladium-catalysed reductive elimination of allylic systems, can be used.

As well as the cleavage site, the linker can comprise a spacer unit. The spacer distances the nucleotide base from the cleavage site or label. The length of the linker is unimportant provided that the label is held a sufficient distance from the nucleotide so as not to interfere with any interaction between the nucleotide and an enzyme.

The modified nucleotides can also comprise additional groups or modifications to the sugar group. For example, a dideoxyribose derivative, lacking two oxygens on the ribose ring structure (at the 2' and 3' positions), can be prepared and used as a block to further nucleotide incorporation on a growing oligonucleotide strand. The ribose ring can also be modified to include a protecting group at the 3' position or a group that can be transformed or modified to form a 3' OH group. The protecting group is intended to prevent nucleotide incorporation onto a nascent polynucleotide strand, and can be removed under defined conditions to allow polymerisation to occur. In contrast to the prior art, there is no detectable label attached at the ribose 3' position. This ensures that steric hindrance with the polymerase enzyme is reduced, while still allowing control of incorporation using the protecting group.

The skilled person will appreciate how to attach a suitable protecting group to the ribose ring to block interactions with the 3'-OH. The protecting group can be attached directly at the 3' position, or can be attached at the 2' position (the protecting group being of sufficient size or charge to block interactions at the 3' position). Alternatively, the protecting group can be attached at both the 3' and 2' positions, and can be cleaved to expose the 3' OH group.

Suitable protecting groups will be apparent to the skilled person, and can be formed from any suitable protecting group disclosed in Green and Wuts, supra. Some examples of such protecting groups are shown in FIG. **3**. The protecting group should be removable (or modifiable) to produce a 3' OH group. The process used to obtain the 3' OH group can be any suitable chemical or enzymic reaction.

The labile linker may consist of functionality cleavable under identical conditions to the block. This will make the deprotection process more efficient as only a single treatment will be required to cleave both the label and the block. Thus the linker may contain functional groups as described in FIG. **3**, which could be cleaved with the hydroxyl functionality on either the residual nucleoside or the removed label. The linker may also consist of entirely different chemical functionality that happens to be labile to the conditions used to cleave the block.

The term "alkyl" covers both straight chain and branched chain alkyl groups. Unless the context indicates otherwise, the term "alkyl" refers to groups having 1 to 8 carbon atoms, and typically from 1 to 6 carbon atoms, for example from 1 to 4 carbon atoms. Examples of alkyl groups include methyl, ethyl, propyl, isopropyl, n-butyl, isobutyl, tert-butyl, n-pentyl, 2-pentyl, 3-pentyl, 2-methyl butyl, 3-methyl butyl, and n-hexyl and its isomers.

Examples of cycloalkyl groups are those having from 3 to 10 ring atoms, particular examples including those derived from cyclopropane, cyclobutane, cyclopentane, cyclohexane and cycloheptane, bicycloheptane and decalin.

Examples of alkenyl groups include, but are not limited to, ethenyl (vinyl), 1-propenyl, 2-propenyl (allyl), isopropenyl, butenyl, buta-1,4-dienyl, pentenyl, and hexenyl.

Examples of cycloalkenyl groups include, but are not limited to, cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadienyl and cyclohexenyl.

The term alkoxy refers to C_{1-6} alkoxy unless otherwise indicated: —OR, wherein R is a C_{1-6}alkyl group. Examples of C_{1-6} alkoxy groups include, but are not limited to, —OMe (methoxy), —OEt (ethoxy), —O(nPr) (n-propoxy), —O(iPr) (isopropoxy), —O(nBu) (n-butoxy), —O(sBu) (sec-butoxy), —O(iBu) (isobutoxy), and —O(tBu) (tert-butoxy).

The term amino refers to groups of type NR^1R^2, wherein R^1 and R^2 are independently selected from hydrogen, a C_{1-6} alkyl group (also referred to as C_{1-6} alkylamino or di-C_{1-6} alkylamino).

The term "halogen" as used herein includes fluorine, chlorine, bromine and iodine.

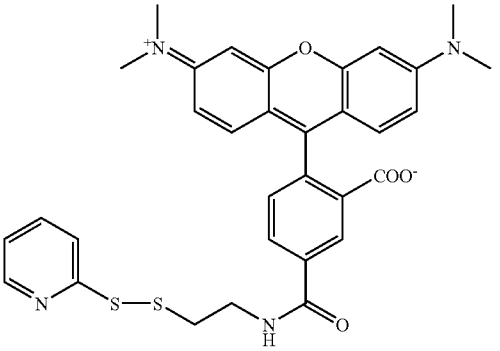
The nucleotide molecules of the present invention are suitable for use in many different methods where the detection of nucleotides is required.

DNA sequencing methods, such as those outlined in U.S. Pat. No. 5,302,509 can be carried out using the nucleotides.

A method for determining the sequence of a target polynucleotide can be carried out by contacting the target polynucleotide separately with the different nucleotides to form the complement to that of the target polynucleotide, and detecting the incorporation of the nucleotides. Such a method makes use of polymerisation, whereby a polymerase enzyme extends the complementary strand by incorporating the correct nucleotide complementary to that on the target. The polymerisation reaction also requires a specific primer to initiate polymerisation.

For each cycle, the incorporation of the labelled nucleotide is carried out by the polymerase enzyme, and the incorporation event is then determined. Many different polymerase enzymes exist, and it will be evident to the person of ordinary skill which is most appropriate to use. Preferred enzymes include DNA polymerase I, the Klenow fragment, DNA polymerase III, T4 or T7 DNA polymerase, Taq polymerase or vent polymerase. A polymerase engineered to have specific properties can also be used.

-continued



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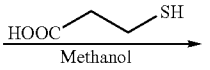

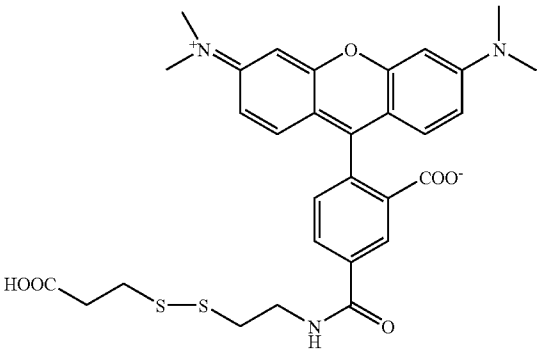

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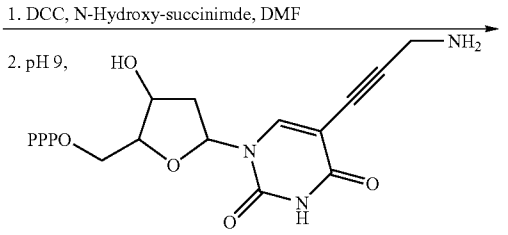

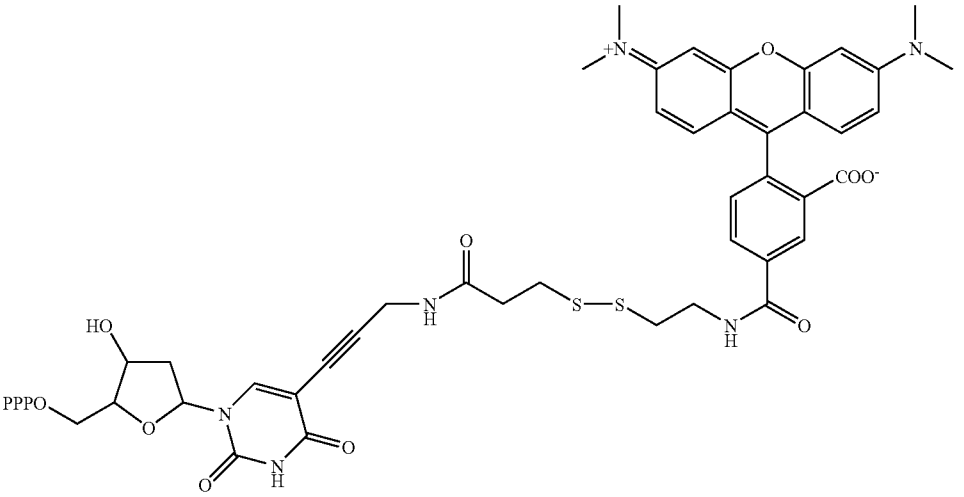

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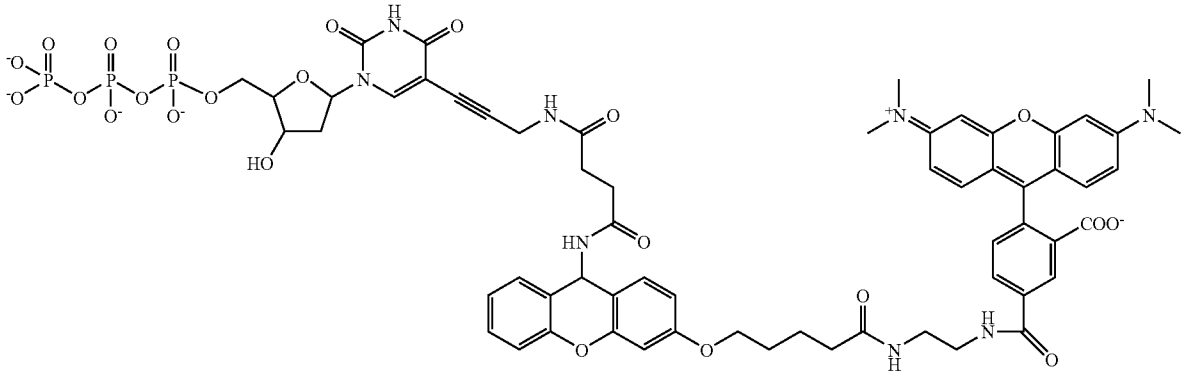
Example 3

Synthesis of TMR-Sieber Linker-dUTP (3)

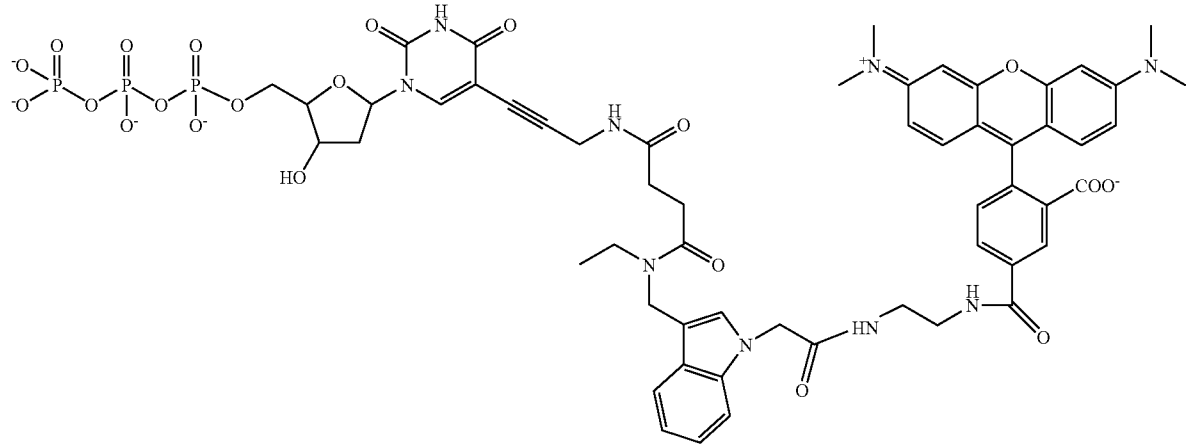


TMR-sieber linker free acid (4.34 mg, 5 μmol) was stirred with disuccinimidyl carbonate (1.74 mg, 7.5 μmol) and N,N-dimethyl aminopyridine (0.92 mg, 7.5 μmol) in DMF (1 mL) at room temperature. After 10 minutes, all the reaction mixture was added to tetra-(tri-butylammonium) salt of 5-(3-aminopropynyl)-2'-deoxyuridine-5'-triphosphate (10 μmol). The reaction was stirred at room temperature for 4 hrs and stored in the fridge overnight. The reaction mixture was then diluted with chilled water (10 mL) and all the resulting solution was applied onto a short column of DEAE A-25. The column was initially eluted with 0.1 M TEAB buffer and then 0.7 M TEAB buffer. The 0.7 M TEAB eluents were collected and evaporated under reduced pressure. The residue was co-evaporated with MeOH (2×10 mL) and then purified by preparative HPLC. The title compound was obtained as triethylammonium salt in 31% yield (based on the quantification of TMR at 555 nm in water (pH 7)). ^{1}HNMR in D_2O indicated two diastereoisomers, due to the sieber linker moiety and there were approximately three triethylammonium count ions. ^{1}HNMR [D_2O]: 8.18 (1H, m), 8.06 (1H, m), 7.76 (0.55H, s), 7.74 (0.45H, s), 7.36-7.09 (5H, m), 6.89-6.72 (3H, m), 6.59-6.37 (5H, m), 6.12 (0.55H, t, J 6.6), 6.05 (0.45H, t, J 6.6), 5.99 (0.45H, d, J 2.5), 5.91 (1.1H, m), 5.88 (0.45H, s), 4.49 (0.55H, m), 4.43 (0.45H, m), 4.00-3.35 (9H, m), 3.30-2.95 (32H, m), 2.65-2.52 (4H, m), 2.25-2.05 (4H, m), 1.62-1.42 (4H, m) and 1.23 (27H, t, J 7.3). ^{31}P [D_2O]: −9.91 ($^{\gamma}$P, d, J 19.2), [−11.08($^{\alpha}$P, d, J20.1) and −11.30 ($^{\alpha}$P, d, J 20.1), due to two diastereoisomers] and −22.57 ($^{\beta}$P, m). MS[(ES(−)], m/z 1369.1 (M^-).

Triphosphate (3) was successfully incorporated using Klenow DNA polymerase. The reaction was performed in the following conditions: 50 mM Tris.HCl (pH 7.5), 10 mM NaCl, 2 mM DTT, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 μM compound 3, 100 nM DNA template (previously labelled with P32 and T4 polynucleotide kinase) and 10 units of commercial exo-Klenow (Amersham Corp. Arlington Heights, Illinois, USA). The DNA templates were self-complementary hairpins (5'-TACCgTCgACgTCgACgCTggCgAgCgT-gCTgCggTTTTT(C6-amino)TTACCgCAgCACgCTCgC-CAgCg; SEQ ID NO:1). The reaction was performed in 100 μL volume at 37° C. with timepoints taken at 0, 1, 3, 5 and 10 min. The reaction products were electrophoresed down a denaturing (8 M urea) 20% polyacrylamide gel and imaged on a typhoon phosphorimager. Complete single base extension was seen in 1 minute indicating efficient polymerase incorporation (Sieber linker gel, FIG. **4**).

Example 4

Synthesis of TMR-Indole Linker-dUTP (4)



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Directly labeled DNA probes using fluorescent nucleotides with different length linkers

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ABSTRACT

Directly labeled fluorescent DNA probes have been made by nick translation and PCR using dUTP attached to the fluorescent label, Cy3, with different length linkers. With preparation of probes by PCR we find that linker length affects the efficiency of incorporation of Cy3-dUTP, the yield of labeled probe, and the signal intensity of labeled probes hybridized to chromosome target sequences. For nick translation and PCR, both the level of incorporation and the hybridization fluorescence signal increased in parallel when the length of the linker arm is increased. Under optimal conditions, PCR yielded more densely labeled probes, however, the yield of PCR labeled probe decreased with greater linear density of labeling. By using a Cy3-modified dUTP with the longest linker under optimal conditions it was possible to label up to 28% of the possible substitution sites on the target DNA with reasonable yield by PCR and 18% by nick translation. A mechanism involving steric interactions between the polymerase, cyanine-labeled sites on template and extending chains and the modified dUTP substrate is proposed to explain the inverse correlation between the labeling efficiency and the yield of DNA probe synthesis by PCR.

INTRODUCTION

Since its invention, (1,2) the technique of *in situ* hybridization (ISH) has become increasingly important in biological and diagnostic research (3). Fluorescent labels have gradually replaced the radioisotopic ones due to safety concerns, high spatial resolution, long shelf life, short detection time and simultaneous detection of multiple sequences in single cells. DNA probes are commonly labeled by nick translation (3, 4, 5). In recent years polymerase chain reaction (PCR) has been introduced to synthesize DNA probes (6, 7).

Most fluorescent *in situ* hybridization (FISH) experiments have been done using indirectly reagents, such as avidins and antibodies, for visualization of probe signals (3, 4). This requires a post-hybridization labeling step, which is time consuming and may increase background fluorescence. Additional signal amplification steps are possible to increase the intensity of the signal (3, 4), but again, background fluorescence may increase. If fluorophores are directly attached to DNA, extra steps can be eliminated and signal-to-background ratio may be increased even if the overall fluorescence intensity is somewhat reduced compared with indirect labeling.

In our previous study (8), Cy3 covalently conjugated through a short linker to dUTP was shown to be incorporated into DNA by nick translation and PCR. Other investigators have shown that if a linker molecule is inserted between biotin and a nucleotide molecule for nick translation, the labeling efficiency and hybridization detectability of Southern blotting experiment are improved (9, 10). Here we report systematic study of linker length on DNA probe labeling efficiency, yield of the PCR synthesis, and FISH signal intensity using Cy3 linked to dUTP.

MATERIALS AND METHODS

Modified dUTPs

Cy3.29.OSu synthesized as described previously (11), contains 6 atoms between the fluorophore ring system and the active ester group. For addition of 7 atoms to the linker, a mixture of 100 mg Cy3.29.OSu and 18 mg 6-amino caproic acid (Aldrich) was dissolved in 15 ml sodium bicarbonate buffer (0.1M, pH = 9.4) and stirred at room temperature overnight. The product Cy3.29-13-OH (13 denotes the number of atoms between fluorophore ring system and carboxylic group) was isolated by reversed-phase (RP) chromatography (C-18) using water-methanol mixture as eluent and dried down.

Cy3.29-13-OH was activated to succinimidyl ester, Cy3.29-13-OSu, by methods used to prepare Cy3.29.OSu (11). The same procedure was repeated to conjugate another 6-amino caproic acid with Cy3.29-13-OSu to obtain Cy3.29-20-OH and to form the activated Cy3.29-20-OSu. The chemical structures were confirmed by ^{1}H NMR. Cy3-x-dUTP (x = 10, 17, 24) was synthesized as described (8) The concentrations were determined by absorbance measurement at 550 nm using the extinction coefficient of Cy3, 150,000 L/mol.cm (11).

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PCR

PCR was generally carried out according to procedure of Yu (8). The PCR reaction mixture contains 1.5mM of $MgCl_2$.

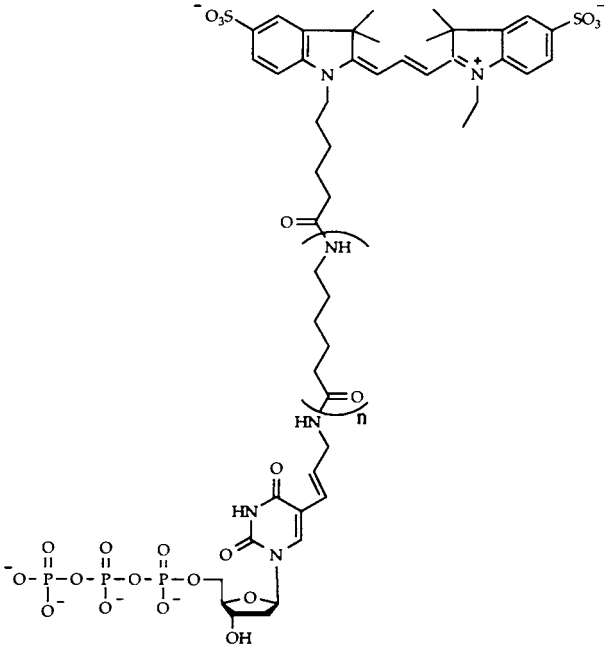
When a certain amount of modified dUTP was used instead of dTTP, the total concentration of modified dUTP and dTTP was kept the same as the concentration of each of other 3 nucleotides because the maximum fidelity of polymerization requires a balanced concentration of each of the 4 dNTPs (12).

The template for all nick translation and PCR experiments was a 900bp chromosome 1 insert (ATCC cat. no. 59863) in plasmid DNA. The primers were two ends of the vector (8), which leads to amplification of the entire 900bp insert. For quantification of DNA synthesis and Cy3 incorporation, the 5′-end of each primer was labeled with Cy5.18.OSu as previously described (13).

PCR was performed in a DNA Thermal Cycler (Perkin Elmer Cetus). The temperature cycling parameters were similar to the ones used by Ried *et al.* (14). After an initial denaturation at 94°C for 4 min, 30 cycles (except cycle number study) of PCR were carried out with denaturation at 94°C for 1 min, annealing at 52°C for 2 min and extension at 72°C for 3 min. Prep-A-Gene method (BioRad) was used for purification to remove unreacted Cy3-x-dUTP and excess Cy5 labeled primers. The molecular weight of PCR product was examined using a 1% agarose gel with 0.5 μg/ml ethidium bromide.

In order to obtain the labeling efficiency and the yield of PCR, the product was dissolved in 140 μl sterile water for fluorescence measurement of Cy3 and Cy5 with a Spex Fluorolog 2 spectrofluorometer (Spex Industries, Inc.). Calibration of fluorescence intensities was accomplished by measuring the fluorescence of known concentration of Cy3 and Cy5, both in a purified carboxylic acid form. Using the calibration data, the Cy3 and Cy5 emission intensities of labeled DNA were converted to Cy3 and Cy5 molar concentrations. Since Cy5 concentration is identical to the concentration of DNA (use of Cy5 labeled primers), it was possible to calculate from fluorescence measurements the amount of DNA produced in PCR, which was taken as the yield of the reaction. The Cy3/Cy5 molar ratio corresponds to the number of Cy3 molecules per DNA chain of PCR product. Assuming 1/4 of bases of this DNA are thymine, the Cy3/Cy5 molar ratio divided by 225 ($\frac{900b}{4}$) can be used as an estimate of the degree of substitution of Cy3-U (henceforth U*) for T in product DNA, defined as S_{Prod}. Thus, $S_{Prod} = \frac{U^*}{U^*+T}$. The quantum yield (Q. Y.) of Cy3-labeled DNA was determined as previously described (15).

Since the 900 bp labeled DNA probes are too long for optimal FISH, the PCR product was incubated with 1/350 unit of DNase I in nick translation buffer at 37°C for 5 min. A 1% agarose gel with 0.5 μg/ml ethidium bromide was used to check the chain length (Optimal length is 200−400 bp (3, 4).). The DNA fragments were ethanol-precipitated and reconstituted in 150 μl sterile water. The ratio of substitution and amount of DNA fragments were determined again by absorption spectroscopy with a HP 8452 diode array spectrophotometer (Hewelett-Packard). The average extinction coefficient of a base in double stranded DNA at 260 nm was 6500 L/mol.cm (16).



n = 0 Cy3-10-dUTP

n = 1 Cy3-17-dUTP

n = 2 Cy3-24-dUTP

Figure 1. Chemical structures of Cy3-modified dUTPs.

Labeling probes by nick translation

Nick translation was carried out under sequential reaction conditions found to be optimal for cyanine-modified dUTPs (8).

In situ hybridization and signal analysis

A common procedure was used for FISH and an imaging microscope system was used to quantify hybridization signals (8). For each slide, at least 24 FISH signals were collected. Hybridization signals were analyzed by 'spot analysis' software. First an algorithm was used to define the boundaries of each hybridization spot. Then fluorescence signals within the spot were quantified and an average background measurement was made in an area outside of the spot boundary where the spot signal was no longer significant. Two parameters were used to describe the FISH signals. The net spot intensity (NSI) was the sum of all pixel intensities within the spot minus the background net intensity of an area equivalent to the spot. The signal-to-background ratio (S/b) is the NSI divided by the background intensity of an area equivalent to the spot.

RESULTS AND DISCUSSION

PCR and nick translation reactions vary from day-to-day depending on the activity of the enzyme and reaction conditions. Therefore experiments presented in the same figure or same table have been done in the same batch for maximum consistency. The same trends were observed for data obtained on different days

The chemical structures of Cy3-modified dUTPs (dU*TPs) are shown in Fig. 1. In this work we are concerned with optimizing the substitution efficiency and the yield of DNA probes directly labeled by PCR or nick translation with dU*TPs. The key variables under our control are the length of the linker arm between the ring structure of the fluorescent label and the uracil

Increasing the linker length also favors greater incorporation of dU*TP (Fig. 2a). It is possible that longer linkers reduce steric interactions between the Cy3 molecule and the polymerase−DNA complex thus making dU*TP with longer linkers better substrates.

Although the efficiency of incorporation of modified bases by PCR improves as linker length or S_{Rxn} increases, the yield of labeled probe generated by multiple PCR cycles is reduced, and when 100% dU*TP was used instead of dTTP, there is no amplification (Fig. 2b). We found that the longer extension time does not raise S_{Prod} and the yield actually decreases. This may be caused by the loss of enzyme activity during long incubation at 72°C. An extension time as short as 1 minute does not affect the labeling efficiency and yield.

Why does the yield of Cy3-labeled probe in a PCR reaction decrease when the labeling density increases? One explanation is based on the steric considerations. When more U* is incorporated into a DNA probe, there is a greater possibility of modified bases appearing in close proximity to one-another. In the next cycle, the labeled chain serves as the template. The presence of several Cy3 fluorophores in a small space on the template (or the template and extending chain) may inhibit the progression of the polymerase and lead to termination of the probe fragment. Since incomplete chains can not be used as templates in the next cycle, the yield is decreased. Finckh *et al.* noted a similar decrease in yield of labeled probe when the substitution of biotin modified dUTP was increased (19).

According to this model, the yield is reduced because of a high labeling density that sterically inhibits translation of the labeled template and that can be generated by either using a high S_{Rxn} or by using probes with longer linkers. However, there is a compensating effect that appears with longer linkers that reduce steric constraints from the fluorophores. This effect shows up in Fig. 2b. Since the labeling density for Cy3-24-dUTP is greater than for Cy3-17-dUTP when S_{Rxn} is in the range of 0.5−0.9 (Fig. 2a), it would be expected that the yield of reaction of Cy3-24-dUTP would be smaller . However, Fig. 2b shows that there is a greater yield of Cy3-24-dUTP than Cy3-17-dUTP over this range of S_{Rxn}. It is apparent that the longer linker facilitates chain extension even when the template is more heavily labeled. Therefore Cy3-24-dUTP is the best substrate for DNA probe labeling by PCR because it can give the highest labeling density with reasonable yield.

Incorporating Cy3-modified dUTP by nick translation

Table 1 shows that under optimal nick translation conditions S_{Prod} values for Cy3-17-dUTP and Cy3-24-dUTP are nearly equivalent but are about two-fold higher than for Cy3-10-dUTP.

Table 1. S_{Prod} and FISH result of DNA probes

	S_{Prod}	NSI (10^4)	S/b
NT.0L	0.11	0.5 ± 0.3	0.12 ± 0.05
NT.1L	0.18	1.5 ± 0.6	0.24 ± 0.07
NT.2L	0.18	1.2 ± 0.6	0.22 ± 0.07
PCR.0L	0.01	0.2 ± 0.1	0.07 ± 0.04
PCR.1L	0.14	1.7 ± 1.3	0.23 ± 0.11
PCR.2L	0.28	2.3 ± 1.6	0.29 ± 0.08

S_{Prod} was determined by absorbance measurement. PCR probes were the same as those for Fig. 3a after DNase digestion. NT = Nick translation; 0L = Cy3-10-dUTP; 1L = Cy3-17-dUTP; 2L = Cy3-24-dUTP. The FISH parameters are described in 'Materials and methods' section.

Our early study of nick translation with a different template (a SstI fragment) yielded similar results (20). This SstI fragment is a moderately repetitive sequence of 2.5 kb appearing in tandem arrays on human chromosome 19 and less frequently on chromosome 4 (21, 22). Others have reported that as the distance between biotin and nucleotide was increased, the labeling efficiency by nick translation first increased and then stayed constant or decreased (9, 10). Apparently steric interactions with the fluorophore on a shorter tether lead to less efficient incorporation of U* in the nick translation reaction.

Since a DNA probe made by nick translation is composed of extending fragments and residual fragments and only extending fragments can be labeled, overall S_{Prod} of a DNA probe depends upon both S_{Prod} of extending fragments and the ratio of extending fragments to residual fragments. In carrying out these reactions dU*TP completely replaces dTTP, so that in the extending fragments all T-sites contain U*. On the other hand, for PCR, the whole probe except for the short primer, can be labeled. Thus, with equally efficient incorporation by the polymerase, PCR should produce probes with an overall higher linear density of labeling. However, we have shown that there can not be complete substitution of dU*TP for dTTP in polymerase reaction. Even though S_{Rxn} is 1 in nick translation and S_{Rxn} is less than 1 in PCR, the optimal S_{Prod} of PCR is still higher than that of nick translation.

Fluorescence *in situ* hybridization (FISH) with Cy3-labeled probes

After having examined the labeling efficiency and yield of fluorescent DNA by PCR and nick translation, we now turn to the utility of such probes in FISH. Target DNA of chromosome 1 in interphase nuclei of HeLa cells was hybridized with Cy3-modified probes. FISH signals were quantified by image cytometry.

Table 1 shows that both the net spot intensity and the signal-to-background ratio of signals follows the same trend as S_{Prod} of probes. This means that the long linker does not seem to affect probe mobility and entry to the nuclei. Since the background is not increased as fast as the signal intensity, the fidelity of

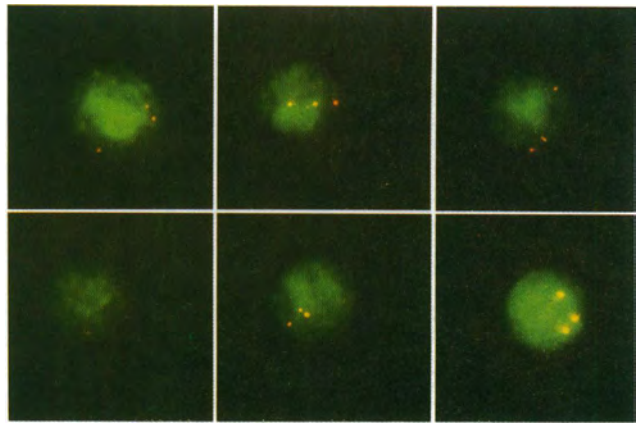


Figure 3. FISH images by different DNA probes. The upper images were obtained using probes made by nick translation. The bottom images were obtained using probes made by PCR. From left to right: Cy3-10-dUTP, Cy3-17-dUTP, Cy3-24-dUTP.

incorporating modified nucleotide is high enough to serve the purpose of preparing DNA probes . Thus improving probe labeling is a way to optimize FISH.

Since the FISH signal intensities obtained with PCR-generated probes are higher than those of probes made by nick translation (Table 1), and since PCR can label and amplify DNA probes simultaneously, PCR is a good labeling technique for preparation of directly labeled fluorescent DNA probes. Even higher labeling densities are achievable by PCR but at the price of low yield.

FISH images of 6 kinds of probe are shown in Fig. 3. The signal intensity of each image is close to the respective average intensity of each kind of probe.

ACKNOWLEDGEMENTS

We would like to thank Dr Ratnakar Mujumdar and Mrs Swati Mujumdar for their help in organic synthesis. We are also grateful to Mr Alasdair Dow for his help in image instrumentation. This work was supported by NSF Grant BIR-8920118 and State of Pennsylvania Contract #93-110-0026 to Center for Light Microscope Imaging and Biotechnology.

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ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK
Patent Owner.

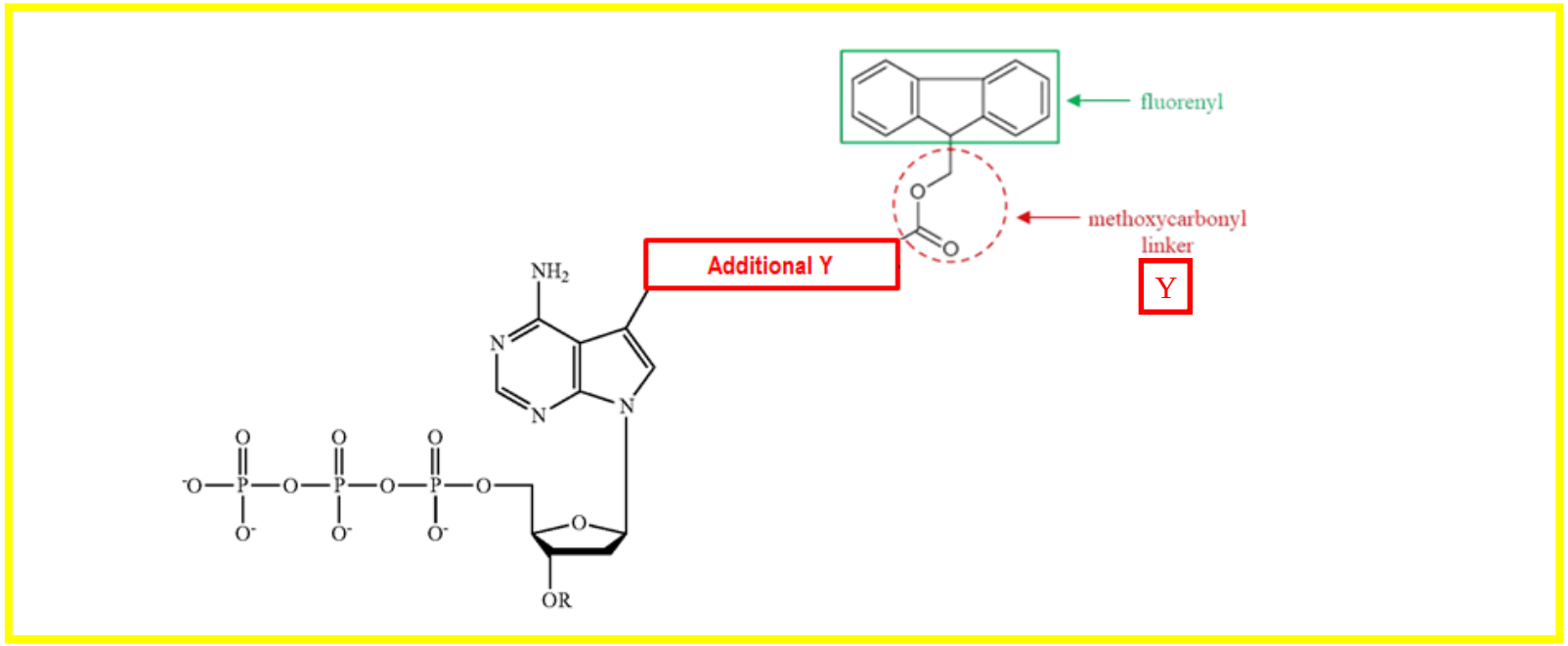
IPR2018-00291, -00318, -00322, -00385, -00797

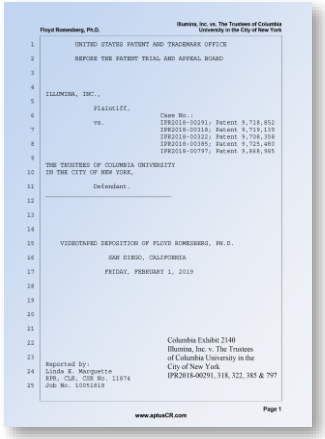
Patent Owner's Demonstratives

U.S. Patent Nos.
9,718,852 9,719,139 9,708,385 9,725,480 9,868,985

Columbia Exhibit No. 2141
Illumina v. Columbia
IPR2018-00291, 318, 322, 385, 797

Illumina's Most Recent Position Requires Adding Another Linker





Floyd Romesberg, Ph.D.
Deposition

Q. But Dower doesn't teach attaching Fmoc to the three bases that he teaches attaching it to through any kind of additional linker, correct?

A. …He didn't attach the Fmoc directly, but it would be an exocyclic amine that you would exactly be able to do that if you chose. Or you would be able to choose an additional linker between them. And that's what Seitz did.

Ex. 2140, Romesberg Dep., 197:16-198:8

Filed on behalf of:

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

INTELLIGENT BIO-SYSTEMS, INC.

Petitioner,

v.

ILLUMINA CAMBRIDGE LTD.

Patent Owner

Case IPR2013-00266 (LMG)
Patent 8,158,346

DECLARATION OF FLOYD ROMESBERG, PH.D., IN SUPPORT OF PATENT OWNER'S MOTION TO AMEND

1. I, Floyd Romesberg, Ph.D., have been retained by Knobbe, Martens, Olson & Bear, LLP, counsel for Illumina Cambridge Limited ("Illumina"). I understand that the U.S. Patent and Trademark Office Patent Trial and Appeal Board has instituted an inter partes review of the patentability of the claims of U.S. Patent No. 8,158,346 ("the '346 patent," Ex. 1001) based upon a petition filed by Intelligent Bio-Systems, Inc. ("IBS"). The following discussion and analysis provides my opinion as to why the proposed claims submitted in Illumina's motion to amend are novel and would have been nonobvious.

I. BACKGROUND AND QUALIFICATIONS, PREVIOUS TESTIMONY, AND COMPENSATION

A. Background and Qualifications

2. I am currently a professor in the Department of Chemistry at The Scripps Research Institute. I have been a faculty member at The Scripps Research Institute since 1998.

3. I earned a Bachelor of Science in Chemistry from the Ohio State University in 1988.

4. I earned a Master of Science in Chemistry in 1990 and a Doctor of Philosophy in Chemistry in 1994 from Cornell University, where Professor David B. Collum served as my thesis advisor.

51. My opinions concerning the proposed claims, as set forth herein, are from the perspective of a person of ordinary skill in the art, as set forth above.

VII. TECHNICAL BACKGROUND AND STATE OF THE ART

52. In 1977, two methods of DNA sequencing were reported. One of these methods was the chemical sequencing method developed by Maxam and Gilbert. Maxam and Gilbert, Proc. Nat'l. Acad. Sci. USA, 74:560-564, 1977 (Ex. 2008). The second method was the dideoxy method developed by Sanger. Sanger *et al*., Proc. Nat'l. Acad. Sci. USA, 74:5463-5467, 1977 (Ex. 2009).

53. The concept of DNA SBS was well established by 1999. *See, e.g.*, Cheeseman U.S. Pat. 5,302,509 (issued Apr. 12, 1994) (Ex. 2010); Metzker et al., Nucleic Acids Res., 22:4259-4267, 1994 (Ex. 2011); and Welch et al., Nucleosides and Nucleotides, 18:197-201, 1999 (Ex. 2012).

VIII. SCOPE AND CONTENT OF THE PRIOR ART

U.S. 7,078,499 ("Odedra, Ex. 1008) and WO 01/92284 ("Odedra PCT", Ex. 1009)

54. U.S. 7,078,499 ("Odedra, Ex. 1008) and WO 01/92284 ("Odedra PCT", Ex. 1009) disclose nucleotide analogues with a reporter moiety and a polymerase enzyme blocking moiety. Odedra at Title; Odedra PCT at Title. The disclosed nucleotide analogues can be used for sequencing nucleic acid molecules. Odedra at col. 7, ll. 57-62; Odedra PCT at page 12, ll. 13-16. In particular, Odedra and Odedra PCT disclose compounds of the following general structure:

incorporating certain modified nucleotides into nucleic acid strands. Short at Abstract.

69. Short discloses that disulfide-based coupling systems can be utilized as linkers. Short at page 22, 24, and FIG. 2. For example, Short shows a dideoxynucleoside triphosphate that contains a disulfide linker. Short at page 22. Short discloses cleavage of a disulfide bond using "mercaptoethanol dithiothreitol." Short at page 24. Short does not provide any experimental results reporting the overall yield of a disulfide linker cleavage reaction. Accordingly, Short does not provide a person of skill in the art with an expectation that the disulfide cleavage efficiency would improve upon the results reported by Ruby (~86% yield) or Herman (87% yield).

Jung *et al.*, Journal of the Chemical Society, Chemical Communications, 7:315-316, 1978 ("Jung," Ex. 1005)

70. Jung *et al.*, Journal of the Chemical Society, Chemical Communications, 7:315-316, 1978 ("Jung," Ex. 1005) discloses the reaction of alkyl carbamates with trimethylsilyl iodide. Jung at 315. Trimethylsilyl iodide is a reagent that was known to hydrolyze phosphate esters. *See* Bystrom and Branchaud *et al.*, Bioorganic & Medicinal Chemistry Letters, 7:2613-2616, 2614, 1997 ("Ester hydrolysis [of a phosphate ester (*i.e.*, compound 5)] was readily accomplished by treatment with trimethylsilyl iodide (TMSI) followed by aqueous

hydrolysis and recrystallization of the cyclohexylamine salt.") (Ex. 2021); *see also* "Iodotrimethylsilane," in Handbook of Reagents for Organic Synthesis, Reagents for Silicon-Mediated Organic Synthesis, Philip L. Fuchs, ed., John Wiley & Sons, West Sussex, United Kingdom, p. 325-335, 326, 2011 ("phosphate esters are cleaved even more readily with TMS-I than carboxylic esters") (Ex. 2022). Tetramethylsilyl iodide would not be a reagent suitable for cleaving protecting groups or linkers on nucleotides intended for use in a SBS context because TMSI was known to hydrolyze phosphate esters. For example, the use of TMSI in a SBS method would result in cleavage of the phosphate ester backbone of the DNA. Cleavage of the phosphate ester backbone would degrade the target DNA and would not "permit further nucleotide incorporation into the complement of the target single stranded polynucleotide," as required in step (d) of claim 20. Therefore, a person of ordinary skill in the art would not have considered TMSI to be a reagent that is compatible with the method of claim 20.

IX. SECONDARY CONSIDERATIONS SUPPORT THE NONOBVIOUSNESS OF THE PROPOSED CLAIMS

71. I understand that Illumina has evaluated the incorporation of nucleotides having disulfide linkers and the cleavage efficiency of such linkers. *See* Vermaas Decl. (Ex. 2023). Illumina has demonstrated that disulfide linkages can be efficiently cleaved using tris(hydroxymethyl)phosphine, which can cleave

86. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Dated: Dec 30, 2013

By: _____
Floyd Romesberg, Ph.D.

16905973

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner.

Case IPR2020-00988
Patent 10,407,458

DECLARATION OF FLOYD ROMESBERG, PH.D., IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 10,407,458

Illumina Ex. 1038
IPR Petition - USP 10,407,458

fragment to "reduce size and minimize steric interference." *Id*. at 26:35-27:1. Thus, Tsien plainly evidences a concern about nucleotide incorporation by polymerase due to the size of the 3′-capping group and suggests avoiding "large and bulky" groups at this position.

59. The crystal structure of a typical DNA polymerase was published in 1994 by Pelletier. Ex. 1044 (Pelletier) at 1897 (Table 3); *id.* at 1903, note 101 ("Full coordinates for both ternary complex structures are available from the Brookhaven Protein Data Bank and are designated 1bpf and 1bpg for the $P6_1$ and $P2_1$ structures, respectively."). Pelletier's publication would have been relevant to the methods of Dower and Tsien.

60. Before the year 2000, skilled artisans routinely considered the three-dimensional structure of enzymes in order to better understand the type of substrates that might be used. For example, when I carried out work designing non-natural nucleotides in the late 1990s, I examined three-dimensional structures of DNA polymerases, and from those structures I understood that the space in the active site was limited. This was understood to be at least partially responsible for the specificity of polymerases that use deoxyribose substrates over ribose substrates. The difference between these two substrates (dNTPs and NTPs) is a single oxygen atom at the 2′ position. Based on this ability to discriminate between very similar

XI. CONCLUSION

283. For the reasons described above, a person of ordinary skill in the art would have found Claims 1 and 2 of the '458 patent obvious over (1) Tsien in view of Prober and Hiatt and (2) Dower in view of Prober and in further view of Hiatt. Additionally, my opinions and prior testimony concerning the obviousness of the claim in U.S. 9,725,480 have not changed.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: 5-26-20 By: _____
Floyd Romesberg, Ph.D.

Notice of Allowability	Application No. 15/167,917	Applicant(s) JU ET AL.	
	Examiner Jezia Riley	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *amdt filed 5/26/17*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *49*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 5/26/17
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
5. ☐ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Jezia Riley/
Primary Examiner, Art Unit 1637

The present application is being examined under the pre-AIA first to invent provisions.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance:

The terminal disclaimers filed 5/26/17 have been approved and entered

The declaration of Jingyue Ju submitted May 26, 2017 explaining what is meant by "small" and applicant's argument that the skilled artisan would understand the definitions of R and Y are persuasive. Therefore the rejections of claim 49 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite and under 35 U.S.C. 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, are hereby withdrawn.

Additionally, applicant's arguments regarding the Stemple reference and the 2-nitrobenzyl group are sufficient to overcome the rejection of claim 49 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Stemple and Tsien in view of Prober and Anazawa.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

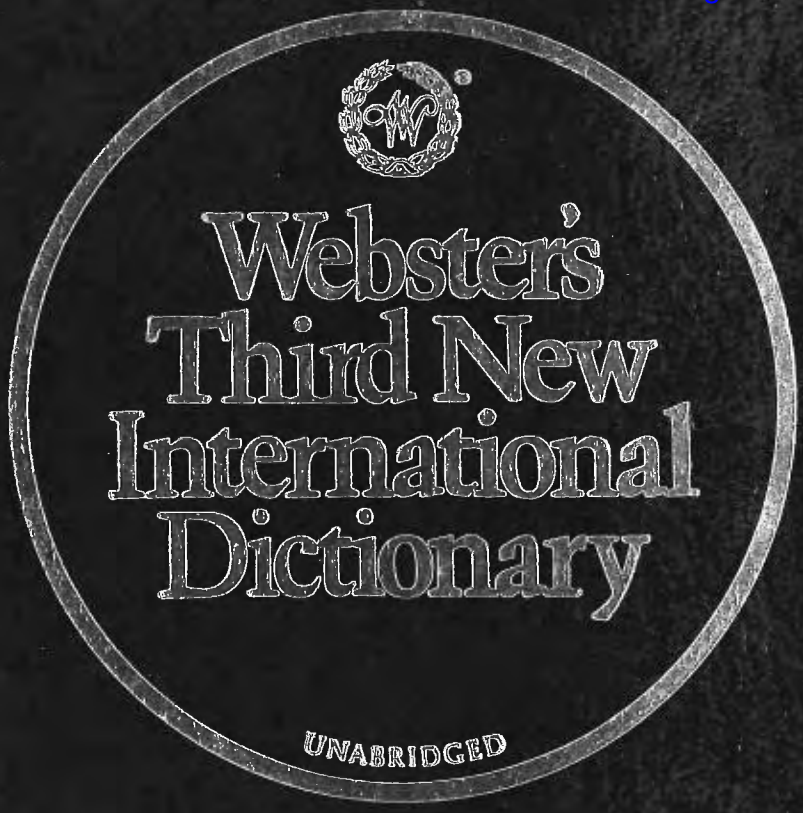
Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jezia Riley whose telephone number is (571)272-0786. The examiner can normally be reached on 9:30AM - 5:00PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Jezia Riley/
Primary Examiner, Art Unit 1637
12 June 2017


Webster's
Third New
International
Dictionary
UNABRIDGED

A GENUINE MERRIAM-WEBSTER

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PE1625.W36 1993
423–dc20 93-10630
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454647AG/H959493

cumpolar distribution belonging to the genera *Lemmus* and *crostonyx*, being four or five inches long with a very short l, furry feet, and small ears, usu. colored tawny yellowish ried with black and reddish, and best known for the recur- it mass migrations of a European form (*L. lemmus*) which en continue into the sea where vast numbers are drowned

ming mouse *n* **1** : any of several small rodents of the genus *naptomys* that are confined to northern No. America and semble the voles (genus *Microtus*) but have a very short tail d peculiar teeth — see BOG LEMMING **2** : any of a number No. American voles (genus *Phenacomys*) related to the iskrat

mo- *comb form* [Gk *lemma* rind, husk + E *-o-*] : neuri- nma ⟨*lemmo*blastic⟩ ⟨*lemmo*cyte⟩

·mus \'leməs\ *n, cap* [NL, fr. *lemmus* lemming, fr. Norw *nming*] : a genus of myomorph rodents consisting of the pical lemmings

·na \'lemnə\ *n, cap* [NL, fr. Gk, a water plant] : a genus ie type of the family Lemnaceae) of very small aquatic herbs ving simple fronds with a single root

·na·ce·ae \lem'nāsē,ē\ *n pl, cap* [NL, fr. *Lemna*, type nus + *-aceae*] : a family of aquatic plants (order Arales) nsisting of a single flat or thickened frond bearing a root or ots below and one or two naked flowers on the upper surface see DUCKWEED — **lem·na·ceous** \(')lem'nāshəs\ *adj*

·nad \'lem,nad\ *n* -s [NL *Lemna* + E *-ad*] : DUCKWEED

m·ni·an \'lemnēən\ *adj, usu cap* [L *Lemnius* Lemnian (fr. k *Lēmnios*, fr. *Lēmnos* Lemnos, island in the Aegean Sea) + *-an*] : of or relating to the Greek island of Lemnos

mnian \"\ *n* -s *cap* : a native or inhabitant of Lemnos

nnian bole *or* **lemnian earth** *n, usu cap L* : a gray to yellow red clay obtained from Lemnos and used formerly in medi- ne as an adsorbent and protective

·nis·cate \lem'niskət\ *n* -s [NL *lemniscata*, fr. fem. of L *mniscatus* with hanging ribbons, fr. *lemniscus* pendent ribbon . Gk *lēmniskos*) + *-atus* -ate] *math* : the locus of the foot of e perpendicular from the center of a conic on its tangent

·nis·cus \-iskəs\ *n, pl* **lemnis·ci** \-i,sī, -i,skē\ [NL, fr. L, bon] **1** : a form ÷ of the obelus **2** : a band of fibers; *ecif* : a band of nerve fibers of the second neurons in the nsory path terminating in the thalamus — called also *fillet*, *queus;* compare LATERAL LEMNISCUS, MEDIAL LEMNISCUS : either of two club-shaped organs hanging into the body vity from the base of the proboscis in the Acanthocephala

m·on \'lemən\ *n* -s [ME *lymon*, fr. MF *limon*, fr. ML *non-*, *limo*, fr. Ar *laymūn*] **1 a** (1) : an acid fruit that is tanically a syncarpous polycarpellary many-seeded pale llow berry of oblong form usu. with a nipple at the apex and yellow rind that contains the fragrant lemon oil and is often ndied or preserved — compare ⁶LIME (2) : the stout thorny ee (*Citrus limon*) that bears this fruit — see CITRON **b** : any of merous trees and shrubs of families other than Rutaceae aving fruit similar to the lemon — used with a qualifying word **a** : LEMON YELLOW **b** : CHLOR **3** : something (as a float for ring buoy) shaped like a lemon **4** : something or someone at proves to be unsatisfactory or undesirable : DUD, FAILURE t is quite possible that one manufacturer . . . may find at he has created an all-around ~ —*Atlantic Bull.*⟩ ⟨being uck with a ~ on the dance floor —William Irish⟩

mon \"\ *adj* **1** : containing lemon : having the flavor or ent of lemon ⟨~ tea⟩ ⟨~ bitters⟩ ⟨sipping a ~ drink⟩ **2** : of e color lemon yellow (sense 1) ⟨the winter afternoons glowed ith a hazy ~ light —Carson McCullers⟩

n·on·ade \,lemə'nād\ *n* -s [F *limonade*, fr. *limon* lemon (fr. F *lymon*) + *-ade*] : a beverage of sweetened lemon juice ixed with plain or carbonated water

nonade berry *n* : an evergreen shrub or small tree (*Rhus tegrifolia*) of southern California with simple obtuse leaves, hite or pinkish flowers in dense panicles, and dark red landular hairy fruit — called also *sourberry*

nonade bush *or* **lemonade sumach** *or* **lemon bush** : SQUAWBUSH 2

nonado *n* -s [Sp *limonada*] *obs* : LEMONADE

mon balm *n* : a bushy perennial Old World mint (*Melissa fficinalis*) often cultivated for its fragrant lemon-flavored aves — called also *garden balm, sweet balm*

mon chrome *n* **1 a** : a pale chrome yellow pigment **b** : bari- m chromate used as a pigment **2** : a brilliant yellow that is edder and deeper than butter yellow or jasmine yellow

mon chrome yellow *n* : LIGHT CHROME YELLOW

mon cucumber *n* : MANGO MELON

mon curd *n* : lemon juice and rind, sugar, butter, and eggs ooked together until thick and used as a spread or tart filling

mon dab *n* : SMEAR DAB

monfish \'=-,=\ *n, South* : COBIA

mon geranium *n* : a common garden pelargonium (*Pelar- onium limoneum*) having lemon-scented foliage

mongrass \'=-,=\ *n* : any of several grasses of the genus *Cymbopogon:* as **a** : a grass (*C. citratus*) of robust habit that as a large compound inflorescence and is cultivated in tropi- al regions (as the West Indies) as a source of lemongrass oil : a similar East Indian grass (*C. flexuosus*) that is also a ource of lemongrass oil **c** : CITRONELLA 2

mongrass oil *n* : a yellow to reddish brown essential oil that as an odor of lemon or verbena, is obtained esp. from either f two lemongrasses (*Cymbopogon citratus* or *C. flexuosus*), nd is used chiefly as a perfume (as in soap) and as a source of he aldehyde citral

m·on·ish \'lemənish\ *adj* : somewhat resembling a lemon

monlike \'=-,=\ *adj* : resembling or suggestive of lemon

mon lily *n* : a day lily (*Hemerocallis flava*) with lemon yellow lowers

mon mint *n* : an annual horsemint (*Monarda citriodora*) with lensely pubescent foliar bracts

mon oil *n* : a fragrant yellow essential oil obtained from the eel of lemons usu. by expression and used chiefly as a flavor- ng agent and in perfumes

mon scab *n* : citrus scab of the lemon

mon-scent \'=-,=\ *n* : LEMONWEED 2

mon-scented gum \'=-,=-\ *n* : a fragrant-leaved ornamental potted gum (*Eucalyptus maculata citriodora*)

mon shark *n* : a moderate-sized shark (*Negaprion brevi- ostris*) of the warm Atlantic that is yellowish brown to gray bove with yellow or greenish sides and is sought for its hide nd oily liver but in some areas is feared as a man-eater

mon sole *n* : a small European sole (*Solea lascaris*); *broadly* any of several other flatfishes (as a Georges Bank flounder or negrim)

mon thyme *n* : a lemon-scented wild thyme (*Thymus serpyl- um vulgaris*)

mon verbena *n* : a small shrub (*Lippia citriodora*) of Chile and Argentina that has narrow verticillate lemon-scented eaves and is used in gardens

mon vine *n* : BARBADOS GOOSEBERRY 1

mon walnut *n* : BUTTERNUT 1a

monweed \'=-,=\ *n* **1** : SEA MAT **2** : any of several lemon- scented composite herbs of the genus *Pectis* in the southwestern U.S.

monwood \'=-,=\ *n* **1** *in New Zealand* : TARATA **2** : a southern African evergreen tree (*Psychotria capensis*) with hard tough elastic wood used for making bows **3** : DAGAME

m·ony \'lemənē\ *adj* : suggestive of lemon : LEMON ⟨all the more palatable for the ~ taste —Christopher Morley⟩ ⟨we had cold fried chicken and . . . ~ iced tea —Jean Stafford⟩

mon yellow *n* **1 a** : a variable color averaging a brilliant greenish yellow **b** : a brilliant yellow — called also *Cassel yellow, Chinese yellow* **2** : a pigment of the color lemon yel-

lem·pi·ra \lem'pirə\ *n* -s [AmerSp, after *Lempira*, Indian chief who opposed the Spanish conquest] **1** : the basic monetary unit of Honduras — see MONEY table **2** : a coin or note representing one lempira

le·mur \'lēmə(r) *sometimes* -,myu̇(ə)r *or* -u̇ə\ *n* [NL, fr. L *lemures*, pl., nocturnal spirits, ghosts; fr. its nocturnal habits; akin to Gk *lamia* devouring monster, *lamyros* gluttonous, Latvian *lamāt* to rail at, *lamatas* mousetrap; basic meaning: open jaws] **1** *cap* : the type genus of Lemuridae **2** -s : any of numerous arboreal chiefly nocturnal mammals formerly widespread but now largely confined to Madagascar that are related to the monkeys but are usu. regarded as constituting the distinct superfamily Lemuroidea and that resemble monkeys in general form and habits but usu. have a muzzle like a fox, large eyes, very soft woolly fur, and a tail which is sometimes rudimentary but usu. long and furry and never prehensile — see AYE-AYE, LORIS, POTTO, TARSIER

lemur 2

lem·u·res \'lemyə,rēz\ *n pl* [L] : hostile spirits of the unburied dead exorcised from homes in religious observances of early Rome — compare LAR, MANES

¹**le·mu·ri·an** \lə'myu̇rēən\ *adj* [NL *Lemur* + E *-ian*] : lemuroid

²**lemurian** \"\ *adj, usu cap* [*Lemuria*, hypothetical former continent in the Indian ocean supposed to be now represented chiefly by Madagascar (fr. NL *Lemur*) + E *-an*] : MALAGASY

¹**lem·u·rid** \'lemyərəd, -,rid, 'lēmə-\ *adj* [NL *Lemuridae*] : of or relating to the Lemuridae

²**lemurid** \"\ *n* -s : a lemur of the family Lemuridae

le·mu·ri·dae \lə'myu̇rə,dē\ *n pl, cap* [NL, fr. *Lemur*, type genus + *-idae*] : a family comprising the typical lemurs

le·mu·ri·form \lə'myu̇rə,fȯrm\ *adj* [NL *Lemur* + E *-iform*] : of, relating to, or resembling lemurs

le·mu·ri·for·mes \lə,myu̇rə'fȯr,mēz\ *n pl, cap* [NL, fr. *Lemur* + *-iformes*] *in some classifications* : a division of Lemuroidea comprising the typical lemurs, the aye-aye, and sometimes the tree shrews

lem·u·rine \'lemyə,rīn, 'lēmə-\ *adj* [NL *Lemur* + E *-ine*] : LEMUROID

¹**lem·u·roid** \-,rȯid\ *adj* [NL *Lemuroidea*] : of, relating to, or resembling the lemurs or the Lemuroidea

²**lemuroid** \"\ *n* -s [NL *Lemuroidea*] : one of the Lemuroidea

lem·u·roi·dea \,=-'rȯidēə\ *n pl, cap* [NL, fr. *Lemur* + *-oidea*] **1** *in some classifications* : a suborder of Primates including lemurs, lorises, tarsiers, and living and extinct related mammals **2** : a superfamily or other division of Primates comprising the typical lemurs

lenape \lə'näpē, lə'napē, 'lenə(,)pē, lə'nap, lə'näp\ *n, pl* **lenape** *or* **lenapes** *usu cap* [Delaware, lit., person, Indian] : ¹DELAWARE

le·nard rays \'lā,närt-, lə'närd-\ *n pl, usu cap L* [after Philipp *Lenard* †1947 Ger. physicist] : a mixture of cathode rays that have emerged from a vacuum tube into the outside space through a window consisting of a piece of thin metal foil and rays emitted by the foil as a result of the incidence of the cathode rays

len·ca \'leŋkə\ *n, pl* **lenca** *or* **lencas** *usu cap* [Sp, of AmerInd origin] **1 a** : an Indian people of central Honduras and Salvador **b** : a member of such people **2** : the Lencan language of the Lenca people

len·can \-kən\ *n* -s *usu cap* : a language family of uncertain relationships comprising the Lenca language

¹**lend** \'lend\ *vb* **lent** \-nt\ **lent; lending; lends** [ME *lenden*, alter. (influenced by past *lende*, after such pairs as ME *sende* sent: *senden* to send) of *lenen*, fr. OE *lǣnan*, fr. *lǣn* loan — more at LOAN] *vt* **1 a** : to give into another's keeping for temporary use on condition that the borrower return the same or its equivalent ⟨the purser has kindly *lent* us excellent binoculars —W.R.Benet⟩ ⟨some 46 works *lent* by museums and private collectors —*Harvard Foundation Newsletter*⟩ **b** : to let out (money) for temporary use on condition that it be repaid with interest at an agreed time ⟨it is sometimes said that the capitalists who *lent* the government the money for the war deserve the hire of it —G.B.Shaw⟩ ⟨commercial banks were obliged . . . to reduce their investments in securities in order to ~ more in advances to customers —*World Economic Survey*⟩ **c** : to place (a subordinate) at the disposal of another for temporary service ⟨he was *lent* by the army to the Institute of Inter-American Affairs as a specialist —*N.Y. Times*⟩ — compare ¹LOAN 2b **2 a** : to give the assistance or support of : ADD, AFFORD, FURNISH, PROVIDE, SUPPLY ⟨his teaching . . . had *lent* to Oxford thought much of its early originality and distinction —G.G.Coulton⟩ ⟨contributed much to the development of the cog railroad . . . ~*ing* his mechanical ability to the problems encountered —*Amer. Guide Series: N.H.*⟩ **b** : to devote the use or effort of (as a part of the body or a faculty of the mind) ⟨the young king seemed to ~ a willing ear —George Eliot⟩ ⟨~ a hand to those in charge of these schools —J.B.Conant⟩ ⟨*lent* eager attention to these hopeful projections⟩ ⟨~*s* a courteous arm to woman or child⟩ **c** : to adapt or apply (oneself or itself) : ACCOMMODATE, OFFER ⟨the peoples *lent* themselves to the nearest leader in their quest for salvation as a group —Francis Hackett⟩ ⟨a buggy exhibit did not ~ itself to much exciting variation —Ben Riker⟩ ⟨hypotheses which ~ themselves to the straining of facts in their support —Edward Clodd⟩ **3** *dial chiefly Brit* : to deal or deliver (a blow) to someone ~ *vi* : to make a loan

²**lend** \"\ *n* -s *dial* : temporary possession and use by a borrower : LOAN ⟨the ~ of her brass fender —Mary Lavin⟩

lend·able \-dəbəl\ *adj* : available for lending ⟨will tend to further reduce the supply of ~ funds —L.H.Olsen⟩

lend·er \-də(r)\ *n* -s [ME *lendare*, alter. (influenced by ME *lenden* to lend) of *lenere*, fr. OE *lǣnere*, fr. *lǣnan* to lend + *-ere* -er] : one that lends

lending *n* -s [ME *lendinge*, alter. (influenced by ME *lenden* to lend) of *lenninge, leninge*, gerund of *lenen* to lend — more at LEND] **1** : a giving or letting out for temporary use **2** : something lent

lending library *n* **1** : RENTAL LIBRARY **2** *chiefly Brit* **a** : the lending department of a public library **b** : PUBLIC LIBRARY

¹**lend-lease** \'=-'=\ *n* [after the title of the U.S. *Lend-Lease* Act (1941)] : the transfer of goods and services to an ally to aid in a common cause (as the winning of a war) with payment being made by a return of the original items or their use in the common cause or by a similar transfer of other goods and services ⟨*lend-lease* . . . will stand forth as the most unselfish and unsordid financial act of any country —Sir Winston Churchill⟩ ⟨the two-way *lend-lease* in ideas —*New Republic*⟩

²**lend-lease** \"\ *vt* : to provide by means of lend-lease ⟨have returned most of the naval craft *lend-leased* them during the war —*Christian Science Monitor*⟩

len·du \'len(,)dü\ *n, pl* **lendu** *or* **lendus** *usu cap* **1 a** : a people of Uganda **b** : a member of such people **2** : a central Sudanic language of the Lendu people

lenes *pl of* LENIS

leng·en·bach·ite \'leŋən,bä,kīt\ *n* -s [*Lengenbach*, Switzerland, its locality + E *-ite*] : a mineral $Pb_6(Ag,Cu)_2As_4S_{13}$ consisting of a sulfide of lead, silver, copper, and arsenic

length \'leŋ(k)th, 'len(t)th\ *n, pl* **lengths** \-ths, 'leŋks\ [ME *lengthe*, fr. OE *lengthu* (akin to OFris *lengethe* length, MD *lengede, lengde*, ON *lengd*), fr. *lang, long* long + *-thu* -th — more at LONG] **1 a** : the longer of the 2 straight-line dimen-

tracted duration of stress of a sound in speech, prosody, or music ⟨the long *a* gives the word *sale* its ~⟩ **c** *archaic* : prolixity or excess in expression ⟨there is such ~ in grief —Shak.⟩ **3 a** : distance or extent in space ⟨it would be hard, even in New England, to match Main Street for its ~ of 18th century square houses —Elizabeth Coatsworth⟩ ⟨appeared dimly white round a distant bend of the dusty road, a weary ~ behind —Haldane Macfall⟩ **b** (1) : the measure of something taken as a unit of distance ⟨darted across the highway scarcely two car ~*s* ahead of me⟩ ⟨kept most of his acquaintances at arm's ~⟩ (2) : the length of a competitor (as a horse or boat) taken as a unit in stating the margin of a lead or of victory in a race ⟨he led by three ~*s* at the top of the stretch⟩ (3) : the fully extended body ⟨stretched her ~ lazily on the warm earth⟩ ⟨took a hard right on the jaw and measured his ~ on the floor⟩ **4 a** *chiefly Scot* : an indicated or specified distance ⟨I'll go with you the ~ to the hall⟩ **b** : the degree, limit, or extreme to which a course of action or a line of thought or discussion is carried ⟨tended to carry his policy of masterly inactivity to dangerous ~*s* —Harvey Graham⟩ ⟨even went the ~ of reading the play . . . to ascertain what it was all about —G.B.Shaw⟩ ⟨here we see the foolish ~*s* to which human malevolence will go —Norman Douglas⟩ **5 a** : a long expanse or stretch ⟨brushed her ~*s* of lustrous hair⟩ ⟨large ~*s* of seas and shores between my father and my mother lay —Shak.⟩ **b** : a piece constituting or usable as part of a whole or of a connected series : SEGMENT, SECTION ⟨steel bars are furnished in standard shapes and sizes, in both coils and straight ~*s* —*advt*⟩ ⟨short ~*s* of film with both ends spliced together to permit continuous repetition —W.F. Mackey⟩ **6 a** : FLUIDITY **b** : ability to yield a fluid mixture — compare OIL LENGTH **7** *archaic* : a 42-line portion of an actor's part **8** : the volume of wort drawn from a quantity of malt during brewing **9** : the holding of more than a player's proportionate share of the cards of one suit in a card game (as four or more at bridge) ⟨he had ~ in trumps⟩ **10 a** : the distance an esp. well pitched ball in cricket travels before hitting the ground ⟨bowled a good ~⟩ ⟨pitched the ball a fraction short of a ~ —Ray Robinson⟩ **b** : the distance to be shot in archery **11** : the vertical dimension of an article of clothing esp. with reference to the part of the body it reaches or its height above the floor ⟨stockings are made in three ~*s*⟩ ⟨evening dresses in short and long ~*s*⟩ ⟨knee-*length* pants⟩ ⟨a hip-*length* jacket⟩ ⟨a floor-*length* gown⟩ — **at length** *adv* **1** : COMPREHENSIVELY, FULLY ⟨an important clash of principles which have to be debated *at length* —*London Calling*⟩ **2** : at last : FINALLY ⟨events looked forward to with trepidation, when *at length* they occur, often fall flat —George Santayana⟩

length–breadth index *n* : CRANIAL INDEX

length·en \-thən\ *vb* **lengthened; lengthened; lengthening** \-th(ə)niŋ\ **lengthens** *vt* **1** : to extend in length : make longer : ELONGATE, PROLONG, PROTRACT ⟨~*ed* their skirts halfway to their shoe tops —Mary Austin⟩ ⟨overhauls and renewals which would undoubtedly have ~*ed* her life —F.W. Crofts⟩ **2 a** *obs* : to eke out (provisions) : STRETCH **b** : to increase by diluting ⟨standing by the counter ~*ing* out a short supply of wine —Charles Dickens⟩ ~ *vi* : to grow longer ⟨fall ~*ed* out into winter —Laura Krey⟩ ⟨faces ~*ed* as the news became more certain⟩ **syn** see EXTEND

length·en·er \-th(ə)nə(r)\ *n* -s : one that lengthens

length·ful \-thfəl\ *adj* [*length* + *-ful*] *archaic* : LONG

length–height index *n* **1** : the ratio of the auricular height of the head to its length **2** : the ratio of the distance between basion and bregma on the skull to its length

length·i·ly \-thəlē, -li\ *adv* : in a lengthy or prolix manner

length·i·ness \-thēnəs, -thin-\ *n* -ES : the quality or state of being lengthy : PROLIXITY

length·ways \-th,wāz\ *adv* : LENGTHWISE

¹**lengthwise** \'=,=\ *adv* [*length* + *-wise*] : in the direction of the length : LONGITUDINALLY ⟨opened his newspaper, folded it ~ —Nathaniel Benchley⟩

²**lengthwise** \"\ *adj* : moving, placed, or directed on or toward the long axis ⟨tiers of planks had been fashioned into ~ seats —Agnes M. Cleaveland⟩

lengthy \-thē,-thi\ *adj* -ER/-EST **1 a** : protracted excessively or tediously : OVERLONG — used of written or spoken expression ⟨~ and histrionic discussions —*N.Y. Times*⟩ **b** : unduly copious : PROLIX — used of a speaker or writer ⟨must not be ~⟩ **2** : EXTENDED, LONG ⟨no very ~ journey was involved —Allan Fraser⟩ ⟨twirled his ~ key chain —Don Davis⟩ ⟨a ~ debate⟩

len·gua \'leŋgwə\ *n, pl* **lengua** *or* **lenguas** *usu cap* [Sp, lit., tongue, fr. L *lingua;* fr. its custom of wearing labrets — more at TONGUE] **1 a** : a group of Amerind peoples of Gran Chaco, Paraguay, including the Macá and Mascoi **b** : a member of any such people **2** : a language of a Lengua people

le·nien·cy \'lēnēənsē, 'lēnyən-, -si *sometimes* 'len-\ *or* **le·nience** \-ən(t)s\ *n, pl* **leniencies** *or* **leniences** : a lenient disposition or practice : MERCY ⟨had the ~ of the unprincipled —Francis Hackett⟩

le·nient \-ənt\ *adj* [L *lenient-, leniens*, pres. part. of *lenire* to soften, fr. *lenis* soft, mild; akin to Latvian *lēns* mild, slow, lazy, OSlav *lēnŭ* — more at LET (to permit)] **1** *archaic* : relieving pain (as a medicine) or stress (as soothing influence) : ASSUASIVE, EMOLLIENT **2** : of mild and tolerant disposition or effect : INDULGENT, MERCIFUL ⟨strict legality was mitigated by his ~ understanding⟩ ⟨contiguous states were attracting capital and enterprise through ~ laws —Broadus Mitchell⟩ **syn** see FORBEARING, SOFT

le·nient·ly *adv* : in a lenient manner

leni·fy \'lenə,fī, 'lēn-\ *vt* -ED/-ING/-ES [MF or LL; MF *lenifier*, fr. LL *lenificare*, fr. L *lenis* soft, mild + *-ficare* -fy] *archaic* : ALLEVIATE, ASSUAGE, MITIGATE, SOFTEN

leni–lenape *or* **lenni–lenape** \'lenē *pronunc at* LENAPE\ *n, usu cap both Ls* [Delaware, lit., real person, fr. *leni, lenni* real + *lenape* person, Indian] : ¹DELAWARE

len·in·grad \'lenən,grad, -raa(ə)d\ *adj, usu cap* [fr. *Leningrad*, U.S.S.R.] : of or from the city of Leningrad, U.S.S.R. : of the kind or style prevalent in Leningrad

len·in·grad·er \-də(r)\ *n* -s *cap* [*Leningrad*, U.S.S.R. + E *-er*] : a native or resident of Leningrad

len·in·ism \'lenə,nizəm\ *n* -s *usu cap* [*Lenin* (real name, Vladimir Ilich Ulyanov) †1924 Russ. Communist leader + E *-ism*] : the political, economic, and social principles and policies advocated by Lenin; *esp* : the theory and practice of communism developed by or associated with Lenin ⟨the application of the principles of *Leninism* to an entirely new situation —D.J.Dallin⟩ ⟨appreciate *Leninism* as an organizational technique —B.I.Schwartz⟩ ⟨the . . . development of Marxism through *Leninism* and Stalinism —Francis Conklin⟩ — compare BOLSHEVISM, MARXISM, MARXISM-LENINISM, STALINISM

¹**len·in·ist** \'lenənəst\ *n* -s *usu cap* [*Lenin* + E *-ist*] : a follower of Lenin : an adherent of Leninism ⟨our party has always been a party of *Leninists* —*Russian Information & Rev.*⟩ ⟨a *Leninist* . . . on the question of power —Lucjan Blit⟩

²**leninist** \"\ *adj, usu cap* : of, relating to, or having the characteristics of Leninism or Leninists ⟨the *Leninist* form of communism —*Brit. Book News*⟩ ⟨backing a party with *Leninist* organization —M.F.Lindsay⟩

len·in·ite \'lenə,nīt\ *adj or n, usu cap* [*Lenin* + E *-ite*] : LENINIST

¹**le·nis** \'lēnəs, 'lān-\ *adj* [NL, fr. LL, smooth (of breathing, as in LL *spiritus lenis*), fr. L, soft, smooth, mild — more at LENIENT] *of one of two homorganic consonants* : produced with more lax articulation and weaker expiration — opposed to *fortis* ⟨\d\ in *doe* is ~, \t\ in *toe* is fortis⟩

²**lenis** \"\ *n, pl* **le·nes** \'lē(,)nēz, 'lā(,)nās\ [NL, fr. LL, adj., smooth] **1** : a lenis consonant **2** : SMOOTH BREATHING

nent or theme of a composition prefixed as a title or introduction; *also* : the heading or theme of a comment or note **3** : a word or phrase glossed in a glossary

lemma \"\ *n -s* [Gk, rind, husk, fr. *lepein* to peel — more at LEPER] : the lower of the two bracts enclosing the flower in the spikelet of grasses — called also *flowering glume*; see WILD OAT illustration

lem·mer \'lemə(r)\ *n -s* [origin unknown] : one that butchers whales — compare FLENSER

lem·ming \-miŋ\ *n -s* [Norw *lemming, lemende, lomund*, fr. ON *lōmundr* (assumed fr. acc. pl. *lōmundi*); akin to ON *lōmr* guillemot, *lō* curlew, Goth *lailoun* they derided, L *latrare* to bark — more at LAMENT] : any of several small rodents of circumpolar distribution belonging to the genera *Lemmus* and *Dicrostonyx*, being four or five inches long with a very short tail, furry feet, and small ears, usu. colored tawny yellowish varied with black and reddish, and best known for the recurrent mass migrations of a European form (*L. lemmus*) which often continue into the sea where vast numbers are drowned

lemming mouse *n* **1** : any of several small rodents of the genus *Synaptomys* that are confined to northern No. America and resemble the voles (genus *Microtus*) but have a very short tail and peculiar teeth — see BOG LEMMING **2** : any of a number of No. American voles (genus *Phenacomys*) related to the muskrat

lemmo- *comb form* [Gk *lemma* rind, husk + E *-o-*] : neuri- emma ⟨*lemmo*blastic⟩ ⟨*lemmo*cyte⟩

lem·mus \'leməs\ *n, cap* [NL, fr. *lemmus* lemming, fr. Norw *emming*] : a genus of myomorph rodents consisting of the ypical lemmings

lem·na \'lemnə\ *n, cap* [NL, fr. Gk, a water plant] : a genus the type of the family Lemnaceae) of very small aquatic herbs having simple fronds with a single root

lem·na·ce·ae \lem'nāsē,ē\ *n pl, cap* [NL, fr. *Lemna*, type genus + *-aceae*] : a family of aquatic plants (order Arales) consisting of a single flat or thickened frond bearing a root or roots below and one or two naked flowers on the upper surface — see DUCKWEED — **lem·na·ceous** \(')lem'nāshəs\ *adj*

lem·nad \'lem,nad\ *n -s* [NL *Lemna* + E *-ad*] : DUCKWEED

lem·ni·an \'lemnēən\ *adj, usu cap* [L *Lemnius* Lemnian (fr. Gk *Lēmnios*, fr. *Lēmnos* Lemnos, island in the Aegean Sea) + *-an*] : of or relating to the Greek island of Lemnos

lemnian \"\ *n -s cap* : a native or inhabitant of Lemnos

lemnian bole *or* **lemnian earth** *n, usu cap L* : a gray to yellow or red clay obtained from Lemnos and used formerly in medicine as an adsorbent and protective

lem·nis·cate \lem'niskət\ *n -s* [NL *lemniscata*, fr. fem. of L *lemniscatus* with hanging ribbons, fr. *lemniscus* pendent ribbon, fr. Gk *lēmniskos*) + *-atus* -ate] *math* : the locus of the foot of the perpendicular from the center of a conic on its tangent

lem·nis·cus \-iskəs\ *n, pl* **lemnis·ci** \-i,sī, -i,skē\ [NL, fr. L, ribbon] **1** : a form ÷ of the obelus **2** : a band of fibers; *specif* : a band of nerve fibers of the second neurons in the sensory path terminating in the thalamus — called also *fillet, aqueus;* compare LATERAL LEMNISCUS, MEDIAL LEMNISCUS **3** : either of two club-shaped organs hanging into the body cavity from the base of the proboscis in the Acanthocephala

lem·on \'lemən\ *n -s* [ME *lymon*, fr. MF *limon*, fr. ML *limon-, limo*, fr. Ar *laymūn*] **1 a** (1) : an acid fruit that is botanically a syncarpous polycarpellary many-seeded pale yellow berry of oblong form usu. with a nipple at the apex and a yellow rind that contains the fragrant lemon oil and is often candied or preserved — compare ⁶LIME (2) : the stout thorny tree (*Citrus limon*) that bears this fruit — see CITRON **b** : any of numerous trees and shrubs of families other than Rutaceae having fruit similar to the lemon — used with a qualifying word **2 a** : LEMON YELLOW **b** : CHLOR **3** : something (as a float for a ring buoy) shaped like a lemon **4** : something or someone that proves to be unsatisfactory or undesirable : DUD, FAILURE ⟨it is quite possible that one manufacturer . . . may find that he has created an all-around ~ —*Atlantic Bull.*⟩ ⟨being stuck with a ~ on the dance floor —William Irish⟩

lemon \"\ *adj* **1** : containing lemon : having the flavor or scent of lemon ⟨~ tea⟩ ⟨~ bitters⟩ ⟨sipping a ~ drink⟩ **2** : of the color lemon yellow (sense 1) ⟨the winter afternoons glowed with a hazy ~ light —Carson McCullers⟩

lem·on·ade \,lemə'nād\ *n* -s [F *limonade*, fr. *limon* lemon (fr. MF *lymon*) + *-ade*] : a beverage of sweetened lemon juice mixed with plain or carbonated water

monade berry *n* : an evergreen shrub or small tree (*Rhus integrifolia*) of southern California with simple obtuse leaves, white or pinkish flowers in dense panicles, and dark red glandular hairy fruit — called also *sourberry*

monade bush *or* **lemonade sumach** *or* **lemon bush** : SQUAWBUSH 2

monado *n -s* [Sp *limonada*] *obs* : LEMONADE

mon balm *n* : a bushy perennial Old World mint (*Melissa officinalis*) often cultivated for its fragrant lemon-flavored leaves — called also *garden balm, sweet balm*

mon chrome *n* **1 a** : a pale chrome yellow pigment **b** : barium chromate used as a pigment **2** : a brilliant yellow that is redder and deeper than butter yellow or jasmine yellow

mon chrome yellow *n* : LIGHT CHROME YELLOW

mon cucumber *n* : MANGO MELON

mon curd *n* : lemon juice and rind, sugar, butter, and eggs cooked together until thick and used as a spread or tart filling

mon dab *n* : SMEAR DAB

monfish \'=,=\ *n, South* : COBIA

mon geranium *n* : a common garden pelargonium (*Pelargonium limoneum*) having lemon-scented foliage

mongrass \'=,=\ *n* : any of several grasses of the genus *Cymbopogon:* as **a** : a grass (*C. citratus*) of robust habit that has a large compound inflorescence and is cultivated in tropical regions (as the West Indies) as a source of lemongrass oil **b** : a similar East Indian grass (*C. flexuosus*) that is also a source of lemongrass oil **c** : CITRONELLA 2

mongrass oil *n* : a yellow to reddish brown essential oil that has an odor of lemon or verbena, is obtained esp. from either of two lemongrasses (*Cymbopogon citratus* or *C. flexuosus*), and is used chiefly as a perfume (as in soap) and as a source of the aldehyde citral

mon·ish \'lemənish\ *adj* : somewhat resembling a lemon

monlike \'=,=\ *adj* : resembling or suggestive of lemon

mon lily *n* : a day lily (*Hemerocallis flava*) with lemon yellow flowers

mon mint *n* : an annual horsemint (*Monarda citriodora*) with densely pubescent foliar bracts

mon oil *n* : a fragrant yellow essential oil obtained from the peel of lemons usu. by expression and used chiefly as a flavoring agent and in perfumes

mon scab *n* : citrus scab of the lemon

mon-scent \'=,=\ *n* : LEMONWEED 2

mon-scented gum \'=,==\ *n* : a fragrant-leaved ornamental potted gum (*Eucalyptus maculata citriodora*)

mon shark *n* : a moderate-sized shark (*Negaprion brevirostris*) of the warm Atlantic that is yellowish brown to gray above with yellow or greenish sides and is sought for its hide and oily liver but in some areas is feared as a man-eater

mon sole *n* : a small European sole (*Solea lascaris*); *broadly* : any of several other flatfishes (as a Georges Bank flounder or megrim)

mon thyme *n* : a lemon-scented wild thyme (*Thymus serpyllum vulgaris*)

mon verbena *n* : a small shrub (*Lippia citriodora*) of Chile and Argentina that has narrow verticillate lemon-scented leaves and is used in gardens

mon wine [illegible]

low: as **a** : a preparation of barium chromate often mixed with zinc chromate **b** : a preparation of lead chromate with lead carbonate

le·mo·si \,lemə'zē, -ə'sē\ *or* **li·mo·si** \,lim-\ *n -s usu cap* [Catal *llemosi*, prob. fr. (assumed) VL *lemovicensis* of the Lemovices, fr. L *Lemovices*, a Gallic people inhabiting what is now the region of Limousin in west central France] **1** : the basic monetary unit of Honduras — see MONEY table **2** : a coin or note representing one lempira

le·mur \'lēmə(r) *sometimes* -,myu(ə)r *or* -ůə\ *n* [NL, fr. L *lemures*, pl., nocturnal spirits, ghosts; fr. its nocturnal habits; akin to Gk *lamia* devouring monster, *lamyros* gluttonous, Latvian *lamāt* to rail at, *lamatas* mousetrap; basic meaning: open jaws] **1** *cap* : the type genus of Lemuridae **2** -s : any of numerous arboreal chiefly nocturnal mammals formerly widespread but now largely confined to Madagascar that are related to the monkeys but are usu. regarded as constituting the distinct superfamily Lemuroidea and that resemble monkeys in general form and habits but usu. have a muzzle like a fox, large eyes, very soft woolly fur, and a tail which is sometimes rudimentary but usu. long and furry and never prehensile — see AYE-AYE, LORIS, POTTO, TARSIER

lemur 2

lem·u·res \'lemyə,rēz\ *n pl* [L] : hostile spirits of the unburied dead exorcised from homes in religious observances of early Rome — compare LAR, MANES

¹le·mu·ri·an \lə'myůrēən\ *adj* [NL *Lemur* + E *-ian*] : lemuroid

²lemurian \"\ *adj, usu cap* [*Lemuria*, hypothetical former continent in the Indian ocean supposed to be now represented chiefly by Madagascar (fr. NL *Lemur*) + E *-an*] : MALAGASY

¹lem·u·rid \'lemyərəd, -,rid, 'lēmə-\ *adj* [NL *Lemuridae*] : of or relating to the Lemuridae

²lemurid \"\ *n -s* : a lemur of the family Lemuridae

le·mu·ri·dae \lə'myůrə,dē\ *n pl, cap* [NL, fr. *Lemur*, type genus + *-idae*] : a family comprising the typical lemurs

le·mu·ri·form \lə'myůrə,fôrm\ *adj* [NL *Lemur* + E *-iform*] : of, relating to, or resembling lemurs

le·mu·ri·for·mes \lə,myůrə'fôr,mēz\ *n pl, cap* [NL, fr. *Lemur* + *-iformes*] *in some classifications* : a division of Lemuroidea comprising the typical lemurs, the aye-aye, and sometimes the tree shrews

lem·u·rine \'lemyə,rīn, 'lēmə-\ *adj* [NL *Lemur* + E *-ine*] : LEMUROID

¹lem·u·roid \-,rôid\ *adj* [NL *Lemuroidea*] : of, relating to, or resembling the lemurs or the Lemuroidea

²lemuroid \"\ *n -s* [NL *Lemuroidea*] : one of the Lemuroidea

lem·u·roi·dea \,=='rôidēə\ *n pl, cap* [NL, fr. *Lemur* + *-oidea*] **1** *in some classifications* : a suborder of Primates including lemurs, lorises, tarsiers, and living and extinct related mammals **2** : a superfamily or other division of Primates comprising the typical lemurs

lenape \lə'näpē, lə'napē, 'lenə(,)pē, lə'nap, lə'nāp\ *n, pl* **lenape** *or* **lenapes** *usu cap* [Delaware, lit., person, Indian] : ¹DELAWARE

le·nard rays \'lā,närt-, lə'närd-\ *n pl, usu cap L* [after Philipp *Lenard* †1947 Ger. physicist] : a mixture of cathode rays that have emerged from a vacuum tube into the outside space through a window consisting of a piece of thin metal foil and rays emitted by the foil as a result of the incidence of the cathode rays

len·ca \'leŋkə\ *n, pl* **lenca** *or* **lencas** *usu cap* [Sp, of Amer-Ind origin] **1 a** : an Indian people of central Honduras and Salvador **b** : a member of such people **2** : the Lencan language of the Lenca people

len·can \-kən\ *n -s usu cap* : a language family of uncertain relationships comprising the Lenca language

¹lend \'lend\ *vb* **lent** \-nt\ **lent; lending; lends** [ME *lenden*, alter. (influenced by past *lende*, after such pairs as ME *sende* sent: *senden* to send) of *lenen*, fr. OE *lǣnan*, fr. *lǣn* loan — more at LOAN] *vt* **1 a** : to give into another's keeping for temporary use on condition that the borrower return the same or its equivalent ⟨the purser has kindly *lent* us excellent binoculars —W.R.Benet⟩ ⟨some 46 works *lent* by museums and private collectors —*Harvard Foundation Newsletter*⟩ **b** : to let out (money) for temporary use on condition that it be repaid with interest at an agreed time ⟨it is sometimes said that the capitalists who *lent* the government the money for the war deserve the hire of it —G.B.Shaw⟩ ⟨commercial banks were obliged . . . to reduce their investments in securities in order to ~ more in advances to customers —*World Economic Survey*⟩ **c** : to place (a subordinate) at the disposal of another for temporary service ⟨he was *lent* by the army to the Institute of Inter-American Affairs as a specialist —*N.Y. Times*⟩ — compare ¹LOAN 2b **2 a** : to give the assistance or support of : ADD, AFFORD, FURNISH, PROVIDE, SUPPLY ⟨his teaching . . . had *lent* to Oxford thought much of its early originality and distinction —G.G.Coulton⟩ ⟨contributed much to the development of the cog railroad . . . ~*ing* his mechanical ability to the problems encountered —*Amer. Guide Series: N.H.*⟩ **b** : to devote the use or effort of (as a part of the body or a faculty of the mind) ⟨the young king seemed to ~ a willing ear —George Eliot⟩ ⟨~ a hand to those in charge of these schools —J.B.Conant⟩ ⟨*lent* eager attention to these hopeful projections⟩ ⟨~*s* a courteous arm to woman or child⟩ **c** : to adapt or apply (oneself or itself) : ACCOMMODATE, OFFER ⟨the peoples *lent* themselves to the nearest leader in their quest for salvation as a group —Francis Hackett⟩ ⟨a buggy exhibit did not ~ itself to much exciting variation —Ben Riker⟩ ⟨hypotheses which ~ themselves to the straining of facts in their support —Edward Clodd⟩ **3** *dial chiefly Brit* : to deal or deliver (a blow) to someone ~ *vi* : to make a loan

²lend \"\ *n -s dial* : temporary possession and use by a borrower : LOAN ⟨the ~ of her brass fender —Mary Lavin⟩

lend·able \-dəbəl\ *adj* : available for lending ⟨will tend to further reduce the supply of ~ funds —L.H.Olsen⟩

lend·er \-də(r)\ *n -s* [ME *lendare*, alter. (influenced by ME *lenden* to lend) of *lenere*, fr. OE *lǣnere*, fr. *lǣnan* to lend + *-ere* -er] : one that lends

lending *n -s* [ME *lendinge*, alter. (influenced by ME *lenden* to lend) of *lenninge, leninge*, gerund of *lenen* to lend — more at LEND] **1** : a giving or letting out for temporary use **2** : something lent

lending library *n* **1** : RENTAL LIBRARY **2** *chiefly Brit* **a** : the lending department of a public library **b** : PUBLIC LIBRARY

¹lend-lease \'=,=\ *n* [after the title of the U.S. *Lend-Lease* Act (1941)] : the transfer of goods and services to an ally to aid in a common cause (as the winning of a war) with payment being made by a return of the original items or their use in the common cause or by a similar transfer of other goods and services ⟨*lend-lease* . . . will stand forth as the most unselfish and unsordid financial act of any country —Sir Winston Churchill⟩ ⟨the two-way *lend-lease* in ideas —*New Republic*⟩

sions of a surface or plane or the longest of the 3 straight-line dimensions of a solid : extent from end to end — distinguished from *width* ⟨the island was three miles in ~⟩ **b : a distance or dimension expressed in units of linear measure ⟨a ~ of 10 inches⟩ **c** : the quality or state of being long — opposed to *shortness* ⟨weariness and boredom exaggerated the ~ of the journey⟩ **d** : WAVELENGTH **2 a** : duration or extent in time ⟨doesn't seem to prove much, considering the ~*s* of the lives of both women —Elizabeth Bishop⟩ ⟨stood weaving on his feet for the ~ of a long breath —F.B. Gipson⟩ ⟨finally the ~ of the high school was standardized . . . at four years —T.H. Briggs⟩ **b** (1) : relative duration of a sound (as a vowel or syllable in speech or prosody or a note in music) (2) : protracted duration or stress of a sound in speech, prosody, or music ⟨the long *a* gives the word *sale* its ~⟩ **c** *archaic* : prolixity or excess in expression ⟨there is such ~ in grief —Shak.⟩ **3 a** : distance or extent in space ⟨it would be hard, even in New England, to match Main Street for its ~ of 18th century square houses —Elizabeth Coatsworth⟩ ⟨appeared dimly white round a distant bend of the dusty road, a weary ~ behind —Haldane Macfall⟩ **b** (1) : the measure of something taken as a unit of distance ⟨darted across the highway scarcely two car ~*s* ahead of me⟩ ⟨kept most of his acquaintances at arm's ~⟩ (2) : the length of a competitor (as a horse or boat) taken as a unit in stating the margin of a lead or of victory in a race ⟨he led by three ~*s* at the top of the stretch⟩ (3) : the fully extended body ⟨stretched her ~ lazily on the warm earth⟩ ⟨took a hard right on the jaw and measured his ~ on the floor⟩ **4 a** *chiefly Scot* : an indicated or specified distance ⟨I'll go with you the ~ to the hall⟩ **b** : the degree, limit, or extreme to which a course of action or a line of thought or discussion is carried ⟨tended to carry his policy of masterly inactivity to dangerous ~*s* —Harvey Graham⟩ ⟨even went the ~ of reading the play . . . to ascertain what it was all about —G.B.Shaw⟩ ⟨here we see the foolish ~*s* to which human malevolence will go —Norman Douglas⟩ **5 a** : a long expanse or stretch ⟨brushed her ~*s* of lustrous hair⟩ ⟨large ~*s* of seas and shores between my father and my mother lay —Shak.⟩ **b** : a piece constituting or usable as part of a whole or of a connected series : SEGMENT, SECTION ⟨steel bars are furnished in standard shapes and sizes, in both coils and straight ~*s* —*advt*⟩ ⟨short ~*s* of film with both ends spliced together to permit continuous repetition —W.F. Mackey⟩ **6 a** : FLUIDITY **b** : ability to yield a fluid mixture — compare OIL LENGTH **7** *archaic* : a 42-line portion of an actor's part **8** : the volume of wort drawn from a quantity of malt during brewing **9** : the holding of more than a player's proportionate share of the cards of one suit in a card game (as four or more at bridge) ⟨he had ~ in trumps⟩ **10 a** : the distance an esp. well pitched ball in cricket travels before hitting the ground ⟨bowled a good ~⟩ ⟨pitched the ball a fraction short of a ~ —Ray Robinson⟩ **b** : the distance to be shot in archery **11** : the vertical dimension of an article of clothing esp. with reference to the part of the body it reaches or its height above the floor ⟨stockings are made in three ~*s*⟩ ⟨evening dresses in short and long ~*s*⟩ ⟨knee-*length* pants⟩ ⟨a hip-*length* jacket⟩ ⟨a floor-*length* gown⟩ — **at length** *adv* **1** : COMPREHENSIVELY, FULLY ⟨an important clash of principles which have to be debated *at length* —*London Calling*⟩ **2** : at last : FINALLY ⟨events looked forward to with trepidation, when *at length* they occur, often fall flat —George Santayana⟩

length-breadth index *n* : CRANIAL INDEX

length·en \-thən\ *vb* **lengthened; lengthened; lengthening** \-th(ə)niŋ\ **lengthens** *vt* **1** : to extend in length : make longer : ELONGATE, PROLONG, PROTRACT ⟨~*ed* their skirts halfway to their shoe tops —Mary Austin⟩ ⟨overhauls and renewals which would undoubtedly have ~*ed* her life —F.W. Crofts⟩ **2 a** *obs* : to eke out (provisions) : STRETCH **b** : to increase by diluting ⟨standing by the counter ~*ing* out a short supply of wine —Charles Dickens⟩ ~ *vi* : to grow longer ⟨fall ~*ed* out into winter —Laura Krey⟩ ⟨faces ~*ed* as the news became more certain⟩ **syn** see EXTEND

length·en·er \-th(ə)nə(r)\ *n -s* : one that lengthens

length·ful \-thfəl\ *adj* [*length* + *-ful*] *archaic* : LONG

length-height index *n* **1** : the ratio of the auricular height of the head to its length **2** : the ratio of the distance between basion and bregma on the skull to its length

length·i·ly \-thəlē, -li\ *adv* : in a lengthy or prolix manner

length·i·ness \-thēnəs, -thin-\ *n -ES* : the quality or state of being lengthy : PROLIXITY

length·ways \-th,wāz\ *adv* : LENGTHWISE

¹lengthwise \'=,=\ *adv* [*length* + *-wise*] : in the direction of the length : LONGITUDINALLY ⟨opened his newspaper, folded it ~ —Nathaniel Benchley⟩

²lengthwise \"\ *adj* : moving, placed, or directed on or toward the long axis ⟨tiers of planks had been fashioned into ~ seats —Agnes M. Cleaveland⟩

lengthy \-thē,-thi\ *adj* -ER/-EST **1 a** : protracted excessively or tediously : OVERLONG — used of written or spoken expression ⟨~ and histrionic discussions —*N.Y. Times*⟩ **b** : unduly copious : PROLIX — used of a speaker or writer ⟨must not be ~⟩ **2** : EXTENDED, LONG ⟨no very ~ journey was involved —Allan Fraser⟩ ⟨twirled his ~ key chain —Don Davis⟩ ⟨a ~ debate⟩

len·gua \'leŋgwə\ *n, pl* **lengua** *or* **lenguas** *usu cap* [Sp, lit., tongue, fr. L *lingua;* fr. its custom of wearing labrets — more at TONGUE] **1 a** : a group of Amerind peoples of Gran Chaco, Paraguay, including the Macá and Mascoi **b** : a member of any such people **2** : a language of a Lengua people

le·nien·cy \'lēnēənsē, 'lēnyən-, -si *sometimes* 'len-\ *or* **le·nience** \-ən(t)s\ *n, pl* **leniencies** *or* **leniences** : a lenient disposition or practice : MERCY ⟨had the ~ of the unprincipled —Francis Hackett⟩

le·nient \-ənt\ *adj* [L *lenient-, leniens*, pres. part. of *lenire* to soften, fr. *lenis* soft, mild; akin to Latvian *lēns* mild, slow, lazy, OSlav *lēnŭ* — more at LET (to permit)] **1** *archaic* : relieving pain (as a medicine) or stress (as soothing influence) : ASSUASIVE, EMOLLIENT **2** : of mild and tolerant disposition or effect : INDULGENT, MERCIFUL ⟨strict legality was mitigated by his ~ understanding⟩ ⟨contiguous states were attracting capital and enterprise through ~ laws —Broadus Mitchell⟩ **syn** see FORBEARING, SOFT

le·nient·ly *adv* : in a lenient manner

leni·fy \'lenə,fī, 'lēn-\ *vt* -ED/-ING/-ES [MF or LL; MF *lenifier*, fr. LL *lenificare*, fr. L *lenis* soft, mild + *-ficare* -fy] *archaic* : ALLEVIATE, ASSUAGE, MITIGATE, SOFTEN

leni-lenape *or* **lenni-lenape** \,lenē *pronunc at* LENAPE\ *n, usu cap both Ls* [Delaware, lit., real person, fr. *leni, lenni* real + *lenape* person, Indian] : ¹DELAWARE

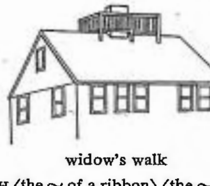
len·in·grad \'lenən,grad, -raa(ə)d\ *adj, usu cap* [fr. *Leningrad*, U.S.S.R.] : of or from the city of Leningrad, U.S.S.R. : of the kind or style prevalent in Leningrad

len·in·grad·er \-də(r)\ *n -s cap* [*Leningrad*, U.S.S.R. + E *-er*] : a native or resident of Leningrad

len·in·ism \'lenə,nizəm\ *n -s usu cap* [*Lenin* (real name, Vladimir Ilich Ulyanov) †1924 Russ. Communist leader + E *-ism*] : the political, economic, and social principles and policies advocated by Lenin; *esp* : the theory and practice of communism developed by or associated with Lenin ⟨the application of the principles of *Leninism* to an entirely new situation —D.J.Dallin⟩ ⟨appreciate *Leninism* as an organizational technique —B.I.Schwartz⟩ ⟨the . . . development of Marxism through *Leninism* and Stalinism —Francis Conklin⟩ — compare BOLSHEVISM, MARXISM, MARXISM-LENINISM, STALINISM

¹len·in·ist \'lenənəst\ *n -s usu cap* [*Lenin* + E *-ist*] : a follower of Lenin : an adherent of Leninism ⟨our party has always been a party of *Leninists* —*Russian Information &*

Rev.⟩
wid·ish \'wīdish, -dēsh\ *adj* [¹*wide* + *-ish*] : somewhat wide ⟨~ shoulders⟩ ⟨a ~ collection⟩
wid·man·stät·ten figures \'widmən,s(h)tet... 'vitmən,s(h)...\ *also* **wid·man·staet·ti·an figures** \¦==¦s(h)ted·ēən-\ *n pl, usu cap W* [*widmanstaetten* after Aloys B. *Widmannstätten* †1849 Austrian mineralogist; *widmanstaettian* fr. Aloys B. *Widmannstätten* + E *-an*] : figures that appear on etched meteoric iron and exhibit its crystalline structure
¹**wid·ow** \'wi(,)dō, -,də; -,dəw, -,dō+V\ *n* -s [ME *widewe, widwe*, fr. OE *widewe, wuduwe;* akin to OHG *wituwa, witawa* widow, Goth *widuwo*, L *vidua* widow, *-videre* to separate, Gk *ēitheos* unmarried youth, Skt *vidhavā* widow, *vidhura* separated from, *vindhate* he lacks] **1 a :** a woman who has lost her husband by death and has not since remarried — often used as if a title before a woman's marriage name ⟨the ~ Jones⟩ ⟨a tavern kept by ~ Smith⟩ **b :** a particular woman identified as having survived her husband and often as having thereby acquired legal rights that are not lost by subsequent remarriage by her though they may sometimes be lost by decree, statute, or construction (as on account of the survivor's desertion or adultery) **c** *dial Brit* **:** WIDOWER **d :** a woman whose husband deserts her or spends much time (as in a sports activity) away from her **:** GRASS WIDOW **2** — usu. used with a qualifying word ⟨poker ~⟩ ⟨fishing ~⟩ ⟨club ~⟩; compare GOLF WIDOW **2 :** one of a special class of women in the early Christian church serving as deaconesses in the performance of works of charity and in some liturgical offices (as the baptism of women) **3 :** an extra hand or part of a hand of cards that is dealt face down and usu. placed at the disposal of the highest bidder — see KITTY, SKAT **4 a :** a short line ending a paragraph and appearing at the top of a printed page or column **b :** a short line at the foot of a page or column
²**widow** \"\ *vt* -ED/-ING/-S **1 :** to cause to become a widow **:** bereave (a person) of a spouse ⟨women ~*ed* by the war⟩ ⟨he hath ~*ed* and unchilded many a one —Shak.⟩ **2 :** to survive as the widow of ⟨let me be married to three kings . . . and ~ them all —Shak.⟩ **3 :** to deprive of something greatly loved or needed **:** make desolate — usu. used with *of* ⟨the ~*ed* isle —John Dryden⟩ ⟨tank supporters . . . ~*ed* of tanks —A.J. Liebling⟩
widow-bench \'=(,)=,=\ *n* **:** the portion besides her jointure allowed to a widow from her deceased husband's estate in English law — compare FREE BENCH
widow bewitched *n, chiefly dial* **:** a woman separated from her husband **:** GRASS WIDOW 2
widow bird *or* **widow finch** *n* [so called fr. its dark plumage and long black tail feathers like a widow's veil] **:** WHYDAH
widow duck *n* **:** a West Indian tree duck (*Dendrocygna viduata*)
wid·ow·er \'widəwə(r), -dōə-\ *n* -s [ME *widewer*, fr. *widewe* widow + *-er*] **1 :** a man who has lost his wife by death and has not married again **2 :** a particular man identified as having survived his wife and often as having thereby acquired legal rights that are not lost by subsequent remarriage by him though they may sometimes be lost by decree, statute, or construction (as on account of the survivor's desertion or adultery)
wid·ow·ered \-ə(r)d\ *adj* **:** made a widower ⟨his ~ father —William Humphrey⟩
wid·ow·er·hood \-ə(r),hu̇d\ *n* **1 :** the quality or state of being a widower **2 :** the period during which a man remains a widower
widowhead *n* [ME *widewehed*, fr. *widewe* widow + *-hed* -hood (akin to ME *-hod, -had* -hood)] *obs* **:** WIDOWHOOD
wid·ow·hood \'=(,)=,hu̇d\ *n* [ME *widewehod, widewehad*, fr. *widewe* widow + *-hod, -had* -hood — more at WIDOW] **1 :** the quality or state of being a widow ⟨destined to an early ~⟩ **2 :** the period during which a person remains a widow ⟨married again after a brief ~⟩
widow lady *n, chiefly dial* **:** WIDOW
wid·ow·ly *adj* **:** of, relating to, or befitting a widow ⟨~ grief⟩
widow-maker \'=(,)=,==\ *n* **:** something dangerous to a worker's life or health; *specif* **:** a loose limb hanging in or falling from a tree in logging
wid·ow·man \-,mən\ *n, pl* **widowmen** *chiefly dial* **:** WIDOWER
widow monkey *n* **:** a So. American titi (*Callicebus torquatus*) that is black except for dull whitish arms, neck, and face and a ring of pure white around the face
widow right *n* **:** a widow's right (as dower, quarantine, or statutory share) in her deceased husband's estate
widows *pl of* WIDOW, *pres 3d sing of* WIDOW
widow's chamber *n* **:** the bedchamber apparel and furniture passing to the widow of a freeman of London by a custom formerly recognized in English law
widow's-cross \'=(,)='=\ *n, pl* **widow's-crosses :** an evergreen fleshy-leaved herb (*Sedum pulchellum*) of the eastern U.S. often cultivated for its rosy purple showy flowers
widow's frill *n* **:** STARRY CAMPION
widow's mite *n* [so called fr. the widow who cast two mites (a farthing) into the Temple treasury (Mark 12:42)] **:** a small contribution that is willingly given and is all one can afford ⟨gave his *widow's mite* to the cause⟩
widow's peak *n* [so called fr. the former belief that it is an omen of early widowhood] **:** PEAK 8
widow's quarantine *n* **:** QUARANTINE 1
widow's-tears \'=(,)='=\ *n pl but sing or pl in constr* **:** SPIDERWORT 1a
widow's walk *n* [so called fr. its use by the wives of seamen] **1 :** a railed observation platform built above the roof of a coastal dwelling for an unobstructed outlook to sea — called also *captain's walk* **2 :** a balustraded roof area



widow's walk

widow woman *n, chiefly dial* **:** WIDOW
width \'width, -itth, *chiefly substand* -ith\ *n, pl* **widths** \"s, -idts, -it(t)s\ [¹*wide* + *-th*] **1 :** a distance from side to side **:** measure taken at right angles to length **:** BREADTH ⟨the ~ of a ribbon⟩ ⟨the ~ of a printed letter⟩ ⟨carpeting available in several ~*s*⟩ ⟨traveled across the ~ of the country⟩ **2 a :** largeness or greatness in extent **:** SPACIOUSNESS, SCOPE, RANGE ⟨gives you no idea of the ~ and the depth of his knowledge —K.C.Wheare⟩ ⟨the ~ of his invective —H.J.Laski⟩ **b :** FULLNESS, AMPLITUDE ⟨give ~ to a sleeve⟩ **c :** freedom from narrowness, constraint, or limitation **:** COMPREHENSIVENESS, LIBERALITY ⟨a ~ of view⟩ ⟨concerned to give medical education a greater cultural ~ —Walter Moberly⟩ **3 :** a measured and cut piece of material ⟨a ~ of calico⟩ ⟨a ~ of board⟩ **4 :** girth at the widest part — used of a shoe last and usu. given by a letter designating a standard size ⟨wears an E ~ shoe⟩
widthways \'=,=\ *adv* **:** WIDTHWISE
widthwise \'=,=\ *adv* **:** in the direction of the width **:** LATITUDINALLY ⟨trimmings placed ~ —*Women's Wear Daily*⟩
wie·de·mann effect \'vēdə,män-, 'wēdəmən-\ *n, usu cap W* [after Gustav H. *Wiedemann* †1899 Ger. physicist] **:** twisting of a ferromagnetic rod due to the joint action of a longitudinal current in the rod and a longitudinal magnetic field — compare MAGNETOSTRICTION
wiedemann-franz law \-'frän(t)s-, -'fran(t)s-\ *n, usu cap W&F* [after Gustav H. *Wiedemann* †1899 and Rudolph *Franz* †1902 Ger. physicists] **:** a statement in physics: at a given temperature the ratio of the thermal to the electrical conductivity has nearly the same value for most metals and approximately is proportioned to the absolute temperature of the metal
wiegela *syn of* WEIGELA
wield \'wēld, *esp before pause or consonant* -ēəld\ *vt* -ED/-ING/-S [ME *welden* to have power over, control, fr. OE *wieldan;* akin to OHG *waltan* to rule, ON *valda* to rule, wield, Goth *waldan* to rule, dominate, OIr *flaith* power, rule, L *valēre* to be strong, power or authority by means of **:** GOVERN, RUN ⟨those who ~*ed* the bureaucratic machine —Hugh Seton-Watson⟩ **b :** to exercise (as power, authority, sovereignty) **:** EMPLOY ⟨~ influence⟩ ⟨a highly centralized executive wing absolute power —Aldous Huxley⟩ **4** *obs* **:** EXPRESS ⟨I love you more than word can ~ the matter —Shak.⟩ **syn** see HANDLE
wield·er \-də(r)\ *n* -s **:** one that wields (as a weapon or implement)
wieldy \-dē, -di\ *adj* **1 :** capable of wielding **:** STRONG ⟨entrusted to ~ hands —Anthony Harris⟩ **2 :** capable of being wielded **:** MANAGEABLE ⟨is a large but ~ book —*New Republic*⟩
wien bridge \'vēn-, 'wēn-\ *n, usu cap W* [after Max *Wien* †1938 Ger. physicist] **:** a bridge for measuring or comparing capacitances — compare BRIDGE 6
wie·ner *or* **wei·ner** \'wēnə(r)\ *n* -s [*wiener* short for *wienerwurst; weiner* alter. of *wiener*] **:** FRANKFURTER
wie·ner schnit·zel \'vēnə(r),shnitsəl, 'wēnə(r),sn-\ *n, usu cap W* [G, lit., Vienna cutlet] **:** a thin breaded veal cutlet served with a garnish (as lemon wedges, capers, or a fried egg)
wie·ner·wurst \'wēnə(r),= — *last syllable as at* LIVERWURST\ *n* [G, fr. *Wiener* of Vienna (fr. *Wien* Vienna) + *wurst* sausage, fr. OHG — more at WURST] **1 :** VIENNA SAUSAGE **2 :** FRANKFURTER
wie·nie *or* **wee·nie** \'wēnē, 'winē, -ni\ *n* -s [*wienie* fr. *wiener* + *-ie; weenie* alter. of *wienie*] *slang* **:** WIENER
wien's displacement law \'vēnz-, 'wēnz-\ *n, usu cap W* [after Wilhelm *Wien* †1928 Ger. physicist] **:** a statement in physics: the wavelength of thermal radiation most copiously emitted by a blackbody is inversely proportional to the absolute temperature of the body
wies·ba·den \'vēs,bädᵊn, 'vis-\ *adj, usu cap* [fr. *Wiesbaden*, Germany] **:** of or from the city of Wiesbaden, Germany **:** of the kind or style prevalent in Wiesbaden
wie·sen·bo·den \'vēzᵊn,bōdᵊn\ *n* -s [G, meadow soil, fr. *wiesen* (pl. of *wiese* meadow, fr. OHG *wisa*) + *boden* ground, soil, fr. OHG *bodam* bottom; akin to OE *wāse* mud, mire — more at OOZE, BOTTOM] **:** any of an intrazonal group of dark brown to black meadow soils rich in organic matter with gray underlayers developed through poor drainage in humid or subhumid grassy or sedgy regions
¹**wife** \'wīf\ *n, pl* **wives** \-īvz\ [ME *wif*, fr. OE *wīf;* akin to OHG *wīb* woman, wife, ON *vīf* woman; perh. akin to ON *veipr* head covering — more at WIPE] **1 a** *dial* **:** WOMAN — compare OLD WIFE **b :** a woman acting in a specified capacity — used in combination: as (1) **:** one who sells something **:** VENDER ⟨a fish*wife*⟩ ⟨an oyster*wife*⟩ (2) **:** one who has charge of something **:** KEEPER ⟨hen*wife*⟩ (3) **:** a woman worker ⟨washer*wife*⟩ ⟨hostler*wife*⟩ **2 a :** a married woman ⟨a ~ can take credit for the good in her husband —Lenard Kaufman⟩ **b :** a woman who on the basis of her tribal or societal institutions is married ⟨in sororal polygyny when a man married the eldest daughter each of her sisters became his ~ also⟩ **3 :** the female of a pair of mated animals ⟨a new ~ for the gander is introduced into the pen⟩
²**wife** \"\ *vb* -ED/-ING/-S [ME *wifen*, fr. *wif* wife] **:** WIVE
wife·hood \'wīf,hu̇d\ *n* [ME *wifhod, wifhode*, fr. OE *wīfhād* womanhood, fr. *wīf* woman, wife + *-hād* -hood] **:** the quality or state of being a wife ⟨achieved the status of ~⟩
wife·less \-,ləs\ *adj* [ME *wifles*, fr. OE *wīflēas*, fr. *wīf* wife + *-lēas* -less] **:** having no wife ⟨the only ~ man in the group of old classmates⟩
¹**wifelike** \'=,=\ *adv* **:** in a wifely manner ⟨laid, ~, her hand in one of his —Alfred Tennyson⟩
²**wifelike** \"\ *adj* **:** WIFELY
wife·li·ness \'wīflēnəs, -lin-\ *n* -ES **:** the quality or state of being wifely ⟨vague piecemeal efforts at ~ —Martha Gellhorn⟩
wife·ly *adj* [ME *wifly*, fr. OE *wīflīc*, fr. *wīf* wife + *-līc* -ly] **1 :** of, relating to, or befitting a wife ⟨~ virtues⟩ ⟨~ duty⟩ ⟨a ~ act⟩ **2 :** having the character or look of a wife ⟨stout and ~, in her chaste cambric nightgown —Ellen Glasgow⟩
wife's equity \'wīfs- *sometimes* -īvz-\ *n* **:** the equitable right or claim of a married woman prior to the married women's separate property acts as against her husband or his assignees or creditors to a reasonable provision (as by way of settlement) out of her choses in action or out of any property of hers under the jurisdiction of the court of chancery for the support of herself and her children
wif·ey *or* **wif·ie** \'wīfē, -fi\ *n* **:** WIFE — not often in formal use
wif·ish \-fish, -fēsh\ *adj* **:** WIFELY
¹**wig** \'wig\ *n* -s [ME *wigge*, fr. MLG, wedge, wedge-shaped cake; akin to MD *wegge* wedge, OHG *weggi, wecki* — more at WEDGE] *Brit* **:** a bun flavored with spices and caraway seeds
²**wig** \"\ *n* -s [short for ¹*periwig*] **1 a :** a manufactured covering of hair for the head usu. made of human hair that is woven or attached to a piece of net or a skullcap and worn as a cover for baldness or thin hair or as part of theatrical costume, official or professional dress, or fashionable attire ⟨London barristers wearing ~*s*⟩ ⟨the elaborately curled and powdered ~*s* of the 18th century⟩ — see PERUKE **b :** TOUPEE 2 **2 a :** a person wearing a wig (as a judge or lawyer) **b :** DIGNITARY, BIGWIG **3 :** an act of wigging **:** REBUKE **4 a :** the coarse fur on the shoulders of a large male hooded seal **b :** a male fur seal — **wigs on the green :** a bitter dispute **:** FUSS, CLASH ⟨feared . . . *wigs on the green* at the annual stockholders' meeting —*Time*⟩
³**wig** \"\ *vt* **wigged; wigged; wigging; wigs 1 :** to supply with a wig **2 :** to scold severely **:** CENSURE, REBUKE ⟨*wigged* me for being there the other night —*Delineator*⟩ **syn** see SCOLD
wig·an \'wigən\ *n* -s [fr. *Wigan*, Lancashire, England, where it was orig. manufactured] **:** a plain-weave cotton fabric with a stiff finish used for interlining (as tailored coats or jackets)
wig block *n* **:** a round-topped block for making, dressing, or holding a wig
wigeon *var of* WIDGEON
wigged \¦wigd\ *adj* **:** wearing a wig ⟨the judge, all ~ and robed⟩
wig·gen *or* **wig·gin** \'wigən\ *n* -s [alter. of ²*quicken*] *dial* **:** ROWAN TREE 1
wig·ger \-gə(r)\ *n* -s **:** WIGMAKER
wig·gery \-gərē, -ri\ *n* -ES **1 :** the use of wigs ⟨preferred ~ to baldness —Anthony Trollope⟩ **2 :** a business dealing in wigs ⟨a visit to a nearby theatrical ~ —P.G.Wodehouse⟩
wig·ging \-giŋ, -gēŋ\ *n* -s [²*wig* + *-ing*] **1 :** severe censure from one in authority **:** DRESSING DOWN, SCOLDING ⟨the ~ I received from my editor —C.A.Lejeune⟩ **2** *Austral* **a :** the removal of wool from around the eyes of sheep to prevent obstruction of vision **b :** wool so removed — usu. used in pl. ⟨~*s* . . . mixed with good-quality lambs' wool —R.G. Montgomery⟩
¹**wig·gle** \'wigəl\ *vb* **wiggled; wiggled; wiggling** \-g(ə)liŋ\ **wiggles** [ME *wiglen, wigelen*, of LG or D origin; akin to MLG *wiggelen* to totter, reel, MFlem *wigelen* to totter, reel, rock, MD *wiege* cradle; akin to OHG *wiga* cradle, OE *wegan* to move — more at WAY] *vi* **1 :** to move back and forth or up and down with quick jerky or shaking motions **:** JIGGLE, OSCILLATE ⟨a compass needle *wiggling* crazily⟩ ⟨the screen . . . image ~*s* —M.C.Faught⟩ ⟨high heels that make a woman ~ . . . when she walks —Wolcott Gibbs⟩ **2 :** to proceed with twisting and turning movements **:** WRIGGLE, WORM ⟨~ through a crowd⟩ ⟨has an unimaginable gift of *wiggling* in wherever he wants to —O.W.Holmes †1935⟩ ~ *vt* **:** to cause to wiggle ⟨*wiggled* his eyebrows —John Fountain⟩ ⟨found his toe and *wiggled* it —Winifred Bambrick⟩
²**wiggle** \"\ *n* -s **1 :** the motion of one that wiggles ⟨she was all smiles . . . and ~*s* coming down the broad stairs —Calvin Kentfield⟩ **2 :** shellfish or fish in cream sauce with peas ⟨shrimp ~⟩ — **get a wiggle on** *slang* **:** hurry up **:** HUSTLE ⟨better *get a wiggle on* or we'll be late⟩
wiggle nail *n* **:** CORRUGATED FASTENER
wig·gler \-g(ə)lə(r)\ *n* -s [¹*wiggle* + *-er*] **1 :** one that wiggles **2 :** the larva or pupa of the mosquito — called also *wriggler* **3 :** a tool for positioning work centers accurately by exagger-
suaded to be categorical —Lionel Hale⟩
²**wiggle-waggle** \"\ *vi* [redupl. of ¹*wiggle*] **:** to move jerkily back and forth **:** wiggle and waggle from one thing to another **:** VACILLATE ⟨*wiggle-waggles* between appeals for charity and responsible state action —*Nation*⟩
³**wiggle-waggle** \"\ *or* **wiggle-woggle** \¦==¦==\ *n* **:** something (as an amusement park contrivance) that wiggle-waggles ⟨flip-flaps, switchbacks, *wiggle-woggles* —Rose Macaulay⟩
wig·gly \'wig(ə)lē, -li\ *also* **wiggly-waggly** \¦=(=)=¦=(=)=\ *adj* **:** tending to wiggle **:** WIGGLING, WRIGGLY ⟨follow a ~ course⟩ ⟨~ lines⟩ ⟨~ worms⟩
wig·gy \'wigē\ *adj* -ER/-EST [¹*wig* + *-y*] **1 :** marked by excessive gravity and formality **:** POMPOUS ⟨a dried up, ~ . . . little scandalmonger —Richard Dehan⟩ **2 :** BEWIGGED
¹**wight** \'wīt, *usu* -īd·+V\ *n* -s [ME *wight, wiht* creature, thing, fr. OE *wiht;* akin to OHG *wiht* creature, thing, ON *vættr* creature, being, Goth *waihts* thing, OSlav *veštĭ*] **1 :** a living being **:** CREATURE, MAN ⟨no patriarch he . . . but a withered, anxious, crabbed ~ —Compton Mackenzie⟩ ⟨yonder a maid and her ~ —Thomas Hardy⟩ ⟨one of those benighted ~*s* —Norman Cousins⟩ ⟨any luckless ~ . . . who gets his wife in bad with her boss —G.W.Johnson⟩ **2** *archaic* **:** a preternatural being (as a fairy or witch) ⟨protection against uncouth ~*s* —William Morris⟩
²**wight** \"\ *adj* [ME *wight, wiht*, of Scand origin; akin to ON *vīgr* skilled in fighting, in fighting condition (neut. *vīgt*), *vīg* fight — more at VICTOR] **1** *archaic* **:** VALIANT, STALWART **2** *dial* **a :** STRONG **b :** SWIFT
³**wight** \"\ *or* **wight·ly** *adv* [*wight* fr. ME, fr. *wight, wiht* strong, swift; *wightly* fr. ME, fr. *wight, wiht* strong, swift + *-ly*] *dial* **1 :** STRONGLY **2 :** SWIFTLY
wig·less \'wigləs\ *adj* **:** having or wearing no wig ⟨tumbles headlong and ~ to the floor —Agnes Repplier⟩
wigmaker \'=,==\ *n* **:** one that makes or deals in wigs
wigs *pl of* WIG, *pres 3d sing of* WIG
wig·town·shire \'wigtən,shi(ə)r, -,tau̇n-\ *or* **wigtown** *adj, usu cap* [fr. *Wigtownshire* or *Wigtown*, Scotland] **:** of or from the county of Wigtown, Scotland **:** of the kind or style prevalent in Wigtown
¹**wig·wag** \'wi,gwag, -aa(ə)g, -aig\ *vb* [E dial. *wig* to move, shake (prob. back-formation fr. E ¹*wiggle*) + E ¹*wag*] *vi* **1 :** to signal by waving a flag or portable light according to a code in which movements to the right and left are the elements of the code alphabet and a movement to the front indicates the end (as of a word or message) **2 :** to make a signal (as with the hand or arm) ⟨~*s* through the window of his office —*advt*⟩ ~ *vt* **1 :** to signal (as a message) by wigwagging ⟨the mariner *wigwagged* the necessary directions —*Amer. Guide Series: Conn.*⟩ **2 :** to cause to wigwag ⟨*wigwagged* the white flags —*Blue Bk.*⟩
²**wigwag** \"\ *n, often attrib* **1 a :** the art or practice of wigwagging ⟨no wireless reports . . . and the papers got their news by ~ —Harland Manchester⟩ ⟨the ~ system⟩ **b :** a wigwagged message **2 :** a polishing device used by watchmakers and clockmakers in which the polisher has a back-and-forth motion
wigwag signal *n* **:** a signal at a railway grade crossing that indicates the approach of a train by the horizontal swinging of a disk
wig·wam \'wi,gwäm *also* -gwȯm\ *n* -s [Abnaki & Massachuset *wīkwām*, lit., their dwelling] **1 a :** a hut of the Indians of the region of the Great Lakes and eastward having typically an arched top and consisting of a framework of poles overlaid with bark, rush mats, or hides — compare LODGE 8a **b :** a roughly similar hut ⟨a rough ~ fashioned of fir boughs —F.V. W.Mason⟩ **2 :** a large building serving as the headquarters or meeting place (as convention hall) of a U.S. political organization (as the convention hall of the Republican Party in 1860 or any of the successive buildings housing the Tammany Society of New York) **3 :** a moderate brown that is yellower, lighter, and stronger than chestnut brown, auburn, bay, or tobacco and redder, lighter, and stronger than coffee

wigwam

wiik·ite \'vē,kīt\ *n* -s [Sw *wiikit*, fr. F. J. *Wiik* †1909 Finnish mineralogist + Sw *-it* -ite] **:** a mineral consisting probably of a mixture of samarskite, betafite, and perhaps allanite and occurring in pegmatite in Impilakhti parish on Lake Ladoga, Finland
wijs method \'vīs-\ *n, usu cap W* [after Jacob J. Alexander *Wijs* †1942 Dutch analytical chemist] **:** a method for determining the iodine number (as of an oil or fat) that consists in adding a solution of iodine monochloride in glacial acetic acid and estimating the excess of unused halogen by titration with sodium thiosulfate
wi·ke·no \wə'kā(,)nō\ *n, pl* **wikeno** *or* **wikenos** *usu cap* **1 :** a Bellabella people of British Columbia **2 :** a member of the Wikeno people
wi·ki·wi·ki \¦wēkē¦wēkē\ *adv* [Hawaiian] *Hawaii* **:** QUICKLY, FAST
wik·stroe·mia \wik'strōmēə, -rēm-\ *n, cap* [NL, fr. J. E. *Wikström* †1856 Swed. botanist + NL *-ia*] **:** a genus of chiefly Asiatic shrubs (family Thymelaeaceae) including some (as *W. canescens*) with bark that yields a fiber used in making paper and cloth
wil·bur·ite \'wilbə,rīt\ *n* -s *usu cap* [John *Wilbur* †1856 Am. Quaker preacher + E *-ite*] **:** a member of the Religious Society of Friends (Conservative) formed in the U. S. in 1845 as a protest on behalf of Inner Light against the doctrine of the Gurneyites
wil·co \'wil(,)kō\ *interj* [short for the phrase *will comply*] — used esp. in radio and signaling to indicate that a message received will be complied with
¹**wild** \'wīld, *esp before pause or consonant* 'wīəld\ *adj* -ER/-EST [ME *wilde*, fr. OE; akin to OHG *wildi* wild, ON *villr* wild, gone astray, bewildered, Goth *wiltheis* wild, W *gwyllt*, Corn *guyls*] **1 a** (1) **:** living in a state of nature **:** inhabiting natural haunts (as the forest or open field) **:** not tamed or domesticated ⟨a ~ ox⟩ ⟨~ duck⟩ (2) **:** being one of a kind not ordinarily subjected to domestication ⟨the tame ~ goose finally flew away⟩ — compare FERAL (3) **:** SHY 1a **b** (1) **:** growing or produced without the aid and care of man **:** not cultivated **:** brought forth by unassisted nature or by animals not domesticated **:** NATIVE ⟨~ furs⟩ ⟨the closest ~ relative of cultivated corn —P.C.Mangelsdorf⟩ ⟨~ honey⟩ (2) **:** related to or resembling a corresponding cultivated or domesticated organism — used in vernacular names of plants and animals; see WILD OAT, WILD ONION **c :** not living near or associated with man — used esp. of a mosquito that does not breed near human habitations in distinction from one that habitually does so **d :** of or belonging to organisms in a state of nature **:** typical of undomesticated animals or uncultivated plants ⟨the ~ state⟩ ⟨~ nature⟩ **2 a :** not inhabited or cultivated ⟨the only profit in ~ land was to clear and plant it with one's own hands or to sell it —*Amer. Guide Series: N.Y.*⟩ **b :** not being or appearing amenable to human habitation or cultivation **:** ROUGH, WASTE, DESOLATE ⟨becomes much ~*er* as the trees give place to bare granite crags —S.P.B.Mais⟩ **3 a** (1) **:** not subjected to restraint or regulation **:** UNCONTROLLED, INORDINATE, UNGOVERNED ⟨mobs are ~, unpredictable, vicious, and insanely cruel when aroused —P.I.Wellman⟩ ⟨the ~ frenzy of religious camp meetings —J.T.Adams⟩ ⟨a piano played with a ~ exuberance —Louis Bromfield⟩ (2) **:** abandoned to or overcome by passion, desire, or emotion ⟨the frenzied old man, ~ with hatred and insane with baffled desire —W.M. Thackeray⟩ ⟨~ with grief⟩; *also* **:** passionately eager, enthusiastic, desirous, or angry ⟨he was ~ to own a toy train —J.C.Furnas⟩ ⟨his sponsors . . . are ~ about him as a salesman —Howard Taubman⟩ ⟨boys ~ for the venture —Marjory S. Douglas⟩ ⟨straining and ~ to take to the air —Kay Boyle⟩ ⟨was ~ at people talking, and upsetting him —Sheila Kaye-Smith⟩ (3) **:** not amenable to control, restraint, or domestication **:** UNRULY, UNGOVERNABLE, RECKLESS ⟨bars and bowling

SHOP MATHEMATICS

A TREATISE ON APPLIED MATHEMATICS DEALING WITH VARIOUS MACHINE-SHOP AND TOOL-ROOM PROBLEMS, AND CONTAINING NUMEROUS EXAMPLES • ILLUSTRATING THEIR SOLUTION. AND THE PRACTI-CAL APPLICATION OF USEFUL RULES AND FORMULAS

BY

ERIK OBERG
EDITOR OF MACHINERY
EDITOR OF MACHINERY'S HANDBOOK AND MACHINERY'S ENCYCLOPEDIA.
AUTHOR OF "HANDBOOK OF SMALL TOOLS," ETC.

AND

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AUTHOR OF "TURNING AND BORING," "PLANING AND MILLING," "MECHANISMS AND MECHANICAL MOVEMENTS," "THREAD-CUTTING METHODS," ETC.

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then

$$V = \frac{H}{6} \times (A_1 + 4\,A_m + A_2).$$

As this formula applies to all regular solid bodies, it is useful to remember. For ordinary calculations, however, the formulas previously given for each kind of solid should be used because of greater simplicity.

Volume of a Cylinder. — A solid body having circular and parallel end faces of equal size is called a *cylinder*. (See Fig. 6.) The two parallel faces are called *bases*. The height or altitude H of a cylinder is the distance between the bases measured at right angles to the base surfaces.

The volume of a cylinder equals the area of the base multiplied by the height. The area of the base, therefore, must be

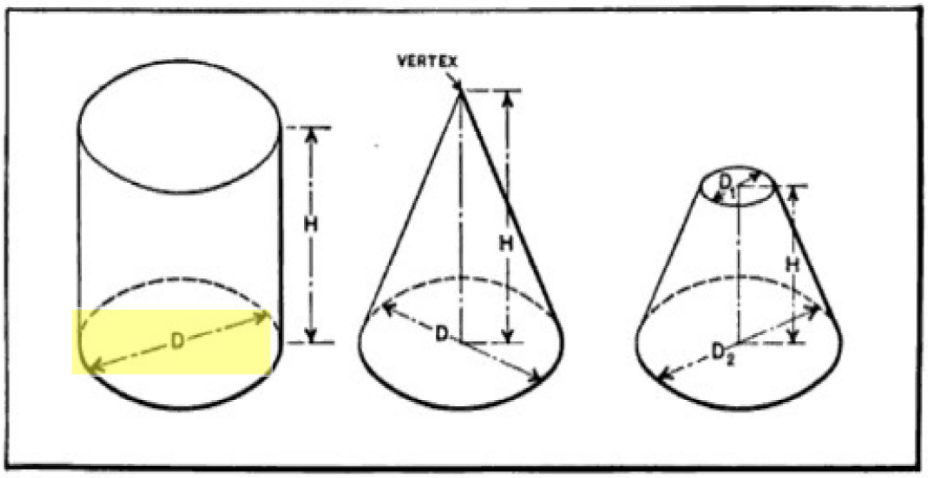

Fig. 6. Cylinder Fig. 7. Cone Fig. 8. Frustum of Cone

found before the volume can be obtained. If the diameter of the base is D, the area of the base equals $0.7854\,D^2$. The volume of the cylinder then equals:

$$0.7854 \times D^2 \times H.$$

If $D = 3$ inches and $H = 5$ inches, then the volume equals $0.7854 \times 3^2 \times 5 = 0.7854 \times 3 \times 3 \times 5 = 35.343$ cubic inches.

Volume of a Cone. — A solid body having a circular base and the sides inclined so that they meet at a common vertex, the same as in a pyramid, is called a *cone*. (See Fig. 7.) If a line is drawn from the vertex of the cone at right angle to

Filed January 23, 2018

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

COMPLETE GENOMICS, INC.

Petitioner

v.

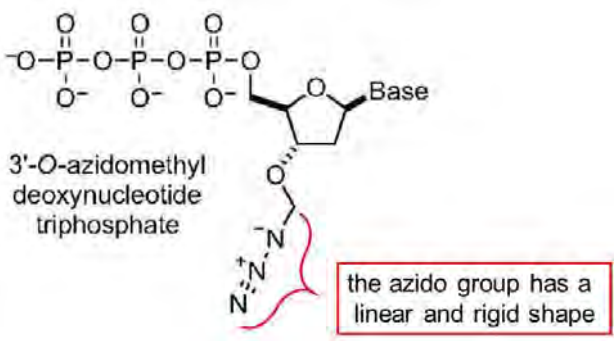
ILLUMINA CAMBRIDGE LTD.

Patent Owner

IPR2017-02172
Patent 7,566,537

PRELIMINARY RESPONSE OF PATENT OWNER ILLUMINA CAMBRIDGE LTD.

discriminates against nucleotide analogues based on size and shape"). An azidomethyl group has a linear shape. Petition at 39. The linear shape of an azidomethyl group is rigid along the three nitrogen atoms:



Ex-1101 ¶95 (page 69) (annotated); Ex-2024 ¶25. All three nitrogen atoms lie in essentially the same plane, as shown below:

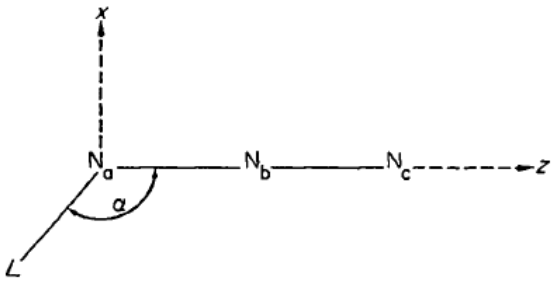


FIGURE 1. The geometry of covalent azides (*L* is the atom to which N_a is bound; all the nuclei lie in the *xz* plane).

Ex-2027 at 4. The three azido nitrogen atoms are severely restricted in their degree of freedom—the three atoms are limited to rotation about a single axis. *Id.*; Ex-2024 ¶25.

BGI argues "the linear shape of the azido group allows it to adopt rotameric states" that "lessens steric interference." Petition at 39. The linear azido group,

however, behaves essentially like a cylinder. Ex-2024 ¶25. This cylinder rotates along only one axis (the z-axis shown in the above Figure 1 from Ex-2027). *Id.* The linear and rigid cylindrical shape of the azido group would not be expected to allow each nitrogen atom to be independently repositioned within the active site to lessen steric hindrance. *Id*. Instead, the long and linear cylindrical shape of the azido group would occupy a large amount of space and provides significant steric bulk that must be accommodated within a polymerase active site. Ex-2028 at 71:1-8 ("I would think that with an azide -- an azide is a very long molecule. It's a long, narrow molecule. It's three atoms linear. That means you've got to have -- oh, about, I'm guessing, five angstroms of linear space; half a nanometer. For an enzyme, that's a lot of space, and you have to have the space to accommodate that and it can't wrap around.").

BGI relies on 3'-moieties that Hovinen and Metzker demonstrated were incorporable to assert that the allegedly "very small" azidomethyl group would also be expected to be incorporated. Petition at 38-39 (citing Ex-1101 ¶95). The linear and rigid shape of an azido group is very different from the flexible and adaptable 3'-moieties that Hovinen and Metzker demonstrated were incorporated by polymerases. These differences are as shown in the following annotated figure:

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK and QIAGEN SCIENCES, LLC,	)	
Plaintiffs,	)	Civil Action No. 19-1681-CFC
v.	)	
ILLUMINA, INC.,	)	
Defendant.	)	

DECLARATION OF JOHN KURIYAN, PH.D. IN SUPPORT OF PLAINTIFFS' OPENING CLAIM CONSTRUCTION BRIEF

TABLE OF CONTENTS

I. Professional Experience and Qualifications

1. I am Professor and Howard Hughes Medical Institute investigator in the Department of Molecular and Cell Biology at the University of California, Berkeley, California. I am also a Professor in the Department of Chemistry at Berkeley. I joined the faculty of the University of California, Berkeley in 2001. I have been a Howard Hughes Medical Institute investigator since 1990. I am also a faculty scientist at Lawrence Berkeley National Laboratory, Berkeley, California (2001 to present).

2. Prior to the appointments in Berkeley, I was a Professor at The Rockefeller University, New York, New York (1987-2001). From 1993 to 2001, I was also the Patrick E. and Beatrice Haggerty Professor at the Rockefeller University.

3. I received my Ph.D. in Chemistry in 1986 from the Massachusetts Institute of Technology, Cambridge, Massachusetts. I was a post-doctoral fellow from 1986 to 1987 at Harvard University, Cambridge, Massachusetts.

4. I received my Bachelor of Science degree in Chemistry from Juniata College, Huntington, Pennsylvania in 1981. I also attended the University of Madras from 1977 to 1979.

5. I have extensive experience in the fields of molecular biology, biochemistry and structural biology. My laboratory's focus is on the structure and function of proteins involved in cellular signal transduction and DNA replication. My laboratory utilizes x-ray crystallography and electron microscopy, as well as biochemical, biophysical, and computational analyses to understand how various proteins function.

6. My research mainly focuses on the atomic-level structure and mechanism of the enzymes and molecular switches that carry out critical cellular regulatory processes, using x-ray crystallography and electron microscopy to determine the three-dimensional structures of proteins involved in those processes, as well as biochemical, biophysical, and cell biological analyses to elucidate protein mechanisms of action. One area of my special expertise involves the molecular structure of DNA polymerases.

7. Research in my laboratory has resulted in fundamental contributions to understanding the structural basis for high-speed DNA replication. Other breakthroughs include determining the auto-inhibited structures of several tyrosine kinases, including Src family kinases and elucidating the mechanism of allosteric activation of the kinase domains of the EGF receptor,

which have provided new insights that directly resulted in the development of novel therapies used in cancer and immune diseases.

8. My curriculum vitae, which describes in greater detail my professional experience and qualifications, is attached as Exhibit 1 (JA0176–94).

I. Prior Expert Testimony

9. During the preceding five years, I have not testified at deposition or at trial.

II. Compensation

10. I am being compensated for my work in connection with this litigation at my rate of $650 per hour for the time I spend working on this matter.

II. Materials Considered

11. The opinions and conclusions I express in this report are based on my review of the patents-in-suit, U.S. Patent Nos. 10,407,458 ("'458 Patent"), 10,407,459 ("'459 Patent'"), 10,435,742 ("'742 Patent"), 10,457,984 ("'984 Patent") and 10,428,380 ("'380 Patent"); portions of the prosecution file histories of those patents ("prosecution history") that relate to the size of the capping groups; and materials listed in Exhibit 2 (JA0195–96).

12. My opinions and conclusions are also based on (1) my general knowledge of protein chemistry, structural biology and biochemistry, (2) my

experience and training as a scientist, consultant, and advisor in the biotech industry as it relates to discovery and development of new medicines, and (3) my experience working with others in the fields of chemistry, molecular biology, biochemistry and structural biology that provides me insight as to the thinking of a person of ordinary skill, as set forth below.

III. Scope of Testimony

13. I have been asked to provide my opinion on the meaning of "diameter" as used in the prosecution history, particularly in view of the diameter measurements listed in the prosecution history. Specifically, I have been asked whether the diameter of the chemical groups discussed in the prosecution history as being less than 3.7 Å in diameter would be understood by a person of ordinary skill in the art to correspond to their widths or lengths.

14. For the reasons set forth below, it is my opinion that a person of ordinary skill in the art would readily understand that diameter refers to width, not length (or longest dimension) of each chemical group.

IV. Level of Ordinary Skill in the Art

15. For the subject matter of the patents-in-suit, it is my opinion that the person of ordinary skill in the relevant time period (October 6, 2000) would have a Ph.D. in chemistry, structural biology, biochemistry, molecular biology or

a closely-related discipline, as well as two or more years of experience with DNA sequencing technology.

16. My opinions provided herein are based on an interpretation of diameter as understood by a person of ordinary skill in the art.

V. Meaning of "Diameter"

17. I understand that the parties have submitted proposed claim constructions on the term "small" as used in the claims of the patents-in-suit, and that the proposed constructions are as follows:

Claims	**Plaintiffs' Proposed Construction**	**Defendant's Proposed Construction**
'458 Patent: Claims 1, 2 '459 Patent: Claims 1, 2 '742 Patent: Claims 1, 2 '984 Patent: Claims 1, 2 '380 Patent: Claims 1, 3	"A chemical group that has a diameter, i.e., width, that is less than 3.7Å"	"A chemical group that fits within the rat DNA polymerase active site shown in Fig. 1 of the patent, i.e. has a longest dimension less than 3.7Å, including the 3′ oxygen"

18. I understand that the parties agree that "small" means a chemical group having a dimension less than 3.7 Å.

19. I have been advised that the parties disagree as to which dimension of the chemical group must be less than 3.7 Å. Plaintiffs assert that the dimension is "diameter, *i.e.*, width," whereas, defendant argues that it must be the "longest dimension . . . including the 3' oxygen" (i.e., length).

20. As explained below, it is my opinion, that a person of ordinary skill reading the patent specification and prosecution history would readily understand that the "diameter" of the chemical group, as that term is used in the prosecution history, is the width of the chemical group and cannot be "the longest dimension . . . including the 3' oxygen".

21. The patent specification explains that the chemical groups used to cap the 3' oxygen of the nucleotide ribose ("3'-O") must be small. The specification provides two examples of small chemical groups, MOM ($–CH_2$-OCH_3) and allyl ($–CH_2$-$CH=CH_2$). ('458 Patent (JA0011 at 3:41-42).) Thus, a person of ordinary skill would understand that MOM and allyl meet the size requirement for a small chemical group.

22. The meaning of a small chemical group is further discussed in the prosecution history of the patents. Specifically, a Declaration submitted by Dr. Ju, a named inventor, explains that, based on the disclosures in the patents-in-suit, a person of ordinary skill would have understood that the chemical group capping

the 3'-O had to be less than 3.7 Å in "diameter" to fit within the active site of the benchmark polymerase discussed in the specification. The Declaration also explains that the two exemplified groups, MOM and allyl, had diameters that were less than 3.7 Å. ('458 Patent PH, 5/9/19 Supplemental Communication at 4 (JA0031); Ju Decl. (JA0062–64 at ¶¶ 10-18).)

23. In addition, accompanying the Declaration is an "Analysis" section that provides additional details on the meaning of diameter, as it applies to small chemical groups. ('458 Patent FH, Ju Decl., Ex. C (JA0082–84)). Specifically, the Analysis gives the diameters for specific chemical groups, as follows:

> The calculated diameter (D) for each group is as follows:
>
> 1. Allyl ($–CH_2$-CH=CH_2): D = 3.0 Å
>
> 2. Methoxymethyl (MOM; $–CH_2$-OCH_3): D= 2.1 Å
>
> 3. Methylthiomethyl ($–CH_2$-SCH_3): D= 2.4 Å
>
> 4. Azidomethyl ($–CH_2$-N_3): D= 2.1 Å
>
> 5. 2-Nitrobenzyl ($–C_7H_6O_2N$): D = 5.0 Å

24. Based on the prosecution history, including those listed dimensions, a person of ordinary skill would have understood that the "diameter" of a small chemical group is the width of the chemical group (Plaintiffs' construction) and not its longest dimension (Defendant's construction).

25. The widths and lengths of chemical groups are derived from the lengths and angles of the bonded atoms in the group, which are reported in chemical treatises (e.g., STEREOCHEMISTRY OF ORGANIC COMPOUNDS – PRINCIPLES AND APPLICATIONS, Nasipuri, D., Revised 2nd Ed., New Age International Publishers, 1994 (JA0198–212); Nielsen and Sjögren, The Vibrational Spectra, Molecular Structure and Conformation of Organic Azides. Part IV, J. Mol. Struct. (Theochem), 150:361-379, 1987 (JA0214–32). Chemical bond lengths and angles are fundamental chemical properties that do not change. A person of ordinary skill, well before 2000, would have been able to ascertain those values without trouble for at least two reasons.

26. First, the calculations of molecular dimensions can be made manually based on the lengths and angles of the bonds in the chemical groups, which information is provided in references dating back over 50 years. Indeed, a person of ordinary skill would have been able to arrive at estimates of the lengths of the listed chemical groups without performing precise manual calculations, based on that person's knowledge of atom sizes. Those length estimations would clearly exceed the diameter values listed in the Analysis.

27. Second, since well before 2000, chemical graphics programs have been available and used to calculate the lengths and widths of chemical groups. These programs all use the same underlying principles and properties (e.g., bond

angles and bond lengths) to arrive at the same end results as a manual calculation. These calculations would be the same regardless of the software package used to make the calculations, as they are all based on chemical principles that do not change.

28. As disclosed in the prosecution history, Dr. Ju used such software to determine the diameters of the various chemical groups listed above. ('458 Patent PH, Ju Decl. (JA0063–64 at ¶¶ 16-17).)

29. I was asked to determine the widths and lengths of the four groups identified in the prosecution history as having diameters less than 3.7 Å. To do this, I used PyMOL, a commercially available and widely used software package to display the structure of each capping group (with each group attached to oxygen for purposes of making the various measurements discussed below). In doing so, I used the formulae and structures of the allyl, MOM, methylthiomethyl, and azidomethyl chemical groups reproduced in the patent prosecution history ('458 Patent PH, Ju Decl., Ex. C, p.3 (JA0084)). For example, for the allyl chemical group I used the software PRODRG (A.W. Schuttelkopf and D.M.F. van Aalten (2004) Acta Crystallograph. D60, 1355-1363), available on a website, to specify the structure O-CH_2-CH=CH_2 which the software converts into a format for viewing by PyMOL. The dimensions of the group would be determined by selecting the farthest opposing atoms in the length and width dimensions. As

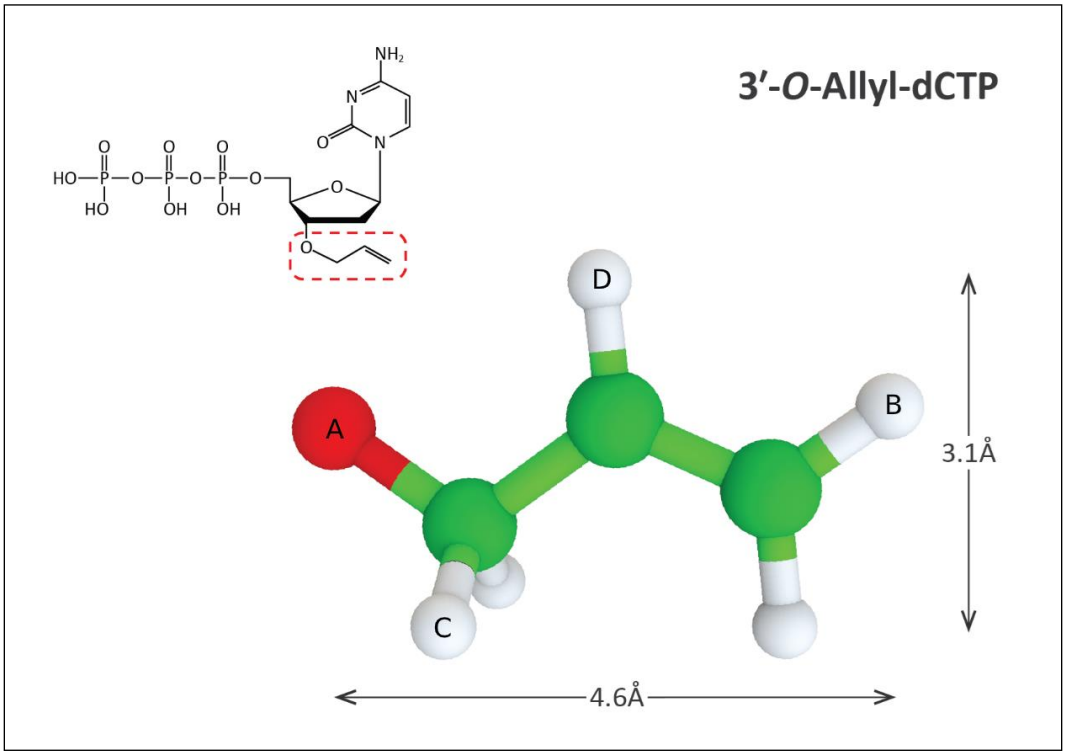
stated above, the software calculates the distance between those selected atoms using the fundamental chemical principles of bond lengths and angles.

30. I created an illustration showing the length and width dimensions of the allyl chemical group. In making the illustration I first reproduced the 2-dimensional ("2-D") structure of 3'-O-allyl-dCTP nucleotide from the prosecution history ('458 Patent PH, Ju Decl., Ex. C, p.3 (JA0084)). I then drew the 3'-O-allyl chemical group portion of the nucleotide, indicated by the dashed circle in the 2-D drawing, in 3-D, displaying the group in the ball and stick format. In the 3-D rendering, the red represents the oxygen atom attached to the 3' carbon of the nucleotide. The green represents the three carbon atoms of the allyl chemical group shown in the 2-D drawing. The white represents the hydrogen atoms that are attached to the carbon atoms. Hydrogen atoms are typically not shown in 2-D chemical drawings, but their positions are defined based on chemical principles.

31. When employing the chemical graphics program, the skilled artisan is able to view the chemical group in three dimensions, rotating the structure in space to observe the lengths and widths of the groups from different perspectives. The illustrations below, presented necessarily in two dimensions, provide a single perspective for each chemical group, which allows the lengths and widths to be viewed together. Regardless of the perspective displayed, each chemical group has a length dimension, which is the group's longest dimension,

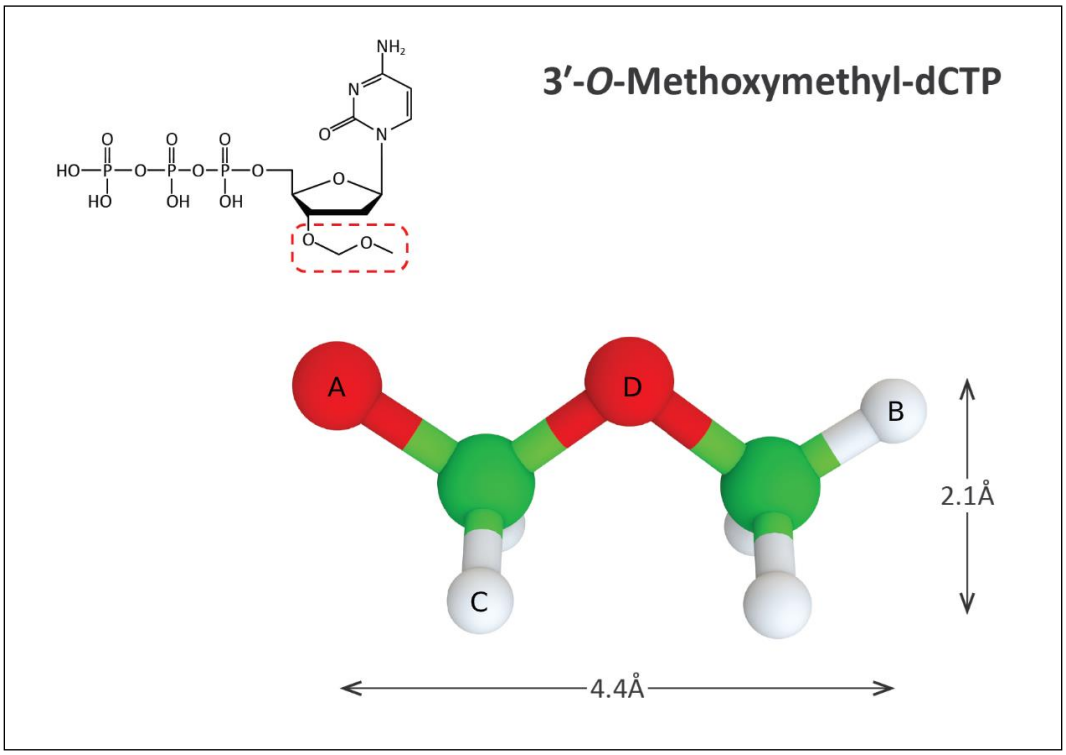
and a dimension substantially perpendicular to the length dimension, which is the chemical group's widest (i.e., width) dimension.

32. As shown in the illustration below, the allyl chemical group has a length of 4.6 Å from the 3' oxygen (from A to B, indicated by the horizontal line), and a width of 3.1 Å, (from C to D, indicated by the vertical line), with or without a 3' oxygen.

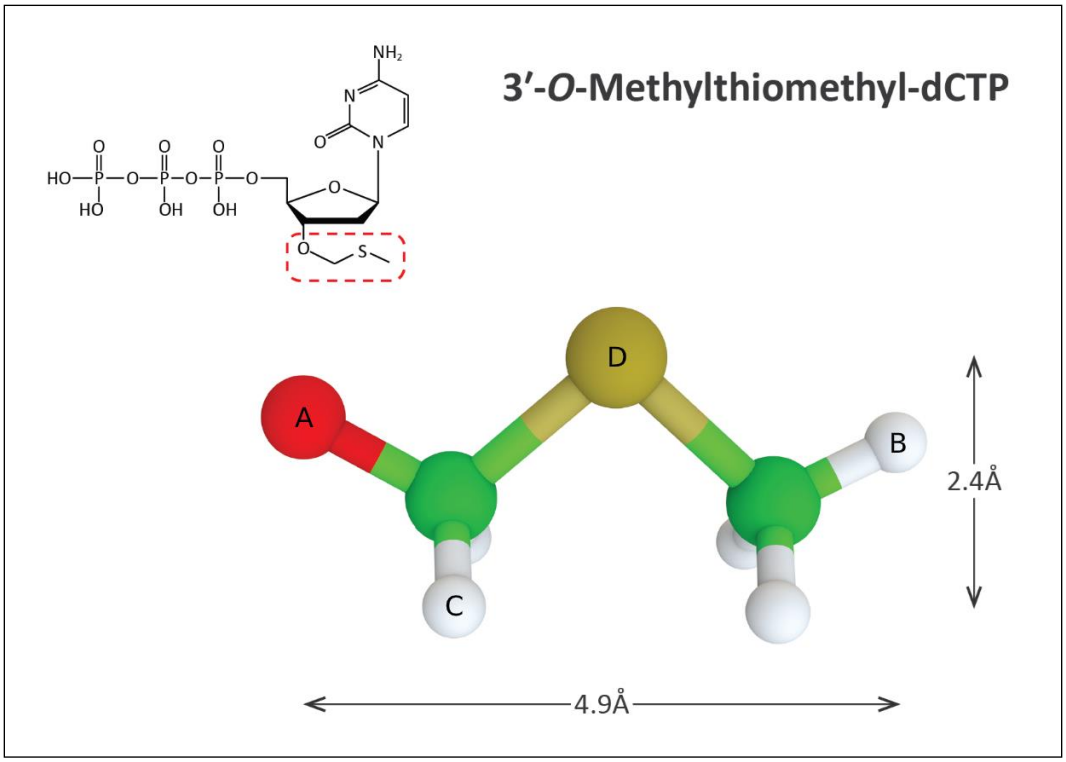


33. I followed the same format for illustrating each of the other chemical groups disclosed in the prosecution history. As shown in the illustration below, the methoxymethyl (MOM) chemical group, has a length of 4.4 Å from the

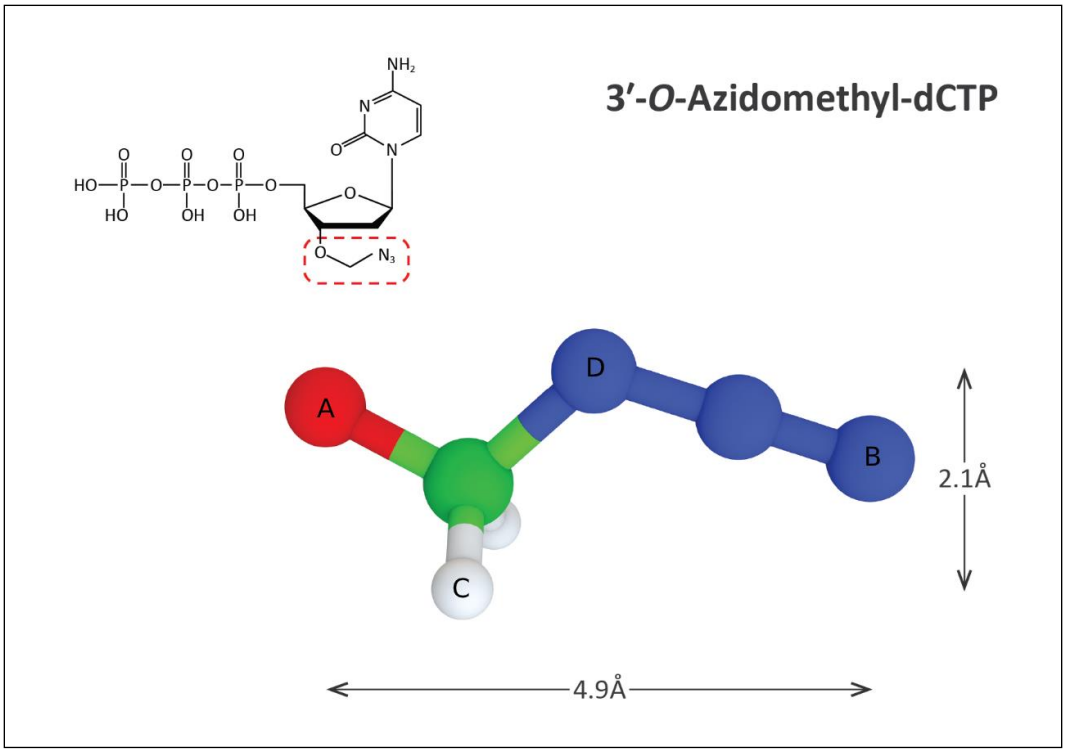
3’ oxygen (from A to B, indicated by the horizontal line), and a width of 2.1 Å (from C to D, indicated by the vertical line), with or without a 3’ oxygen.



34. As shown in the illustration below, the methylthiomethyl chemical group has a length of 4.9 Å from the 3’ oxygen (from A to B, indicated by the horizontal line), and a width of 2.4 Å (from C to D, indicated by the vertical line), with or without a 3’ oxygen. In this drawing, yellow represents a sulfur atom.



35. As shown in the illustration below, the azidomethyl chemical group has a length of 4.9 Å from the 3' oxygen (from A to B, indicated by the horizontal line), and a width of 2.1 Å (from C to D, indicated by the vertical line), with or without a 3' oxygen. In this drawing, blue represents nitrogen atoms.



36. My measurements, along with the listed diameters from the prosecution history (highlighted in yellow), are summarized in tabular form below.

Chemical Group	Diameter in the prosecution history	Measured width[1]	Measured longest dimension (from the 3' oxygen)
Allyl	3.0 Å	3.1 Å	4.6 Å
MOM	2.1 Å	2.1 Å	4.4 Å
Methylthiomethyl	2.4 Å	2.4 Å	4.9 Å
Azidomethyl	2.1 Å	2.1 Å	4.9 Å

[1] The measured width is the same regardless of whether or not the oxygen is included, as the oxygen adds only to the length dimension.

37. As can be seen, the widths that a person of ordinary of skill would have obtained consistently match (with a minor deviation for the allyl, possibly due to rounding) with the listed diameters in the inventor's Declaration. As can also be seen, none of the lengths/longest dimensions obtained matches the stated diameters in the inventor's Declaration. Indeed, the lengths are much larger than the diameter values provided in the Declaration. Therefore, a person of ordinary skill at the time would have readily understood that the "diameter" of the chemical groups cannot be its longest dimension as that measurement exceeds the "diameters" of the chemical groups provided in the prosecution history. Instead, diameter clearly refers to the width, which matches the diameters listed in the prosecution history. ('458 Patent PH, Ju Decl., Ex. C, p.3 (JA0084).) A person of ordinary skill would have arrived at this conclusion by calculating the dimensions manually or by using software existing at the time.

38. Moreover, Defendant's understanding of the relevant measurement—that the diameter would refer to the "longest dimension . . . including the 3' oxygen"—would exclude the chemical groups designated as small in the patent specification. Specifically, the patent identifies both allyl and MOM as being embodiments of the invention (i.e., small chemical groups). Under Defendant's proposal of using the longest dimension of these chemical groups

(including the 3' oxygen) as the relevant measurement, those two embodiments would far exceed 3.7 Å, and consequently, be excluded from the meaning of a small chemical group. Given that the patent identifies those groups explicitly as representative of suitably small chemical groups, a person of ordinary skill would disagree with that conclusion.

39. I declare under penalty of perjury that the foregoing is true and correct.

Executed on: June 1, 2020

John Kuriyan, Ph.D.

Exhibit 1

John Kuriyan

Date of Birth: July 24, 1960 **Place of Birth:** Mavelikera, Kerala, India

Citizenship: USA

Present Position:

Professor
University of California, Berkeley
Department of Molecular and Cell Biology
Department of Chemistry

Investigator, Howard Hughes Medical Institute

Mailing and Contact Information:

Molecular & Cell Biology
176 Stanley Hall, MC 3220
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Berkeley, CA 94720-322

Phone: 510 643 0137
E-mail: kuriyan@berkeley.edu

Education:

1. Juniata College, Huntingdon, Pennsylvania. 1981. B.S., Chemistry; (transferred after two years at the University of Madras, India).
2. Massachusetts Institute of Technology, Cambridge, MA. Department of Chemistry. Ph. D. (Physical Chemistry) February 1986. Supervisors: Gregory A. Petsko and Martin Karplus (Harvard University). Title of Thesis: The Structure and Flexibility of Myoglobin: Molecular Dynamics and X-ray Crystallography.
3. Harvard University, Cambridge, MA. March 1986 - May 1987. Department of Chemistry. Post-doctoral Fellow with Professors Martin Karplus and Gregory A. Petsko (MIT).

Positions:

1. June 1987 - February 1992: Assistant Professor. The Rockefeller University, New York, NY.
2. March 1992 - March 1993: Associate Professor. The Rockefeller University, New York, NY.
3. April 1993 - June 1997: Professor. The Rockefeller University, New York, NY.
4. July 1997 - July 2001: Patrick E. and Beatrice M. Haggerty Professor. The Rockefeller University, New York, NY.
5. November 1995 - June 1997. Associate Dean of Graduate Studies, The Rockefeller University, New York, NY.
6. September 1990 - August 1993: Assistant Investigator. Howard Hughes Medical Institute.
7. August 1993 – present: Investigator, Howard Hughes Medical Institute.
8. July 2001 – present. Professor of Molecular and Cell Biology and Chemistry. University of California, Berkeley.

9. July 2001- September 2004: Divisional Deputy for Structural Biology at the Advanced Light Source, Physical Biosciences Division. Lawrence Berkeley National Laboratory.
10. September 2004 - present: Faculty Scientist, Physical Biosciences Division, Lawrence Berkeley National Laboratory.
11. July 2007 – December 2012. Head, Division of Biochemistry and Molecular Biology, Department of Cell and Molecular Biology, University of California, Berkeley.

Selected Honors:

Member, US National Academy of Medicine, 2018
Stein & Moore Award, The Protein Society, 2017
Foreign Member of The Royal Society, London. Elected April 2015.
Doctor of Humane Letters, *honoris causa*, Juniata College, Huntington, PA. May 2014.
Merck Award, American Society of Biochemistry and Molecular Biology, 2009.
Fellow, American Academy of Arts and Sciences, Elected 2008.
Richard Lounsbery Award, US National Academy of Sciences, 2005.
Member, US National Academy of Sciences, Elected 2001.
Eli Lilly Award of the American Chemical Society, 1998.
DuPont-Merck Award of the Protein Society, 1997.
Schering-Plough Award of the American Society of Biochemistry & Molecular Biology, 1994.
Pew Scholar in the Biomedical Sciences, 1989-1993.

Professional Service (selected)

1. BBCA (Biophysical Chemistry) study section, National Institutes of Health, *Ad hoc* member, 1991. Charter Member, 1995 - 1999.
2. Member, Standing Advisory Committee (Overseas), Department of Biotechnology, Government of India, 1997-2001.
3. Member, Board of Scientific Counselors, National Cancer Institute, 2001-2005.
4. Member, Board of Trustees, Juniata College, Huntingdon, PA. 2004-2007.
5. Member, Scientific Management Board, National Center for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, July 2006-present.
6. Member, Board of Scientific Advisors, Jane Coffin Childs Memorial Fund, July 2007-2014.
7. Member, National Advisory Committee, Pew Scholars Program in the Biomedical Sciences. 2007 – present.
8. Member, Advisory Committee, Burroughs Wellcome Fund Career Awards at the Scientific Interface. 2007–2015.
9. Editorial Board, *FEBS J. (formerly European Journal of Biochemistry)*, 2000-2008.
10. Editor, *Advances in Protein Chemistry,* 2001-2009.
11. Associate Editor, *Cell*. 1995-2012.
12. Member, Editorial Board, *Proceedings of the National Academy of Sciences (USA)*. 2005- present.
13. Co-Head of Structural Biology Faculty (with David Eisenberg), *Faculty of 1000*, 2001-2012.
14. Senior Editor, *eLife.* 2012-present.
15. Scientific Advisory Board, Skirball Institute, NYU Medical Center. 2013 - present.
16. Member, Committee on Budget and Interdepartmental Relations, University of California, Berkeley. 2015 – 2017.
17. Member, Scientific Advisory Board, Amgen. 2016 – present.

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Exhibit 2

Exhibit 2
Materials Considered

1. Patents-in-suit

2. Portions of the prosecution file histories

3. STEREOCHEMISTRY OF ORGANIC COMPOUNDS – PRINCIPLES AND APPLICATIONS, Nasipuri, D., Revised 2nd Ed., New Age International Publishers, 1994 (Exhibit 3)

4. Nielsen and Sjögren, The Vibrational Spectra, Molecular Structure and Conformation of Organic Azides. Part IV, J. Mol. Struct. (Theochem), 150:361-379, 1987 (Exhibit 4)

Exhibit 3

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Principles and Applications

D. Nasipuri

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Chapter 1

Molecular Geometry and Chemical Bonding

1.1 Introduction

Literally, stereochemistry means *chemistry in space* and describes chemistry as a function of molecular geometry. Historically, it happened the other way round—certain molecular phenomena were observed and in order to explain them, the stereochemical principles had to be developed. One such observation which marked the beginning of organic stereochemistry was made by the French physicist Biot (1815) who found that certain organic molecules rotate the plane of a polarised light in solution or in the gaseous phase—a property known as optical activity. Later, Louis Pasteur (1848), who may be regarded as the *father* of organic stereochemistry, ascribed this property to the presence of some *dissymmetric grouping* of atoms in a molecule. It was, however, van't Hoff and Le Bel* (1874), working independently of each other, who laid the foundation of organic stereochemistry by postulating the *tetrahedral* geometry of carbon compounds and thus added a third dimension to the two-dimensional chemistry of earlier days. With the help of the new structural hypothesis, they not only explained optical activity in terms of asymmetric atoms but also made certain predictions which have since then been experimentally verified, e.g., the existence of optical activity in substituted allenes and spiranes. The tetrahedral disposition of the four carbon bonds has now received firm support from physical measurements of a more direct nature, e.g., electron diffraction and X-ray diffraction experiments and also from theoretical calculations.

The next important event which brought a revolutionary change in the field of stereochemistry was the introduction of the concept of conformational analysis during the early nineteen-fifties by Barton and Hassel. This concept not only helped chemists to appreciate certain detailed aspects of molecular structure in relation to physical and chemical properties but added another dimension, a time-dependent (temporal) one to the three-dimensional stereochemistry and extended its scope to reaction processes marking the advent of dynamic stereochemistry.

1.2 Molecular structure and chemical bonding

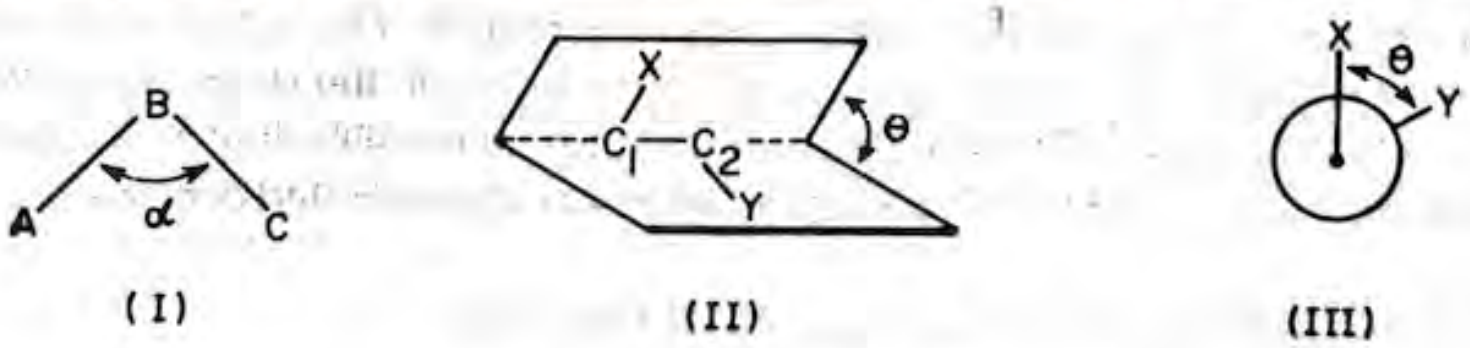
To gain an insight into the molecular structure, one must know the nature of

*Actually, Le Bel never advocated the tetrahedral theory although it was indirectly implied in his treatment (see Ramsay 1981).

chemical bondings which hold the atoms in a molecule together. For organic compounds, chemical bonding is relatively simple since it usually involves stable covalent linkages of carbon atoms with other carbon atoms, with atoms of a few other common elements like hydrogen, halogens, oxygen, and nitrogen, with other heteroatoms, and occasionally with some metals. What makes the chemistry of carbon compounds so fascinating is the unique *catenating* property of carbon atoms which combine among themselves by single and multiple covalent bonds to give almost infinite varieties of structural patterns. The non-planar geometry of a tetrahedral carbon having four ligands (connected atoms or groups) and the planar geometry of a trigonal carbon having three ligands permit the existence of structural isomerism and stereoisomerism by multiplying the possible arrangements of the ligands. Chemical bonding in carbon compounds has been adequately dealt with in numerous textbooks in organic chemistry and books on reaction mechanism and theoretical chemistry. Only a few salient features of the chemical bonding of carbon are included here which are pertinent to stereochemical discussion.

1.2.1 Bond length, bond angle, and dihedral angle

Three basic parameters are of primary importance in defining the bonding geometry of a molecule. They are bond length (l), bond angle (α), and dihedral angle (θ)*. The bond length is measured by the distance between two atomic nuclei joined together by single or multiple covalent bonds, the bond angle (α) by the angle subtended by two atoms covalently linked to a third atom as in A—B—C (I), and the dihedral angle (θ) by the angle between the two planes containing X—C_1—C_2 and C_1—C_2—Y respectively in a molecule, X—C_1—C_2—Y as shown in (II) (Figure 1.1). The dihedral angle is best seen in a Newman projection formula (III) in which the molecule is viewed along C_1—C_2 bond, the dot in the front indicating C-1 and the circle behind it indicating C-2 (the remaining four bonds are not shown). The bond length, bond angle, and dihedral angle are one-dimensional, two-dimensional, and three-dimensional parameters of a molecule involving two atoms, three atoms, and four (or more) atoms respectively. Because of vibration along a bond axis (stretching and compression) and of scissoring motion (bending in and out) across the plane of an angle, mean or equilibrium values of bond lengths and bond angles are used The deformation of a bond is a high energy process and seldom needs to be taken into consideration. On the other



(I) (II) (III)

Figure 1.1 Bond angle and dihedral angle

*Different symbols are used by different authors for these parameters.

hand, deformation of bond angles is relatively easy requiring approximately 0.04 kJ mol^{-1} for a change of 1° and may be sizeable thus causing substantial deviation in the molecular geometry from the *idealised* model based on the equilibrium values of bond lengths and bond angles. These deviations are, however, not sufficiently high to invalidate the usefulness of describing molecules in their idealised forms (see Chapter 9).

1.2.2 Covalent radii and van der Waals atomic radii

A useful concept has been introduced to express the bond length between two atoms, as in A—B, in terms of the hypothetical radii r_A and r_B, known as covalent radii of atoms A and B respectively, so that $r_A + r_B$ is equal to the equilibrium bond length. The covalent radius of an atom is independent of the nature of the other atom to which it is bonded. The bond lengths and the covalent radii of a few common elements are given in Table 1.1.

Bond energy is another very important parameter of a bond but we are not concerned with it at the moment.

Table 1.1 Bond lengths and covalent radii

Bond	Bond lengths (nm)	Element	Coordination number	Covalent radii (nm)
C—C	0.154	C	4	0.077
C=C	0.133	C	3	0.0665
C≡C	0.121	C	2	0.0605
C—H	0.110	H	1	0.033
C—O	0.143	O	2	0.074
C=O	0.121	O	1	0.062
C—N	0.147	N	3	0.074
C=N	0.127	N	2	0.062
C≡N	0.115	N	1	0.055
C—Cl	0.177	Cl	1	0.100
C—Br	0.191	Br	1	0.114
C—I	0.210	I	1	0.133

To each atom or neutral grouping corresponds a definite distance within which it resists penetration by other atoms. Pauling has estimated this distance, known as van der Waals atomic or group radius for a number of atoms and groups (Table 1.2). When two non-bonded atoms approach each other, weak attractive forces, known as van der Waals attraction (or London forces) operate until at a certain distance (r), an energy minimum is reached. Beyond this, the attractive forces are replaced by a very strong repulsive force (van der Waals repulsion or Born force). The sum of the attractive and repulsive forces is known as non-bonded interaction and the distance r is the sum of the van der Waals radii of the two atoms. It corresponds to the optimal approach between two non-bonding atoms or groups and plays an important role in determining steric strain in a molecule. The values given in Table 1.2 are from crystallographers' data which are slightly lower (by approximately 0.03 nm) than the more realistic values applied to isolated atoms (Allinger 1976) in which, unlike in crystals, intermolecular packing forces are absent.

Table 1.2 van der Waals atomic and group radii (nm)

H	0.120	O	0.140	N	0.150	Cl	0.180
C	0.150	S	0.185	P	0.190	Br	0.195
CH_3	0.200	Se	0.200	F	0.135	I	0.215

1.3 Hybridisation and chemical bonding

The electronic configuration of carbon in the ground state is $1s^2 2s^2 2p^2$ which suggests bivalency (2 empty p orbitals) for carbon. In order to provide a simple rationalisation of the bonding in carbon compounds, Pauling suggested that the four L-shell orbitals (2s, $2p_x$, $2p_y$, $2p_z$) be mixed together and then split into a set of four equivalent hybrid orbitals, designated sp^3—a process known as hybridisation. These hybrid orbitals, under idealised condition are directed towards the four corners of a regular tetrahedron. The two important consequences of hybridisation are: four bonds instead of two may be formed to carbon; and secondly, the highly directional sp^3 orbitals provide more effective overlap during bond formation which more than compensates for the extra energy required in placing the valence electrons in the hybrid orbitals. Methane and carbon tetrachloride with four identical ligands form a perfect tetrahedron with valency angles of 109.5° as shown in structure (IV) (Figure 1.2). H's joined to carbon by full lines are in the plane of the paper, H joined by a dotted line is below the plane, and H joined by a thick line is above the plane. During bonding between two sp^3 hybridised carbons as in ethane (V), two sp^3 orbitals overlap forming a C—C σ bond while the remaining six sp^3 orbitals form bonds with hydrogens by overlapping with their s orbitals. Because of the cylindrical symmetry of electron distribution in a σ bond, free rotation about a single bond might be expected.

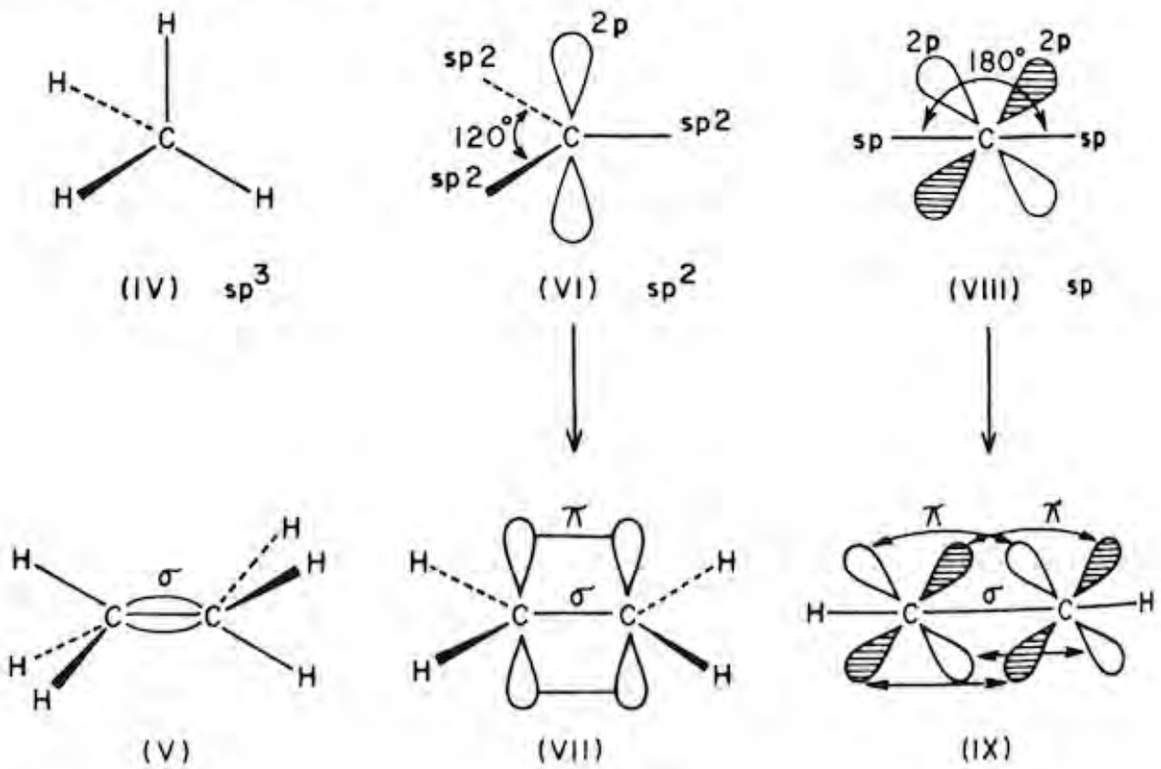


Figure 1.2 Hybridisation and bonding (only axes of hybrid orbitals are shown).

In a similar manner, one 2s and two 2p orbitals may be hybridised to give three equivalent sp^2 hybrid orbitals. They lie in a plane and are equidistant from one another making an interorbital angle of 120° (VI). Two of the sp^2 hybrid orbitals can undergo axial overlap forming a C—C σ bond as in ethylene (VII) with the remaining four hybrid orbitals forming σ bonds with four hydrogens. Each carbon is left with one 2p orbital (containing an electron) perpendicular to the $—CH_2$ plane; these orbitals can undergo lateral overlap with each other forming a π bond. The two carbons are thus doubly linked which shortens the bond length and confers extra stability to the bond. In order to provide most effective overlap between the two p orbitals, the molecule should necessarily be planar and any deviation from planarity would weaken the π bond. As a result, rotation around a double bond is highly restricted.

Hybridisation of one 2s and one 2p orbital similarly gives two equivalent sp hybrid orbitals which are linearly oriented (maximum separation) and the interorbital angle is 180° (VIII). In acetylene (IX), a σ bond is formed between the two carbons by axial overlap of an sp orbital of each and two more σ bonds to hydrogen are formed by overlap of carbon sp and hydrogen s orbitals. In addition, two π bonds are formed due to lateral overlap of two sets of mutually perpendicular 2p orbitals. The two carbon atoms are triply bonded which causes a further shortening of the bond length and an increase in the bond energy. Because of the radial distribution of electron density, rotation about the triple bond is expected to be free; in any case, it does not alter the shape of the molecule. The geometries of sp^3, sp^2, and sp hybridised carbon are tetrahedral, trigonal, and linear with four, three, and two ligands respectively.

Nitrogen with an electronic configuration, $1s^2 2s^2 2p_x^1 2p_y^1 2p_z^1$ and oxygen with an electronic configuration, $1s^2 2s^2 2p_x^2 2p_y^1 2p_z^1$ are also assumed to form bonds through similarly hybrid orbitals with the difference that one (in nitrogen) and two (in oxygen) of the hybrid orbitals are occupied by a lone pair or pairs of electrons. In Table 1.3, the characteristic properties of a few common bonds are summarised.

Table 1.3 Characteristic properties of some C—C, C—N, and C—O bonds

Type of bond	State of hybridisation	Molecular geometry	Bond length[1] (nm)	Bond angle (idealised)	Bond energy (kJ mol^{-1})	Rotation
C—C	sp^3	tetrahedral	0.154	109.5°	350	"free"[2]
C=C	sp^2	trigonal	0.134	120°	670	restricted
C≡C	sp	linear	0.120	180°	960	free
C—N	sp^3	tetrahedral	0.147	109.5°	305	"free"[2]
C=N	sp^2	trigonal	0.127	120°	616	restricted
C≡N	sp	linear	0.115	180°	893	free
C=O	sp^2	trigonal	0.121	120°	750	restricted[3]

[1] The bond lengths are already included in Table 1.1 but reproduced here for the sake of comparison.
[2] Not really free; see later.
[3] As seen in complexes.

1.3.1 Hybridisation and bond angles

In Table 1.3, the idealised values of bond angles are given. However, deviation of bond angles is rather a rule than an exception. Thus in propane (X) (Figure 1.3), the C—C—C angle is 112° and the H—C—H bond angle is 107°. Similarly, in compounds containing trigonal carbons, the bond angle is seldom 120°. Two factors are generally responsible for the deformation of bond angles: steric and electronic. Thus in propane, the bulky methyl groups interact with each other sterically and the angle between them increases with a simultaneous decrease in H—C—H angle (Thorpe-Ingold effect). In contrast, in dichloromethane (XI) the Cl—C—Cl angle is smaller (108°) and H—C—H angle is larger (112°). Here the electronic factor predominates. The C—H bond being shorter than C—Cl, the bonding electrons in the former are nearer than in C—Cl bond and exert greater electrostatic repulsion. In general, there is a balance between the two factors and it is not often easy to predict which will dominate. An alternative explanation is based on the change of hybridisation of the bonding orbitals to accommodate steric and electronic effects. In a p orbital, the electron distribution is away from the centre of a bond while in an s orbital, it is directed towards the centre. So any factor which moves the bonding electrons away from the centre (e.g., electronegativity of Cl) would increase the p character of the orbital and decrease the bond angle. This is more strikingly exhibited in H_2O and H_2S molecules where the

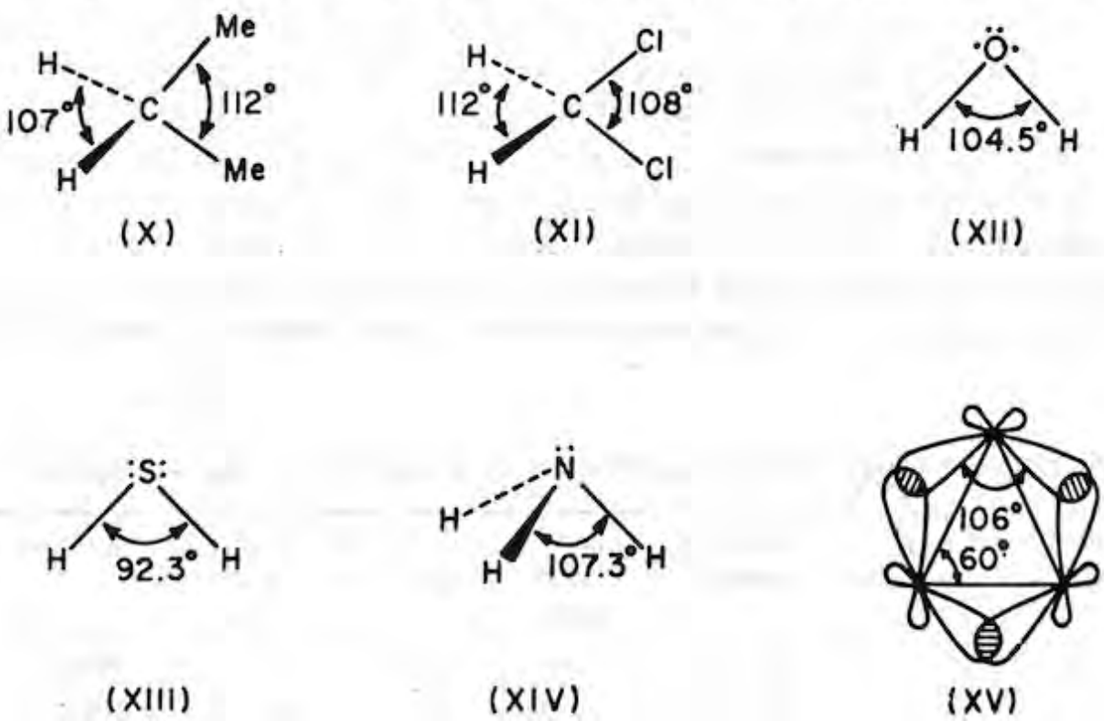


Figure 1.3 Deviation of bond angles

bond angles are respectively 104.5° and 92.3° shown in (XII) and (XIII). The bonding electrons are closer to oxygen in H_2O than to sulphur in H_2S because of higher electron affinity of oxygen which explains the difference in bond angles. Alternatively, the repulsive interactions of the lone pairs of electrons occupying the non-bonding hybrid orbitals increase the interorbital angle on their side and decrease the angle between the two hydrogens, the effect being stronger in H_2O

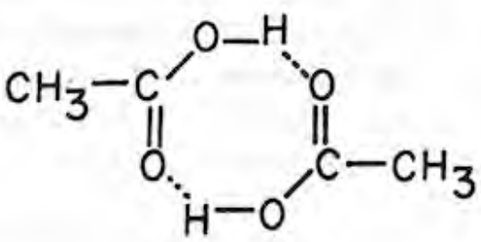
because of higher electronegativity of oxygen. The order of the repulsive interaction between pairs of electrons is: lone pair-lone pair > lone pair-bond pair > bond pair-bond pair. The H—N—H angle in ammonia (XIV) is 107.3° and may be ascribed to the interaction between lone pair and bond pair electrons.

1.3.2 Bond angle deformation in small ring compounds

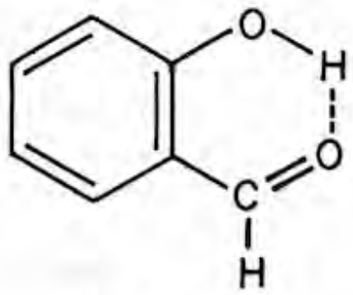
Bond angle deformation plays a more important part in cyclic compounds. Thus in cyclopropane, the internuclear angle is, by necessity, 60° much smaller than the ideal interorbital angle, 109.5°. This gives rise to serious angle strain (Baeyer strain) in the molecule which can be partially relieved by rehybridising the endo ring orbitals (increasing the p component) so that the *ideal interorbital angle* is reduced to 106° or even less. The orbitals forming C—C bonds overlap partly along the axial and partly in the lateral direction. This type of bond is known as a *banana* or *bent* or τ bond and is intermediate between a pure σ and a pure π bond. The region of maximum overlap (see XV in Figure 1.3) does not correspond to the internuclear axis and cyclopropane behaves like an unsaturated compound in certain respects (e.g., addition of bromine). In cyclobutane, the internuclear and interorbital angles are 90° and 109.5° respectively and the angle strain is considerably less. The different strains in other ring systems will be discussed in a later chapter.

1.4 Hydrogen bonding

No discussion of chemical bonding can be complete without the consideration of hydrogen bonding. When a proton donor group (A—H, A being an electronegative element) interacts with an electron donor (:B) having a lone pair of electrons or a π bond, a weak bond is formed represented by A-H · · · B known as hydrogen-bond (H-bond). If the two groups belong to two different molecules, the H-bond is called intermolecular and association between the two molecules occurs. If they form part of the same molecule, the H-bond is called intramolecular and, by default, opposes association. Examples are shown in acetic acid dimer (XVI) and salicylaldehyde (XVII) respectively (Figure 1.4). The molecules of



(XVI)



(XVII)

Figure 1.4 Hydrogen bonding

water and alcohols are highly associated due to intermolecular H-bonds. The bond angle in A-H · · · B is preferably 180° (linear) but may vary (especially in crystals or when the bond is intramolecular) depending on the requirements of the

molecular geometry. The bond length is typically 0.3 nm and the bond energy lies within 8-40 kJ mol^{-1} but can be as high as 170 kJ mol^{-1}, e.g., in F-H $\cdots$ F . The bond energy depends on the electronegativity of the acceptor centre (B), the bond angle, and the acidity of the donor group. Because of its directional property and its ability to connect groups which are many bonds apart, H-bonds play a very important part in shaping molecular geometry; they are particularly significant in molecules of biological importance. Thus the secondary structure of protein molecules, for example, arises out of H-bonds formed between N—H (donor) and C=O (acceptor) groups of non-adjacent peptide moieties. H-bonding affects the physical, chemical, and spectroscopic properties of the molecules in which it occurs. Being next to covalent bonds in energy, its occurrence profoundly influences the relative stability of molecular shapes (conformations) and is thus an important factor in conformational analysis to be discussed later. For other aspects of H-bonding, the readers are advised to consult the literature cited at the end of this chapter.

1.5 Rotation around bonds and change in dihedral angle

It has previously been noted that rotation about a single bond (e.g., C—C) is relatively free while rotation around a double bond (e.g., C=C) is highly restricted. Between these two extremes, there exist also intermediate bonds with fractional bond order arising out of internuclear delocalisation of electrons. Rotation around these bonds are also more or less restricted. These three types of bonds are discussed in the context of restricted rotation and change of dihedral angle.

1.5.1 Rotation around a single bond

According to the *principle of free rotation* of classical stereochemistry, rotation around a single bond was considered to be free. Strictly speaking, this would mean that the potential energy of the molecule is independent of the dihedral angle. However, calculation of enthalpy and entropy of ethane based on statistical mechanics showed that in order to bring agreement between the calculated and experimental values, an energy barrier of 12.5 kJ mol^{-1} has to be assumed. The diagram (Figure 1.5) shows the change of enthalpy with the change of dihedral angle from 0° to 360°. Three energy minimum conformations*, known as conformers and three energy maximum conformations representing the energy barrier arise during the operation. For ethane, the three conformers are equivalent as are the three energy maxima (barriers to rotation).

In order to specify the conformations, it is necessary to represent them in perspective formulae following certain conventions. Three modes of representations are commonly used, namely, sawhorse formula, Newman projection formula, and flying wedge formula (see Chapter 3). The first two are illustrated by the structures (XVIII) and (XIX) (for ethane) respectively. In sawhorse formula, the C—C bond is viewed sideways while in Newman projection formula, the C—C bond lies

*Spatial orientations of a molecule which differ only in the dihedral angle and are easily interconvertible are called conformations. More detailed definition is given in Chapter 10.

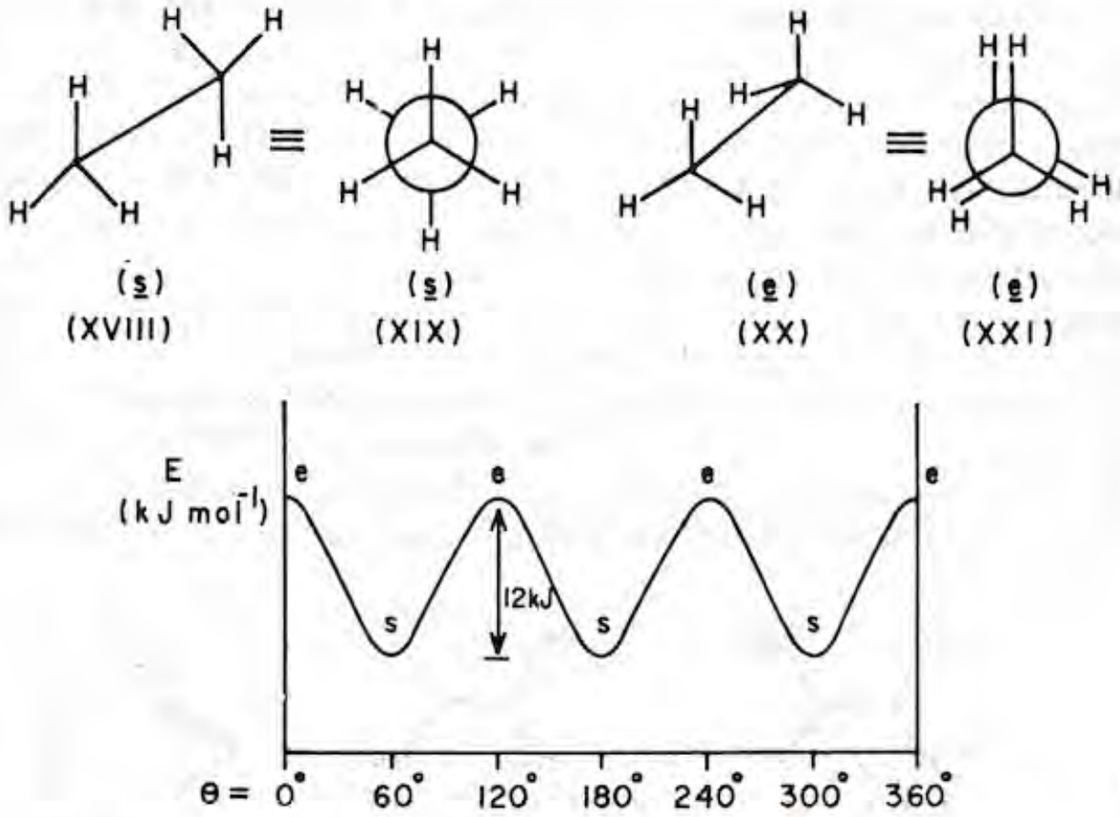


Figure 1.5 Restricted rotation in ethane

along the line of vision and cannot be seen. The other bonds are oriented radially making 120° angle with one another. The 109.5° angle of a tetrahedral carbon and 120° angle of a trigonal carbon would appear as 120° and 180° respectively when projected on a plane. The conformation (XVIII) or (XIX) with the six hydrogen atoms positioned as far apart as possible is called staggered (*s*). The conformation (XX) or (XXI) with the hydrogen atoms in pairwise conjunction is called eclipsed (*e*). The dihedral angles are respectively 60°and 0°in these two conformations. A detailed nomenclature will be given elsewhere.

According to the spectral evidence, the staggered conformations are energetically preferred and contribute to the ground state population of ethane. The eclipsed conformations correspond to the energy maxima in the diagram and serve as barriers between the conformers. Since the energy barrier is low (12.5 kJ mol^{-1}), the interconversion of conformers in ethane is fast even at a comparatively low temperature, i.e., their kinetic stability is very low.

The torsional strain in the eclipsed ethane is believed to originate from the interaction of the eclipsed C—H bonds. Steric contribution due to non-bonded interaction between the vicinal hydrogens is negligible since the internuclear distance (0.23 nm) is almost equal to twice the value of van der Waals atomic radius of hydrogen (0.12 nm) (see Chapter 9).

1.5.2 Rotation around a double bond

Rotation around a double bond is highly restricted because it disrupts a π bond. 2-Butene can be represented by two isomeric structures (XXII) and (XXIII) (Figure 1.6), both being planar and having dihedral angles of 0° and 180°

respectively (see Newman projection formulae, *a* and *b* viewed along C=C bond). They differ in the relative disposition of the methyl groups and are called cis and trans isomers respectively. This type of stereoisomerism will be discussed in more general terms later. Starting from the structure (XXII, *a*), an increase of dihedral angle to 90° breaks the π bond completely resulting in a high-energy species (XXIV, *c*) in which the two p orbitals are mutually perpendicular. A further rotation of 90° regenerates the π bond giving the trans isomer (XXIII, *b*) (which is more stable than the cis isomer by approximately 4.2 kJ mol^{-1} due to the absence of non-bonded interaction between the two methyls) with a large drop of energy (see the diagram). Another energy maximum occurs at dihedral angle 270° corresponding to a species (*c'*) which is the mirror image of XXIV (*c*) and finally, the molecule returns to the original structure (XXII) after a rotation of 360°. The energy barrier separating the two isomers is very high and as a result, inter-

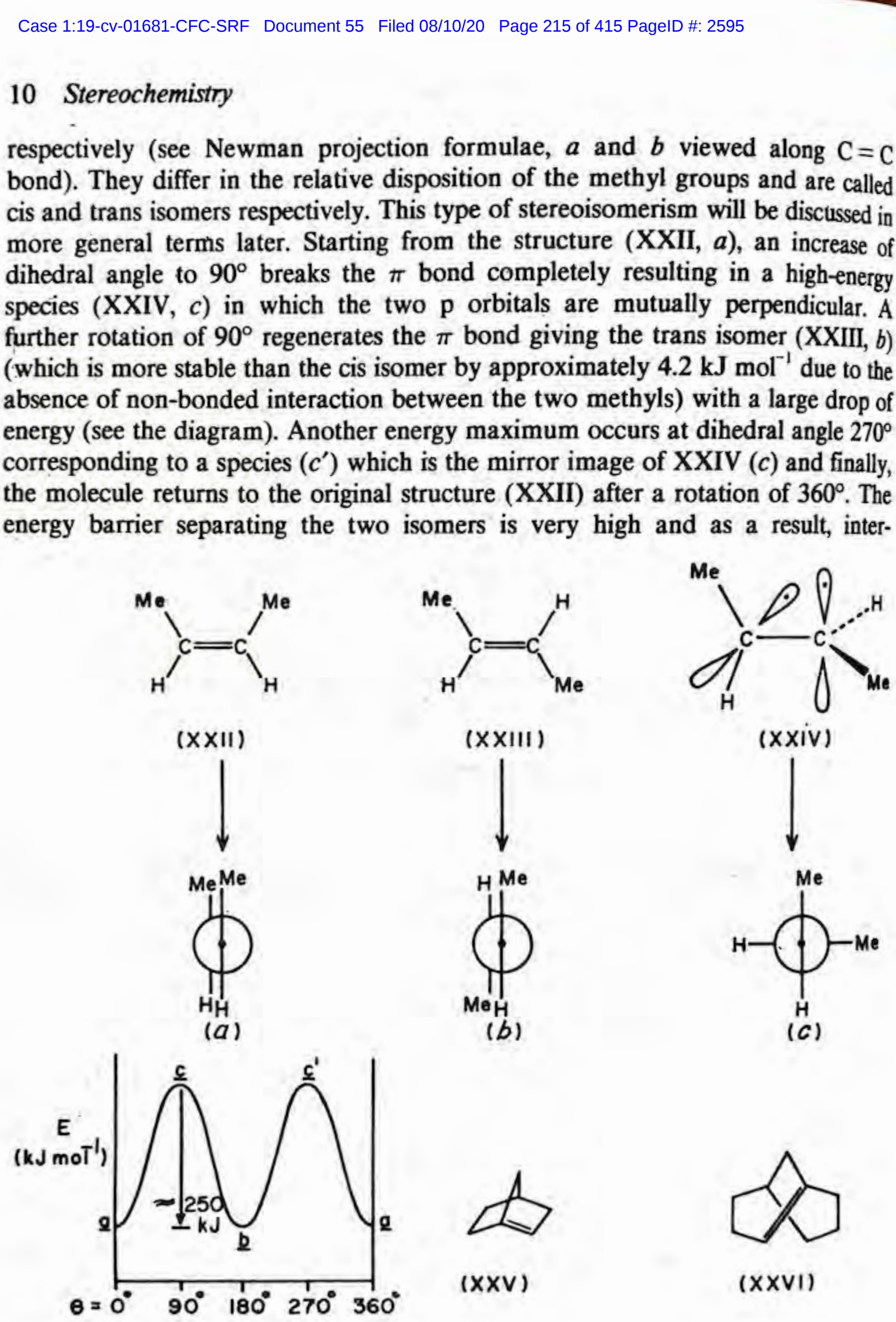


Figure 1.6 Restricted rotation around double bond. Examples of Bredt's rule

conversion between them is not possible under ordinary conditions. Other double bonds, e.g., C = N, N = N behave similarly and may lead to cis-trans isomerism.

Bredt's classical rule which states that double bond cannot exist at a bridgehead position finds its justification in the special geometrical requirement of a double bond. Thus the bicycloheptene (XXV) is not formed because the planarity required

by the π bond cannot be maintained in the rigid ring system. On the other hand, the bicyclononene (XXVI) with a bridgehead double bond is stable. Here the planarity of the π bond is accommodated by the puckering of the large ring. Many exceptions to Bredt's rule are now known*.

1.5.3 Restricted rotation around intermediate (hybrid) bonds

There are molecules in which a particular bond is neither a purely single nor a purely double bond but a hybrid between the two. A common example is 1,3-butadiene (XXVII) (Figure 1.7) in which the bond connecting the second and the third carbon develops some double bond character due to resonance between the two canonical forms (XXVIIa) and (XXVIIb). An alternative explanation based on delocalisation of the four π electrons over the σ framework of the molecule (π-orbital overlap) may be given. Two conformations, cisoid (XXVIII) and transoid† (XXVII) are possible and are separated by an energy barrier of approximately 25 kJ mol^{-1} which is much higher than in ethane but not high enough to permit

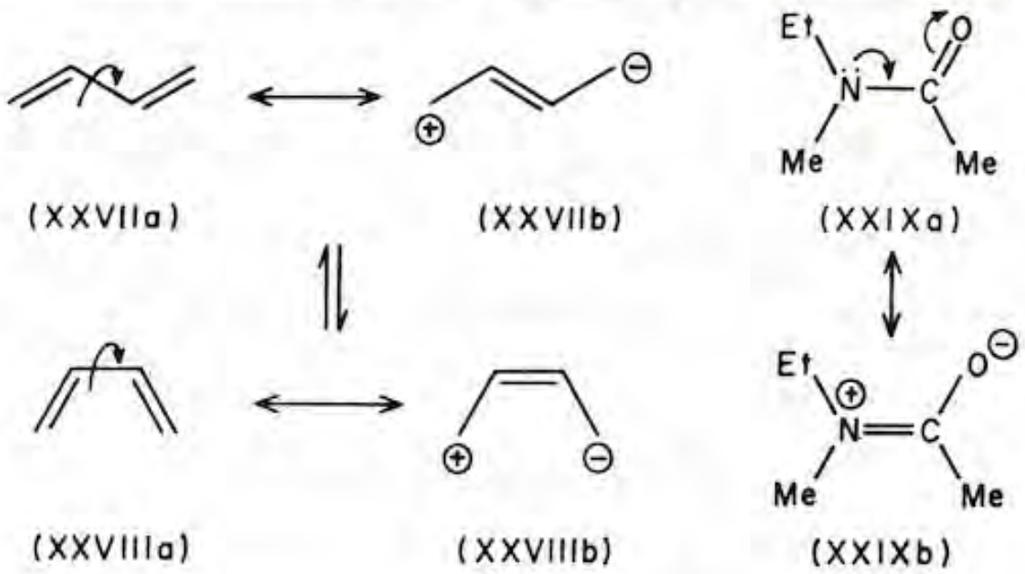


Figure 1.7 Restricted rotation around partial double bond

isolation under ordinary conditions‡. A still higher energy barrier (about 75 kJ mol^{-1}) is encountered in the rotation around C—N bond in N-methyl, N-ethylacetamide (XXIX), the double bond character of the amide bond arising out of delocalisation of the nitrogen lone pair of electrons. Here also, restricted rotation leads to two distinct isomers; however, the rotation is too fast to permit their isolation although they can be distinguished by low temperature NMR. It may be noted that any two species in equilibrium cannot ordinarily be separated at room temperature unless they are separated by a minimum energy barrier of 100

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* See Buchanan (1974) for a review of Bredt's rule and Keese (1975) for a review on bridgehead olefins.

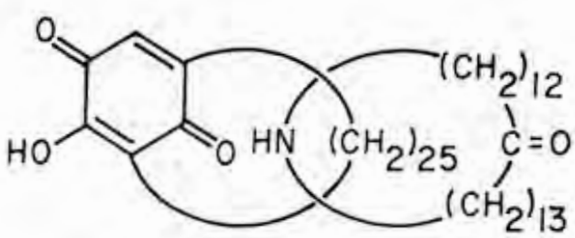
† Also called *s*-cis and *s*-trans respectively (*s* stands for single bond).

‡ The *s*-cis form has been trapped on CsI plate and studied by UV and IR (Squillacote et al 1979) by suddenly cooling a hot vapour (400-900°C) of butadiene to 30 K (matrix separation): λ_{max} of *s*-cis and *s*-trans is 226 and 220 nm respectively.

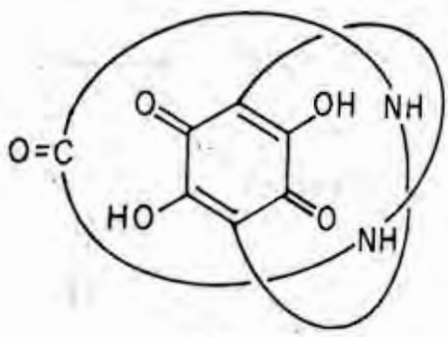
kJ mol^{-1}. Below that, they can be detected by various physical measurements (IR, NMR etc.) depending on the time-scale of observation, instrument frequency, and the average lifetime of the species in equilibrium.

1.6 Catenanes

An interesting class of compounds in which two or more rings are held together not by any chemical bond but through interlock between rings (Figure 1.8) is known as *catenanes*. One of the earliest catenane synthesised is represented by structure (XXX). Some DNA's provide examples of naturally occurring catenanes, the two closed strands being interlocked with each other. A catenane may be called a *topological isomer* of the two isolated composite rings. The catenanes pose certain intriguing problems regarding their physicochemical properties (Dmitriev 1981). The trefoils are another interesting type of molecules in which a single chain is knotted, e.g., XXXI. They are topological isomers of the corresponding unknotted molecules (Schill 1971). Topological stereochemistry has been recently reviewed (Walba 1985, Sauvage and Dietrich 1991).



(XXX)



(XXXI)

Figure 1.8 Catenane and trefoil

1.7 Summary

1. Three basic parameters, namely, bond length, bond angle, and dihedral angle which affect the molecular geometry have been defined. Deformation of a bond length is a high energy process but change of bond angle is relatively easy and of general occurrence. Fairly drastic change in bond angles is seen in small ring compounds like cyclopropane and cyclobutane in which the internuclear and interorbital bond angles are widely different. This leads to angle strain also known as Baeyer strain.

2. Bond length can be expressed as the sum of the covalent radii (a hypothetical parameter) of the two atoms forming the bond. Each atom and group has a definite radius within which it resists penetration by other atoms and groups. These radii are known as van der Waals atomic or group radii and define the optimal approach that two non-bonding atoms or groups can make. They determine the intra- and intermolecular non-bonded interactions which are responsible for steric effects in stereochemistry.

3. The formation of covalent bonds (single, double and triple) in carbon compounds has been rationalised using the concept of hybridisation of bonding

orbitals. The geometry and other characteristic properties of bonds formed by overlap of sp^3, sp^2, and sp hybrid orbitals have been discussed with particular reference to stereochemistry. Deviations of bond angles from the mean or equilibrium values expected from bond hybridisation (*idealised* values) have been explained on the basis of steric (Thorpe-Ingold effect) and electronic factors. Alternative explanation based on the change of hybridisation of bonding orbitals to accommodate steric and electronic factors is also thought to be important. The sp^3, sp^2 and sp hybridised carbons are called tetrahedral, trigonal, and linear with bond angles of 109.5°, 120°, and 180° respectively.

4. When a proton donor group (A—H) and electron donor group (:B) interact, a weak bond known as H-bond, A-H···B is formed with an average energy of 8-40 kJ mol^{-1} and a typical bond length of 0.3 nm. In view of its directional property and its ability to join two groups many bonds apart, this bond plays an important part in stereochemistry. Depending on whether the two groups, A—H and :B belong to the same molecule or to different molecules, the H-bond is called intramolecular or intermolecular. The two types affect molecular properties differently.

5. When two atoms in a molecule are joined by a single bond, the molecule behaves as a dynamic system in which a few species with different geometries (conformers) exist in equilibrium. They are usually separated by low energy barrier. Nevertheless, this affects the physical and chemical behaviour of the molecule. If the energy barrier separating the conformers is sufficiently high (>100 kJ mol^{-1}), stable stereoisomers may be expected. Rotations around single, double, and intermediate bonds have been discussed with the help of energy diagrams.

6. The different strains and interactions encountered during the discussion are weak attractive van der Waals forces (London forces), non-bonded interaction (van der Waals repulsion), angle or Baeyer strain, coulombic or electrostatic interactions, torsional strain, and interaction due to H-bond. Other interactions such as dipole-dipole and dipole-induced dipole are not separately discussed but may be collectively considered along with the H-bond, under the general term, electrostatic interactions.

7. A class of compounds in which two or more rings are interlocked without any chemical bonds between them is called catenanes.

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Exhibit 4

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THE VIBRATIONAL SPECTRA, MOLECULAR STRUCTURE AND CONFORMATION OF ORGANIC AZIDES

Part IV. An ab initio study of hydrazoic acid, azidomethane, azidoethane, azidoethene and azidomethanal*

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ABSTRACT

Fnlly optimized geometries have been calculated for the title compounds at the Hartree—Fock SCF level and compared with existing experimental data. A basis set of double zeta quality has been employed. For hydrazoic acid, a calcnlation with a larger basis set, expected to give results near the Hartree—Fock limit, has also been performed. All of the calcnlations show the azide group to be slightly bent with a *trans* configuration around the central NN bond. Azidoethane is predicted to exist in two conformations, *gauche* (71°) and *anti*, with a negligible energy difference of 0.26 kJ mol^{-1} between them. Azidoethene and azidomethanal both prefer the *syn* orientation of the azide group with respect to the C═C or C═O bonds, the computed energy difference between the *anti* and *syn* conformations being 3.31 and 30.3 kJ mol^{-1}, respectively.

The barrier to rotation around the C—N bond has been calculated to be 3.75 kJ mol^{-1} in azidomethane while in azidoethane it was 3.30 and 9.40 kJ mol^{-1} in the eclipsed *anti-clinal* (120°) and *syn* positions, respectively.

Complete harmonic force fields and dipole moment derivatives have been calculated for hydrazoic acid, azidomethane and for the two stable conformations of azidoethane. For azidoethane and azidomethanal only the azide part of the harmonic force field has been calculated. The theoretical harmonic force fields have been modified through scaling by a least squares refinement to the observed wavenumbers of hydrazoic acid, azidomethane and azidoethane (*anti* and *gauche*). Infrared vapour phase intensities have been calculated and theoretical spectra are presented for azidomethane and azidoethane.

INTRODUCTION

Early investigations of the molecular structures of organic covalent azides in the vapour phase, as for instance methyl azide [1], reported a linear azide group with a shorter terminal and a longer middle NN bond. An X-ray study of the crystal structure of cyanuric triazide [2] also reported a linear N—N—N chain, although a small deviation from linearity could not be ruled out.

*Part III of this series is given as ref. 11.

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More recent investigations of hydrazoic acid [3], methyl azide [4] and vinyl azide [5] by microwave spectroscopy, as well as an electron diffraction study of methyl azide [6], all retain the assumption of a linear azide configuration.

A subsequent electron diffraction analysis of gaseous cyanogen azide [7], on the other hand, made no such assumption and determined the N—N—N angle to be 175.3°. Later, a reinvestigation of the microwave spectra of hydrazoic acid [8] also concluded that the azide chain is non-linear, and electron diffraction measurements performed in this laboratory on a series of organic azides [9—11] all indicate that the azide group is slightly bent, with a *trans* configuration around the middle NN bond.

Two more recent ab initio calculations on HN_3 [12, 13] both confirm the non-linear geometry, and a combined electron diffraction and ab initio study of propargyl azide [14] presents the same conclusion.

In order to investigate the structures and preferred conformations of both saturated and olefinic azides, quantum chemical calculations were performed on hydrazoic acid (HN_3), azidomethane (methylazide, hereafter designated MeN_3), azidoethane (EtN_3) and azidoethene (vinylazide, VN_3).

Very little is known about the conformational properties of the industrially important acyl azides, R—CO—N_3. Microwave studies of methyl azidoformate (CH_3OCON_3) [15] and ethanoyl azide (acetyl acide, CH_3CON_3) [16] indicate that the azide group prefers the *syn* position with respect to the carbonyl group, and a calculation on the model compound azidomethanal (formyl azide, FoN_3) was also carried out.

COMPUTATIONAL DETAILS

In order to compute the equilibrium structures and harmonic force fields of the various azides, Hartree—Fock level calculations were performed using a gradient version of the program MOLECULE [17, 18]. The geometry relaxation was continued until all Cartesian forces on the atomic coordinates were less than 0.001 a.u.

A 7*s*3*p* basis set developed for the first row atoms [19] has proved its merit as a medium-sized basis set able to give equilibrium geometries in good agreement with experimental values. This set was therefore chosen as a starting set for carbon, nitrogen and oxygen. For hydrogen, a 4*s* primitive set [20] was employed and the primitive sets were contracted to double zeta quality, 4*s*2*p* on C, N and O, and 2*s* on H, the orbital exponents on hydrogen being scaled by a factor of $(1.2)^2$ [21]. This basis set is hereafter refered to as DZ.

In order to test the effect of a larger basis set flexibility on the computed azide geometries and force constants, a series of calculations were carried out on HN_3 using a relatively large basis set, expected to give results near the Hartree—Fock limit. A primitive 10*s*6*p* set [20] was chosen for nitrogen, which was contracted to 5*s*4*p* [22] and augmented by a set of *d*-functions

with exponent $\zeta = 0.95$. For hydrogen a 5*s* set [20] contracted to 3*s* was employed. The hydrogen *s*-exponents were scaled by a factor of $(1.49)^2$ [22] and a set of *p*-orbitals with exponent $\zeta = 0.80$ was added to the 3*s* set. This basis set is hereafter referred to as HF.

The force constants were calculated by numerical differentiation of the gradient vector in a number of distorted geometries. The bond lengths and angles were displaced ±2.5 pm and ±2° from their equilibrium values. For the torsional coordinates, an 8° deviation from equilibrium was chosen.

By-products of the calculations are the dipole moment derivatives with respect to the internal coordinates. The dipole moment derivatives were calculated by numerical methods imposing the Eckart conditions on the individual molecules during the deformation of the bond lengths or angles.

RESULTS AND DISCUSSION

Geometries

The computed equilibrium azide geometries are shown in Figs. 1–5 and the calculations were all consistent with a slightly bent azide structure having a shorter terminal NN distance and a *trans* configuration around the middle NN bond. All structures were optimized and the computed energy difference between the conformers is listed in the figure captions. The structural parameters of the azide group are collected in Table 1, with the available experimental values included for comparison.

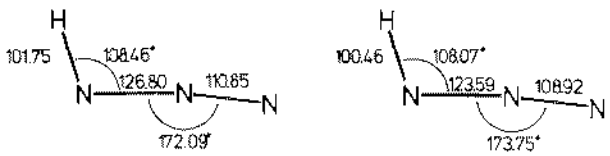

Fig. 1. The ab initio molecular structure of hydrazoic acid (HN_3). (left) Double zeta basis, computed energy: −163.583218 hartree. (right) Near-Hartree–Fock limit basis, computed energy: −163.895796 hartree. Bond lengths in pm and angles in degrees.

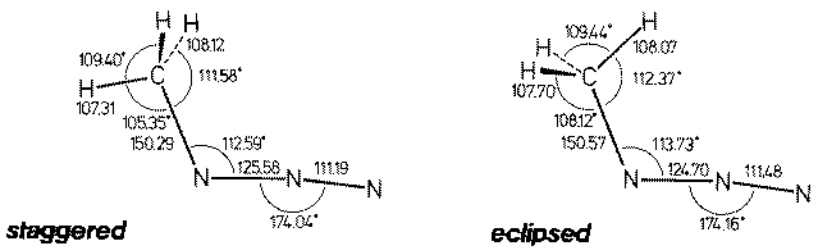

Fig. 2. The ab initio molecular structures of azidomethane (CH_3N_3): double zeta basis, computed energies, −202.567219 hartree (staggered) and −202.565796 hartree (eclipsed); units, see Fig. 1; ΔE (eclipsed–staggered) = 3.30 kJ mol^{-1}.

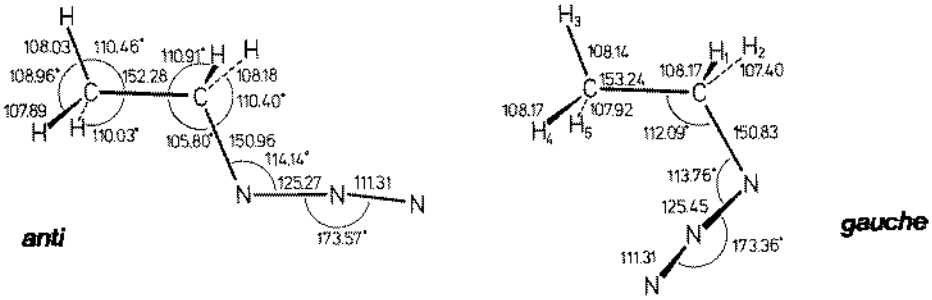

Fig. 3. The ab initio molecular structures of azidoethane ($CH_3CH_2N_3$): double zeta basis; computed energies, −241.559431 hartree (*anti*) and −241.559334 hartree (*gauche*); units, see Fig. 1; ΔE (*gauche*—*anti*) = 0.26 kJ mol^{-1}. Additional bond angles for the *gauche* conformer: $\angle NCH_1$ = 110.71°, $\angle NCH_2$ = 103.41°, $\angle CCH_1$ = 111.17°, $\angle CCH_2$ = 110.78°, $\angle H_1CH_2$ = 108.37°, $\angle CCH_3$ = 110.42°, $\angle CCH_4$ = 111.14°, $\angle CCH_5$ = 109.95°, $\angle H_3CH_4$ = 107.99°, $\angle H_3CH_5$ = 108.78°, $\angle H_4CH_5$ = 108.49°, NNCH$_1$ torsion = 71.29°, NCCH$_3$ torsion = 175.31°.

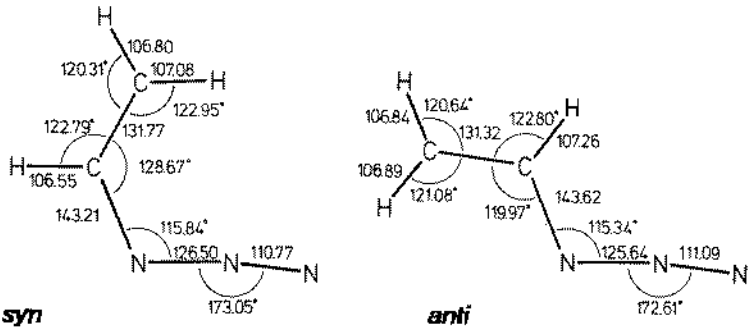

Fig. 4. The ab initio molecnlar structures of azidoethene (CH_2CHN_3): double zeta basis; computed energies, −240.378044 hartree (*syn*) and −240.376783 hartree (*anti*); units, see Fig. 1; ΔE (*anti*—*syn*) = 3.31 kJ mol^{-1}.

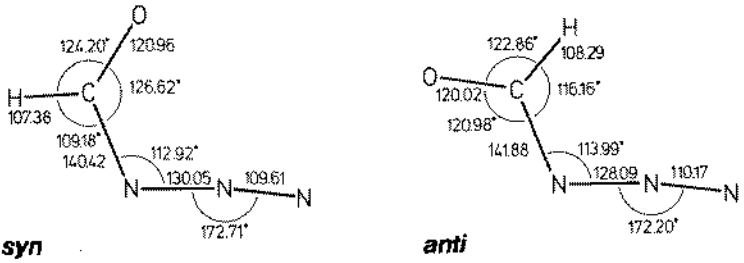

Fig. 5. The ab initio molecular structnres of formyl azide ($CHON_3$): double zeta basis; compnted energies, −276.153270 hartree (*syn*) and −276.141740 hartree (*anti*); units, see Fig. 1; ΔE = (*anti*—*syn*) = 30.3 kJ mol^{-1}.

TABLE 1

Structure (pm, degrees) and gross atomic charges (e) of the azide group in some organic azides

$N_1 \equiv N_2 = N_3 - X$		N≡N	N=N	N–X	N≡N=N	N=N–X	Gross atomic charge				Σq_A	$r_{N\equiv N}/r_{N=N}$
							q_1	q_2	q_3	q_X		
HN_3												
	DZ[a]	110.9	126.8	101.8	172.1	108.5	+0.053	−0.090	−0.290	+0.327	−0.327	0.874
	HF	108.9	123.6	100.5	173.8	108.1	−0.045	+0.235	−0.408	+0.218	−0.218	0.881
	exp[b]	113.4(2)	124.3(5)	101.5(15)	171.3(50)	108.8(40)						
CH_3N_3 (MeN_3)												
	DZ[a]	111.2	125.6	150.3	174.0	112.6	+0.044	−0.070	−0.282	−0.325	−0.308	0.885
	exp[c]	113.0(5)	121.6(4)	146.8(5)	180[d]	116.8(3)						
$CH_3CH_2N_3$ (EtN_3)												
Anti	DZ	111.3	125.3	151.0	173.6	114.1	+0.047	−0.071	−0.281	−0.195	−0.305	0.889
Gauche	DZ	111.3	125.5	150.8	173.4	113.8	+0.041	−0.068	−0.273	−0.200	−0.300	0.887
$HCCCH_2N_3$												
Gauche	DZ[i]	111.1	125.7	151.2	172.6	114.8						0.884
	exp[e]	113.7(6)	124.9(7)	146.4(13)	169.2(41)	114.5						
CH_2CHN_3 (VN_3)												
Syn	DZ	110.8	126.5	143.2	173.1	115.8	+0.090	−0.051	−0.299	+0.045	−0.260	0.876
	exp[f]	114.3(4)	125.3(6)	143.4(7)	176.5(35)	116.8(11)						
	exp[g]	114.1(9)	124.2(12)	143.2(24)	166.7(56)	114.5(23)						
Anti	DZ	111.1	125.6	143.6	172.6	115.3						
$CHON_3$ (FoN_3)												
Syn	DZ	109.6	130.1	140.4	172.7	112.9	+0.151	−0.020	−0.336	+0.491	−0.205	0.843
	exp[h]	113.1	124.8	146.0	180[d]	110.7						

[a]DZ and HF refer to basis sets; see computational details. [b]From ref. 8. [c]From ref. 6. [d]Assumed. [e]From ref. 14. [f]Structural parameters from 2-azido-1,3-butadiene, ref. 10. [g]Structural parameters from 2,3-diazido-1,3-butadiene, ref. 11. [h]Structural parameters from methyl azidoformate, CH_3OCON_3, ref. 15. [i]From ref. 14.

The calculations show that the preferred conformation of MeN_3 is staggered with a rotational barrier of 3.75 kJ mol^{-1}. This result is in good agreement with the experimental value, V_3 = 2.98 ± 0.09 kJ mol^{-1}, from the microwave investigation [4].

The conformation of EtN_3 is obviously also staggered, either *gauche* or *anti*. As seen in Table 2, the computed energy difference between the most stable, *anti*, and the less stable, *gauche*, conformation is negligible. The accuracy of the conformational energy differences, calculated at the Hartree—Fock level with the DZ basis set, is probably 1—1.5 kJ mol^{-1}, and preliminary results from an IR matrix isolation study of EtN_3 indicate an energy difference of ca. 0.6 ± 0.1 kJ mol^{-1} in the nitrogen matrix, with the *gauche* conformation as the more stable [23].

In order to determine the barriers to rotation around the C—N bond, two additional EtN_3 structures were refined: the eclipsed *anticlinal* conformer with the azide group rotated 120° away from the methyl group and the sterically unfavorable *syn* arrangement. A selected set of EtN_3 structural parameters and their variation with the C—N torsional angle are given in Table 2, and the C—N torsion potential energy function, expanded to third order in cosθ is also presented.

It appears from our calculations that the two unsaturated azides, VN_3 and FoN_3, both prefer a *syn* orientation of the azide group with respect to the adjacent C=C and C=O bonds. The computed energy difference between *the anti* and *syn* conformers of VN_3 is 3.31 kJ mol^{-1}, in reasonable agreement with the result, 1.9 ± 0.4 kJ mol^{-1}, from relative intensity measurements by microwave spectroscopy [5]. The only experimental structure parameters of vinylic azides come from recent electron diffraction studies of 2-azido-1,3-butadiene [10] and 2,3-diazido-1,3-butadiene [11]. These results

TABLE 2

Calculated variation of the ethyl azide structure with the C—N conformation (r in pm)[a]

Dihedral angle	0° (*syn*)	71.29° (*gauche*)	120° (*synclinal*)	180° (*anti*)
$r_{N=N}$	111.66	111.31	111.49	111.31
$r_{N=N}$	124.25	125.45	124.77	125.27
r_{N-X}	151.37	150.83	151.17	150.96
N=N=N	172.94°	173.36°	173.69°	173.57°
N=N—C	116.89°	113.76°	114.95°	114.14°
N—C—C	116.56°	112.09°	108.61°	105.80°
Energy (hartree)	—241.555852	—241.559334	—241.558174	—241.559431
ΔE (kJ mol^{-1})	9.40	0.26	3.30	0.0

[a]The C—N torsional potential $V(\theta) = V_0 + V_1 \cos\theta + V_2 \cos 2\theta + V_3 \cos 3\theta$ where V_0 = 2.928(83) kJ mol^{-1}; V_1 = 2.226(94) kJ mol^{-1}; V_2 = 1.734(88) kJ mol^{-1}; V_3 = 2.470(68) kJ mol^{-1}.

have been included in Table 1 for a comparison with the theoretical structure parameters of *syn* azidoethene.

For FoN_3 the *anti—syn* energy difference is calculated to be 30.3 kJ mol^{-1}, which agrees with the observation of only one conformer in the mirowave spectra of both CH_3OCON_3 [15] and CH_3CON_3 [16]. The r_0-structure of the azide group in CH_3OCON_3 [15] is presented with the theoretical structure of FoN_3 in Table 1. Apparently, the basis set employed in our calculations on polar compounds, such as FoN_3, is inadequate, but we are nevertheless confident that the acyl azides in general exist in the C—N *syn* conformation only. This conclusion may be significant for understanding the photolytic and thermal decomposition and the rearrangement of acyl azides to isocyanates.

As apparent from Table 1, the double zeta basis yields computed N—X and N=N distances which are larger than the experimental values, whereas in the Hartree—Fock limit shorter equilibrium bond lengths are, in fact, expected. These systematic deviations, and similar trends in the force constants, harmonic frequencies and charge distributions, lead to the conclusion that the double zeta basis does not provide a near Hartree—Fock limit description of the azide group.

In spite of the systematically longer N=N and N—X bond lengths computed, the calculated moments of inertia agree very well with the available observations [4, 5, 24], generally within 1—2% as apparent from Table 3. However, the *syn* conformer of VN_3 departs from this rule, believed to be due to the exceptionally large computed N=N—C angle, 128.67°. Indeed, a far better agreement with the experimental moments of inertia is obtained with a value of 125°, but it is not known whether the large N=N—C angle is a computational artifact or not. It may be noted that the proposed structures for the *syn* and *anti* conformations of VN_3 have very different C—N bond distances [5], which seems unlikely from our calculations. Further calculations, using different basis sets, would have to be carried out before any firm conclusions can be drawn, but this is beyond the scope of this work. However, the HF basis described above was applied to the HN_3 molecule and the resulting N=N and N—H bond lengths are both shorter than the experimental values, as expected when approaching the Hartree—Fock limit.

Trends within the series of azides, however, may still be revealed by the present double zeta calculations, and significant variations in the azide bond lengths were indeed observed. An increasing electronegativity of the substituent group, R, of a covalent azide, R—N_3, is expected to shorten the R—N bond and to increase the bond length difference between the NN bonds of the azide groups [25]. Table 1 shows how the ratio $r_{N=N}/r_{N=N}$ decreases from MeN_3 and EtN_3 to FoN_3. An estimate of the electronegativity of the R substituent is provided by the calculated total charge of the azide group, and a rough correlation between the total charge and the bond length ratio can be seen in the results of Table 1.

TABLE 3

Comparison between experimental and theoretical moments of inertia (uÅ²) of some organic azides

		I_A	I_B	I_C	Ref.
HN_3					
	DZ[a]	0.840455	41.9696	42.8101	
	Exp.	0.8270956	41.995168	42.895904	24
CH_3N_3 (MeN_3)					
	DZ	12.0846	93.2265	102.171	
	Exp.	11.498	93.664	102.136	4
$CH_3CH_2N_3$ (EtN_3)					
Anti	DZ	15.8907	200.182	209.877	
Gauche	DZ	38.1580	151.488	174.516	
CH_2CHN_3 (VN_3)					
Syn	DZ	33.0434	128.516	161.559	
	Exp.	34.38346	123.611	158.145	5
Anti	DZ	10.7480	183.579	194.322	
	Exp.	10.80759	183.680	194.463	5
$CHON_3$ (FoN_3)					
Syn	DZ	30.6632	122.053	152.716	
Anti	DZ	9.29037	180.008	189.298	

[a] DZ refers to double zeta basis: see computational details.

Dipole moments and charge distributions

Previous gross atomic charge calculations on HN_3 [12] and ClN_3 [26] unambiguously show the charge distribution of the azide group to be

$$R—N^-—N^+\equiv N$$

As is apparent from Table 1, the present double zeta calculations fail to reproduce this scheme, whereas the larger basis set, applied to the HN_3 molecule, agrees with the previous results. In view of the known deficiencies of the double zeta set, which have been discussed above, it is hardly surprising that the computed charges will be affected like the bond lengths and force constants (see later).

Nevertheless, the computed dipole moments given in Table 4 agree quite well with the experimental values [4, 5, 24, 27, 28]. With the exception of FoN_3, the calculations all show the azide group to be the negatively charged end. The dipole moment vector is invariably directed along the R—N bond, as was pointed out as long ago as 1931 from bulk dipole measurements on a series of phenyl azides [29]. Where experimental data are available, the angle between the experimental and computed dipole moments is found to be about 25°, with the computed moments lying nearer to the

TABLE 4

Observed and calculated dipole moments (D)[a] of some organic azides

		$\|\mu_a\|$	$\|\mu_b\|$	$\|\mu_c\|$	$\|\mu_{tot}\|$	Ref.
HN_3	(DZ)[b]	0.14	1.98	—	1.99	
	(HF)	0.09	1.82	—	1.82	
	Exp.	0.837	1.48	—	1.70	24, 27
CH_3N_3 (MeN_3)						
	(DZ)	1.50	1.76	—	2.31	
	Exp.	1.91	1.03	—	2.17	4
$CH_3CH_2N_3$ (EtN_3)						
Anti	(DZ)	1.50	1.69	—	2.26	
Gauche	(DZ)	1.95	0.75	1.14	2.38	
	Exp.				2.12	28
CH_2CHN_3 (VN_3)						
Syn	(DZ)	1.01	0.93	—	1.37	
	Exp.	1.337	0.326	—	1.376	5
Anti	(DZ)	0.72	1.60	—	1.75	
	Exp.	1.156	0.900	—	1.465	5
$CHON_3$ (FoN_3)						
Syn	(DZ)	0.92	1.25	—	1.55	
Anti	(DZ)	3.14	2.40	—	3.95	

[a] 1 D = $3.3356.10^{-30}$ C.m. [b] DZ and HF refer to the basis sets; see computational details.

perpendicular to the NNN chain. This discrepancy is believed to be a correlation effect.

Harmonic force fields and vibrational frequencies

The computed force constants of the azide group are collected in Table 5. It can be seen that the variations in the NN bond lengths are also reflected in the diagonal stretching force constants. As was mentioned above, the calculated N=N and N—X bond lengths are too long, and consequently the corresponding stretching force constants were found to be too small. Complete force fields were only computed for HN_3, MeN_3 and the *gauche* and *anti* conformations of EtN_3; some preliminary results dealing with HN_3 and MeN_3 have been published elsewhere [30].

The too low N=N and N—X stretching frequencies [30] (see also Tables 6—9) are believed to be caused by an insufficient basis set flexibility, giving too weak bonds and too long bond distances. A calculation performed on HN_3 with the HF basis set yielded shorter bond lengths and significantly higher N=N and N—H stretching force constants. The resulting frequencies are all higher than the observed values, as expected of a near-Hartree—Fock limit wavefunction, but, surprisingly, the bending modes are slightly lower than their double zeta counterparts. However, the larger basis confirms

TABLE 5

Valence force fields[a] for the azide group, ab initio results, double zeta basis

N_3—R	—H[b]	—H	—CH_3	—CH_2CH_3		—CH=CH_2	—CHO
constant				*Anti*	*Gauche*		
N≡N	25.428	22.500	21.840	21.656	21.713	22.571	25.098
N≡N/N=N	2.353	2.362	2.420	2.429	2.422	2.283	1.902
N≡N/N—X	—0.198	—0.136	—0.147	—0.171	—0.173	—0.363	—0.611
N=N/N≡N=N	0.091	—0.056	—0.097	—0.088	—0.091	—0.112	—0.059
N≡N/N=N—X	—0.021	—0.058	—0.154	—0.144	—0.133	—0.204	—0.256
N=N	9.570	7.238	7.660	7.894	7.745	7.191	5.320
N=N/N—X	0.059	—0.010	0.230	0.215	0.276	0.563	0.864
N=N/N≡N=N	0.282	0.287	0.303	0.307	0.334	0.343	0.288
N=N/N=N—X	0.941	1.171	1.097	1.038	1.059	1.163	1.319
N—X	7.721	6.746	4.506	4.326	4.371	5.389	5.685
N—X/N≡N=N	0.015	—0.025	—0.003	—0.019	—0.027	—0.037	—0.063
N—X/N=N—X	0.275	0.338	0.719	0.625	0.681	0.816	0.634
N≡N=N	0.693	0.792	0.792	0.798	0.798	0.763	0.640
N≡N=N/ N=N—X	0.102	0.089	0.139	0.135	0.138	0.148	0.151
N=N—X	1.016	1.127	1.367	1.255	1.308	1.447	1.492
$\tau_{N=N}$	0.00975	0.0175	0.0108	0.0116	0.0122	0.0127	0.0132

[a]Units: stretch and stretch/stretch, mdyn/Å; stretch/bend, mdyn/rad; bend and bend/bend, mdyn Å/rad^2. [b]Large basis set (HF); see computational details.

TABLE 6

Observed and calculated fundamental frequencies [cm^{-1}] and IR intensities [km mol^{-1}] of hydrazoic acid (H^1N_3)

Observed[a]	Calculated DZ[b]					Calculated HF					Description
	Harm.	Scaled	Intensity			Harm.	Scaled	Intensity			
			a	b	c			a	b	c	
A′											
3336 m	3488	3336	9.5	0.8	—	3731	3336	37.7	18.7	—	N—H stretch
2129 vs	2330	2132	392.9	0.1	—	2502	2129	659.2	1.1	—	N≡N stretch
1264 m	1465	1263	53.7	1.2	—	1428	1263	4.9	0.8	—	N=N—H bend
1151 vs	1026	1152	214.5	2.7	—	1228	1149	292.1	0.4	—	N=N stretch
537 w	599	531	17.7	1.3	—	569	533	17.6	3.8	—	N=N=N bend
A″											
606 w	683	603	—	—	0.8	657	601	—	—	1.8	N=N torsion

[a]From refs. 33—49: relative intensities (w, m, vs) from ref. 34. [b]DZ and HF refer to basis sets: see computational details.

TABLE 7

Observed and calculated fundamental frequencies [cm^{-1}] and IR intensities [km mol^{-1}] of methyl azide ($Me^{14}N_3$)

Observed[a]	Calculated					Description[d]
	Harm.	Scaled	Intensity			
			a	b	c	
A′						
3023 m,A	3357	3055	8.6	6.9	—	CH_3 asym. stretch
2935 s,A	3188	2901	21.6	18.3	—	CH_3 sym. stretch
2106 vs,A	2288	2106	572.7	24.8	—	N≡N stretch
1456 m,A	1661	1467	24.1	3.7	—	CH_3 asym. def.
1417 m,A	1602	1448	8.0	0.6	—	CH_3 sym. def.
1272 vs,A	1205	1279	184.9	0.7	—	N=N stretch
1132 m,A	1254	1123	8.2	1.2	—	CH_3 rock
910 m,B	890	908	0.4	8.3	—	N—C stretch
666 m,A	745	660	8.8	0.0	—	N≡N=N bend
245 m,A	291	249	6.1	0.1	—	N≡N—C bend
A″						
2962 s,C	3247	2956	—	—	41.3	CH_3 asym. stretch
1465 m,C	1665	1467	—	—	32.8	CH_3 asym. def.
1087[b] vw,C	1186	1069	—	—	6.1	CH_3 rock
560 m,C	712	561	—	—	16.2	N=N torsion
100[c]	135	100	—	—	0.5	CH_3 torsion

[a] From refs. 41—43: relative intensities (vw, m, s, vs) and band types (A, B, C) from ref. 44. [b] From ref. 44. [c] From ref. 4 (calculated using a torsional barrier of 2.98 kJ mol^{-1}). [d] Key: asym., asymmetric; sym., symmetric; def., deformation.

the validity of the double zeta force field, with the exception of the diagonal stretchings and a few small off-diagonal terms mentioned above. All the important interaction constants are calculated to be almost equal using the two basis sets, especially the very large N≡N/N=N and N=N/N=N—X interaction constants (see later).

The higher values for the frequencies calculated from a Hartree—Fock limit wavefunction can be partly attributed to the neglect of anharmonicity, and partly to an overestimation of the harmonic force constants owing to basis set truncation and the neglect of electron correlation [31]. The effect of anharmonicity can be compensated by introducing cubic and higher terms in a perturbation treatment of the vibrational problem, but the computational effort required increases rapidly with the number of internal coordinates. Not only is the perturbation procedure a cumbersome task, but a large number of additional calculations of the energy gradient have to be performed in order to determine all the cubic and higher order force constants (see later).

The aim of the present study was to yield a set of azide force fields which can be used in the assignment of vibrational spectra, and a different ap-

TABLE 8

Observed and calculated fundamental frequencies [cm^{-1}] and IR intensities [km mol^{-1}] of *anti* azidoethane ($Et^{14}N_3$)

Observed[a]	Calculated					Description
	Harm.	Scaled	Intensity			
			a	b	c	
A′						
(2995)	3282	2988	7.4	23.8	—	CH_3 asym. stretch
(2942)	3214	2926	22.5	0.1	—	CH_3 sym. stretch
(2910)	3188	2901	0.5	29.6	—	CH_2 sym. stretch
2114	2282	2106	648.7	8.8	—	N≡N stretch
1471	1672	1490	18.2	1.1	—	CH_2 scissor
1460	1664	1463	1.3	6.5	—	CH_3 asym. def.
1388	1574	1389	4.4	1.4	—	CH_3 sym. def.
1345	1506	1334	27.6	1.0	—	CH_2 wag
1280	1198	1298	254.2	0.4	—	N═N stretch
1124	1245	1116	0.8	3.2	—	CH_3 rock
1022	1055	1023	2.1	5.1	—	C—C stretch
857	891	854	0.6	2.6	—	N—C stretch
630	699	633	9.9	0.0	—	N═N═N i.p. bend
390	419	394	10.5	0.4	—	N—C—C bend
—	217	190	0.6	0.0	—	N═N—C bend
A″						
(2998)	3295	2999	—	—	27.7	CH_3 asym. stretch
(2929)	3228	2938	—	—	39.5	CH_2 asym. stretch
1450	1646	1444	—	—	19.7	CH_3 asym. def.
1244	1419	1259	—	—	0.5	CH_2 twist
1107	1259	1105	—	—	17.9	CH_3/CH_2 rock
807	873	803	—	—	1.0	CH_2/CH_3 rock
562	679	565	—	—	18.7	N═N torsion
—	252	225	—	—	4.7	CH_3 torsion
—	56	42	—	—	1.4	C—N torsion

[a]Preliminary results from ref. 23, frequencies from N_2 matrix IR spectra. Values in parentheses have not been included in the least squares adjustment.

proach, based on the introduction of empirical scale factors [31, 32], was employed. The computed harmonic force constants, F_{ij}, were adjusted according to the scheme

$$F'_{ij} = (x_i x_j)^{1/2} F_{ij} \quad (1)$$

where F'_{ij} is the scaled force constant and x_i, x_j are scale factors for the diagonal force constants F_{ii} and F_{jj}, respectively. Force constants related to the same type of internal coordinates share the same scaling factor; i.e., all the C—H stretching constants are grouped together, all the HCH bending constants are grouped together, etc. We made no effort to use special combinations of valence coordinates as advocated by Pulay et al. [32], as we

TABLE 9

Observed and calculated fundamental frequencies [cm^{-1}] and IR intensities [km mol^{-1}] of *gauche* azidoethane ($Et^{14}N_3$)

Observed[a]	Calculated					Description
	Harm.	Scaled	Intensity			
			a	b	c	
A						
(2995)	3323	3025	8.7	11.4	4.0	CH_2 asym. stretch
(2942)	3282	2988	1.1	0.1	22.4	CH_3 asym. stretch
(2950)	3255	2964	22.3	3.0	0.9	CH_3 asym. stretch
(2950)	3207	2919	4.6	1.8	26.1	CH_2 sym. stretch
(2910)	3197	2910	3.0	18.2	4.1	CH_3 sym. stretch
(2100)	2283	2104	510.2	104.7	6.3	N≡N stretch
1465	1670	1488	1.5	0.7	3.1	CH_2 scissor
1453	1665	1460	4.2	1.1	0.1	CH_3 asym. def
1450	1660	1459	2.8	5.0	7.2	CH_3 asym. def.
1382	1568	1384	2.4	8.0	1.8	CH_2 sym. def.
1341	1506	1337	15.1	2.7	0.1	CH_2 wag
(1280)	1217	1292	172.9	9.1	0.1	N=N stretch
(1244)	1436	1253	34.2	5.5	0.0	CH_2 twist
1140	1266	1124	5.6	1.6	0.4	CH_3/CH_2 rock
1082	1184	1081	3.4	1.2	5.6	CH_3 rock
989	1007	989	5.0	3.8	1.0	C—C stretch
844	855	847	0.0	1.6	0.7	C—N stretch
799	923	807	0.6	0.5	2.7	CH_2/CH_3 rock
657	734	656	8.5	1.8	0.0	N≡N≡N i.p. bend
564	675	561	2.4	8.9	15.8	N=N tors
408	426	405	0.1	4.1	0.9	C—N—N bend
—	293	260	3.2	0.0	0.5	C—N≡N bend/CH_3 torsion
—	199	179	1.4	0.0	0.2	C—N≡N bend/CH_3 torsion
—	78	58	0.3	0.5	1.6	C—N torsion

[a]Preliminary results from ref. 23, frequencies from N_2 matrix IR spectra. Values in parentheses have not been included in the least squares adjustment.

feel that this reduces the credibility of the method. The values of the factors are determined by a least squares fitting to the observed frequencies.

Among the azides studied here, only HN_3 [33—40], MeN_3 [41—44] (and older references) and EtN_3 [23, 45] have been studied by vibrational spectroscopy. Unfortunately, only the spectra and not the wavenumbers of the observed bands of $CH_3CH_2N_3$ and $CD_3CD_2N_3$ are given in ref. 45. The infrared and Raman spectra of EtN_3 are currently being interpreted in detail [23] and only preliminary results have been available in the present study.

The scaling procedure outlined was first tested with the spectroscopic data on HN_3 [33–40]. It was not possible to get any reasonable agreement with the observations by scaling the force field obtained with the DZ basis set unless a separate scale factor was applied to the large off-diagonal term

coupling the N=N stretching and N=N–H bending coordinates of HN_3. Previous experience has shown that this is often necessary with large coupling force constants or terms connecting near-degenerate, zeroth-order frequencies [31]. On the other hand, when the same scaling procedure was applied to the force field obtained with the HF basis set, no separate scaling factor was necessary.

A comparison between the unscaled force constants (Table 5) obtained with the two different basis sets reveals why it is necessary to scale the N=N/N=N–X interaction constant in the "DZ" force field separately. The N=N force constant is overestimated using the HF basis but underestimated with the DZ basis, while the interaction constants, as mentioned before, are calculated to be almost the same with the two basis sets. This, in turn, results in a down-scaling of all "HF" force constants connected with the N=N stretching coordinate but in upscaling of the corresponding "DZ" force constants. The result of introducing an extra scaling factor for the N=N/N=N–X interaction constant in the adjustment of the "DZ" force field is that the scaled value of this constant ends up almost equal to the scaled value in the corresponding "HF" force field. This interesting result leads to the bold assumption that some similar scaling should be used for all the force constants connected with the N=N stretching coordinate. Assuming equal values for the scaled "HF" and "DZ" constants N=N/N=N and N=N/N=N–X leads to a scaling factor $x_{N=N} = 0.70$ for the off-diagonal terms.

A common set of 19 scale factors was adjnsted by a least squares procedure to more than 80 observed frequencies and isotopic shifts of HN_3 (six isotopic species), MeN_3 (two isotopic species) and EtN_3 (two conformers). The spectroscopic data were weighted by σ_i^{-2}, where σ_i was taken as 1% of the observed vibrational frequencies, but not less than 5 cm^{-1}. The scaling factor for the N=N torsional constants had to be treated separately for HN_3, MeN_3 and EtN_3 in order to obtain a satisfactory agreement between observed and calculated fundamental frequencies. The scaling factor, $x_{N=N}$, for the off-diagonal terms, $F_{N=N,j}$, was constrained to the value 0.70 derived above.

The scaled azide force fields and the scale factors applied are shown and compared with "experimental" force constants for HN_3 [35, 46] and MeN_3 [46] in Table 10. The variation of the force constants from HN_3 through MeN_3 to EtN_3 can easily be rationalized in terms of σ-electron donation from the substituted group. Since the computed force fields for the azide group in the *anti* and *gauche* conformations of EtN_3 are almost identical we give only the scaled force field of the *anti* conformer. The calculated frequencies of $H^{14}N_3$, $Me^{14}N_3$ and the *anti* and *gauche* conformations of $Et^{14}N_3$ are included in Tables 6–9, respectively.

The scaled azide force field of VN_3 was transferred to 2-azido-1,3-butadiene [10] and to 2,3-diazido-1,3-butadiene [11] with reasonable sucess. We have further transferred the scaled force field of the $-CH_2N_3$ fragment of

TABLE 10

"Experimental" and scaled quantum mechanical force fields for the azide part of HN_3, CH_3N_3 and $CH_3CH_2N_3$ (*anti*)

Constant[a]	HN_3				CH_3N_3		*Anti* $CH_3CH_2N_3$	Scale factor
	HF	DZ	Exp.[b]	Exp.[c]	DZ	Exp.[c]	DZ	DZ
N≡N	17.905	17.735	16.00[d]	17.181	17.215	16.607	17.073	0.788
N≡N/N=N	1.898	1.755	0.73	1.00[d]	1.797	1.00[d]	1.805	
N≡N/N–X	−0.149	−0.115			−0.129		−0.150	
N≡N/N≡N=N	0.072	−0.044		0.412	−0.076		−0.070	
N≡N/N=N–X	−0.015	−0.044			−0.114		−0.107	
N=N	8.844	8.854	9.86	8.673	9.380	8.189	9.666	1.225[e]
N=N/N–X	0.051	−0.008			0.190		0.177	
N=N/N≡N=N	0.255	0.213		0.412	0.224		0.228	
N=N/N=N–X	0.786	0.834	0.838	0.466	0.765	0.85[d]	0.725	
N–X	6.174	6.169	6.757	6.231	4.407	4.890	4.231	0.915[f], 0.982[g,h]
N–X/N≡N=N	0.013	−0.021			−0.003		−0.017	
N–X/N=N–X	0.214	0.276			0.593	0.35[d]	0.516	
N≡N=N	0.613	0.624	0.611	0.617	0.624	0.758	0.629	0.788
N≡N=N/N=N–X	0.083	0.067	0.048		0.103		0.100	
N=N–X	0.768	0.817	0.729	0.762	0.952	0.628	0.873	0.725[f], 0.696[g,h]
$\tau_{N=N}$	0.0081	0.0136		0.781[i]	0.0067	0.598	0.0080	0.777[f], 0.621[g], 0.687[h]

[a]Units: mdyn A^{-1} (stretch and stretch/stretch), mdyn rad^{-1} (stretch/bend) and mdyn A rad^{-1} (bend and bend/bend). [b]From ref. 35. [c]From ref. 46. [d]Assumed. [e]The off-diagonal terms have been scaled by $x_{N=N} = 0.70$; see text. [f]This scale factor applies to HN_3. [g]This scale factor applies to CH_3N_3. [h]This scale factor applies to $CH_3CH_2N_3$. [i]The corresponding coordinate is defined as a bending of a linear chain.

the *gauche* conformation of EtN_3 to the all *gauche* series $R—CH_2N_3$ (R = —C≡N [47], —C≡CH [14], —C≡C—CH_3 [48] and —C≡C—CH_2N_3 [49]). For all these molecules we have obtained excellent agreement with the observations.

The final interpretation of the IR and Raman spectra of EtN_3 [23], and the identification of the *gauche* and *anti* fundamentals in particular, rests mainly on the force fields developed here, but a detailed account will be given elsewhere [23]. The vibrational spectra of azidopropane have recently been recorded in this laboratory, but they have not yet been analyzed [50]. We hope that it will be possible to determine the conformational properties of this molecule from force field calculations alone combining our scaled $—CH_2N_3$ force field from EtN_3 and the standard hydrocarbon force field of Snyder and Schachtschneider [51].

For the sake of brevity, we do not give the complete harmonic force fields of MeN_3 and EtN_3. The force fields are available from the authors upon request and will be published together with the vibrational spectra [44, 23].

Infrared intensities

The assumptions and approximations involved in calculating infrared intensities from dipole moment first derivatives, obtained numerically from permanent dipole moments in distorted structures, make the results, at best, qualitative. Minute changes in the L-matrix, i.e. in the force field, can have large effects on the calculated intensities. For the azides, the dipole moment derivatives with respect to the N≡N and N=N stretching coordinates are exceptionally large, and small differences in the scaled force fields, arising from different approximations in the scaling procedure or from different weighting of the experimental data, change the calculated, integrated intensities of some bands by up to a factor of two. Nevertheless, when working with highly unstable molecules, a qualitative prediction is far better than none at all. Since IR spectra are most commonly presented as percentage transmission, i.e. in a logarithmic scale, the theoretical predictions often appear better than expected.

The calculated intensities are included in Tables 6—9. No experimental measurements have been carried out, but, for a comparison, the experimental and theoretical IR vapour phase spectra of azidomethane [44] and azidoethane [23] are given in Figs. 6 and 7, respectively. The simulated spectra were obtained by co-adding the (properly scaled) theoretical vapour phase band contours, which were calculated assuming identical rotational constants in the upper and in the ground vibrational states [52]. In this approximation the central Q-branches of the A- and C-type bands are often calculated too sharp, as seen from Figs. 6 and 7, and possible resonances are also neglected. The theoretical spectrum of azidoethane was obtained by applying a statistical weight of two for the *gauche* conformation and assuming an energy difference of 0.6 kJ mol^{-1} between the *gauche* and the *anti* conformations

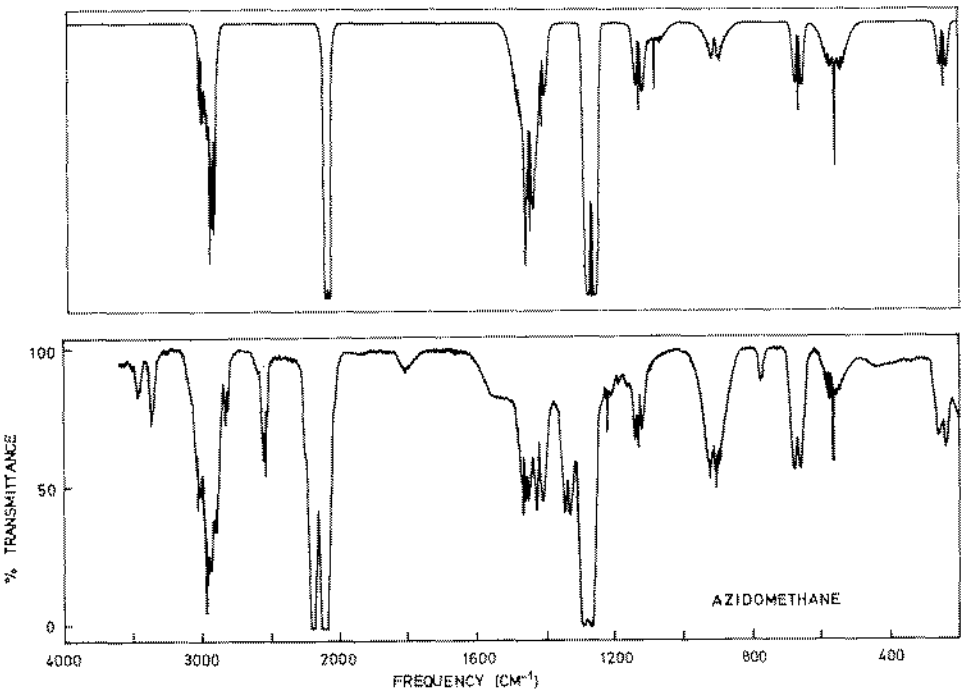

Fig. 6. The theoretical (top) and experimental (bottom) IR vapour phase spectra of azidomethane.

[23]. As seen from Figs. 6 and 7, the correspondence between observation and theory is not overwhelming, but the relative band intensities agree, reasonably, with the observations and the band types are also reproduced quite well.

For HN_3 the calculations predict all the a′-modes to be parallel bands with very weak perpendicular components in the IR in agreement with the observations [33—40]. The a″ mode, ν_6, is predicted to be very weak and in a recent high resolution study [40] the C-type component of ν_6 was barely observed, whereas the Coriolis-induced A-type lines dominate the ν_6 band. The differences in the calculated intensities for HN_3 using the DZ or the HF basis set seem to favour the DZ basis at this level of approximation, but this may be by pure coincidence.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Jan Almlöf and Dr Trygve U. Helgaker for their interest, assistance and valuable suggestions.

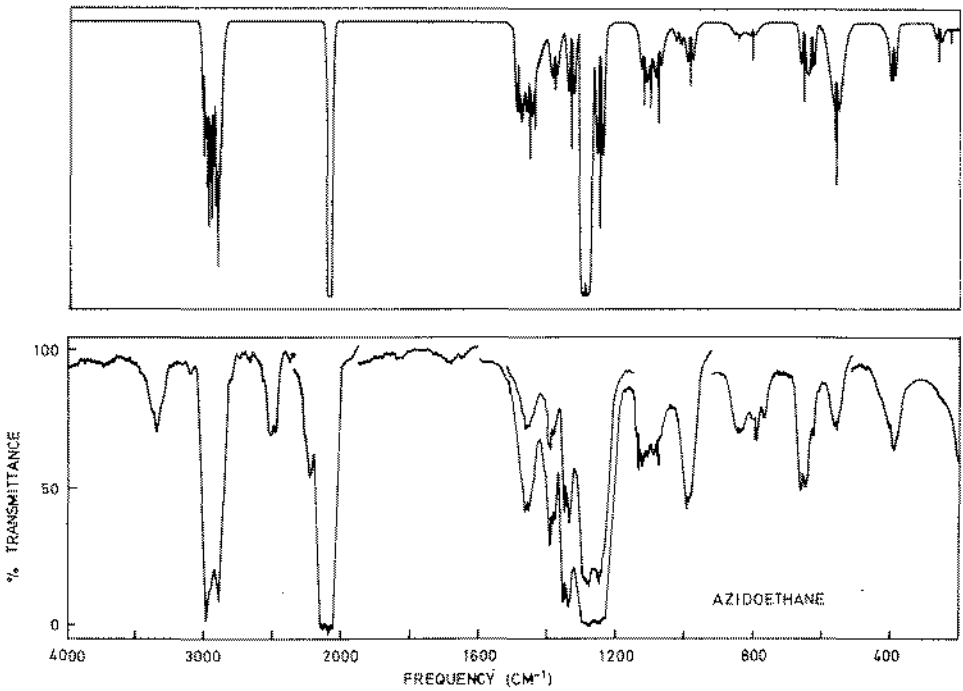

Fig. 7. The theoretical (top) and experimental (bottom) IR vapour phase spectra of azidoethane.

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK,
Patent Owner

Case IPR2018-00291 (Patent 9,718,852 B2)
Case IPR2018-00318 (Patent 9,719,139 B2)
Case IPR2018-00322 (Patent 9,708,358 B2)
Case IPR2018-00385 (Patent 9,725,480 B2)
Case IPR2018-00797 (Patent 9,868,985 B2)

Record of Oral Hearing
Held: March 5, 2019

Before JAMES A. WORTH, MICHELLE N. ANKENBRAND, and BRIAN D. RANGE, *Administrative Patent Judges*.

Case IPR2018-00291 (Patent 9,718,852 B2)
Case IPR2018-00318 (Patent 9,719,139 B2)
Case IPR2018-00322 (Patent 9,708,358 B2)
Case IPR2018-00385 (Patent 9,725,480 B2)
Case IPR2018-00797 (Patent 9,868,985 B2)
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The above-entitled matter came on for hearing on Tuesday, March 5, 2019, commencing at 9:01 a.m., at the U.S. Patent and Trademark Office, 600 Dulany Street, Alexandria, Virginia.

own lab admitting it.

JUDGE WORTH: Are you relying only on an allyl group?

MR. ZIMMERMAN: So --

JUDGE WORTH: From the 3′ blocking group or are you relying in your petition or somewhere else for something other than an allyl group?

MR. ZIMMERMAN: The petition was based on the allyl group of Tsien and the Dower based ground using the allyl group of Metzger 94. So both Ground 1 and Ground 2 rely on an allyl group.

And the patent owner admitted during prosecution that allyl was small so there is no need for a claim construction of small. There is no dispute that allyl is small and satisfies that requirement.

JUDGE WORTH: Can you walk us through the argument and the evidence and specifically where is this in your briefs about Dr. Menchen's patents? It seems like you're relying on maybe deposition testimony but then you're talking about Dr. Menchen's own patents.

MR. ZIMMERMAN: Yes. So there was an argument raised by Columbia that you have the Tsien article, Tsien patent, and then Metzger which discloses specific data with allyl and that nobody then did it until Columbia. That allyl was essentially abandoned as a capping group.

And Dr. Menchen's patents respond directly to that argument. If we look at Slide 12, Dr. Menchen has a 1998 patent that talks about capping at the 2′ or 3′ position and he uses allyloxy, which everybody has admitted in this proceeding is the 3′-O allyl.

So the idea that nobody used allyl as a 3′ blocking group is undercut by their own expert.

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK and QIAGEN SCIENCES, LLC, Plaintiffs, v. ILLUMINA, INC, Defendant.	C.A. No. 19-1681-CFC

EXPERT DECLARATION OF DR. FLOYD ROMESBERG IN SUPPORT OF ILLUMINA'S RESPONSE TO PLAINTIFFS' OPENING CLAIM CONSTRUCTION BRIEF

1. I, Floyd Romesberg, Ph.D., have been retained by Defendant Illumina, Inc. to provide opinions regarding the claim construction of disputed claim terms of U.S. Patent Nos. 10,407,458 (the "'458 Patent"); 10,407,459 (the "'459 Patent"); 10,428,380 (the "'380 Patent"); 10,435,742 (the "'742 Patent"); and 10,457,984 (the "'984 Patent") (collectively the "Patents-in-Suit").

I. SUMMARY OF OPINIONS

2. I have been asked to review the Patents-in-Suit and their prosecution histories, including specifically the Declaration Under 37 C.F.R. §1.132 (dated May 26, 2017) submitted by Dr. Jingyue Ju ("Ju Declaration," also attached as JA0061-0068, the lead inventor of the Patents-in-Suit, and provide my opinion of how it would be interpreted by a person of ordinary skill in the art ("POSITA"). I have also been asked to review the declaration of Dr. Kuriyan submitted with Plaintiffs' opening claim construction brief, and his interpretation of the Ju Declaration.

3. It is my opinion that a POSITA reviewing the materials mentioned above, and specifically the Ju Declaration, would conclude that the "diameter of approximately 3.7 Å" given as the maximum size of a "small" protecting group would be understood to be a sphere with a diameter of 3.7 Å with its surface touching the 3' carbon and extending into the space available. JA0084. It is my opinion that a POSITA reviewing the Ju Declaration would not understand it to be describing a cylinder with a diameter of 3.7 Å as described by Dr. Kuriyan.

II. BACKGROUND AND QUALIFICATIONS, PREVIOUS TESTIMONY, AND COMPENSATION

A. Background and Qualifications

4. I am the scientific founder and Distinguished Fellow of the biopharmaceutical company Synthorx Inc., which is based in San Diego, California, United States of America.

5. I earned a Bachelor of Science in Chemistry from the Ohio State University in 1988.

6. I earned a Master of Science in Chemistry in 1990 and a Doctor of Philosophy in Chemistry in 1994 from Cornell University, where Professor David B. Collum served as my thesis advisor.

7. From 1994 until 1998, I was a National Institutes of Health (NIH) postdoctoral research fellow at the University of California, Berkeley, where I studied under Professor Peter G. Schultz.

8. From 1998 to June 2019, I was a professor in the Department of Chemistry at The Scripps Research Institute.

9. As a principal investigator, I have authored over 140 publications in peer-reviewed journals, including Nature, The Proceedings of the National Academy of Sciences of the United States of America, The Journal of the American Chemical Society, Angewandte Chemie International Edition, Biochemistry, Nucleic Acids Research, and The Journal of Physical Chemistry.

10. I have authored or co-authored at least 33 invited review articles. In addition, I have authored or co-authored at least 17 other publications during my graduate and post-doctoral studies.

11. Over 40 of the publications that I have authored as a principle investigator are related to nucleotide analogues, and specifically the characterization of how nucleotide modifications affect their recognition by DNA polymerases.

12. I taught several graduate courses in the Department of Chemistry at The Scripps Research Institute, including a Spectroscopy course (which included a section of mass spectroscopy) and a course on Bacteria and Antibiotics.

13. I mentored numerous graduate students, post-doctoral researchers, interns, and research associates at The Scripps Research Institute.

14. I have been an invited lecturer at multiple universities, symposiums, and conferences throughout the United States and abroad.

15. I have reviewed manuscripts as part of the peer-review process to determine whether they are acceptable for publication for numerous journals, including the Proceedings of the National Academy of Sciences, Science, Nature, The Journal of the American Chemical Society, Angewandte Chemie, Biochemistry, the Journal of Organic Chemistry, Bioorganic and Medicinal Chemistry Letters, Chemistry & Biology, Nucleic Acids Research, and Nucleosides, Nucleotides, and Nucleic Acids.

16. I am a member of the American Chemical Society and the American Society for Microbiology.

17. I served as a permanent member of the NIH Synthetic and Biological Chemistry (SBCA) study section that handles a significant percentage of the grants dealing with modified nucleotides for four years. Also, I regularly serve on various National Science Foundation (NSF) study sections, and my service on

these study sections involves reviewing and determining the merits of numerous grant proposals.

18. I have been awarded numerous research grants from the NIH, the NSF, the Office of Naval Research, Defense Advanced Research Projects Agency, and several other sources, including federal funding for the synthesis and analysis of nucleotide analogues and for the development of DNA polymerases specifically for sequencing DNA.

19. I have been the recipient of multiple awards and honors, including the Royal Society of Chemistry Award for Bioorganic Chemistry in 2018, the ACS San Diego Chapter 2018 Scientist of the Year, elected as a National Academy of Inventors Fellow in 2018, the ACS Nobel Laureate Signature Award for Graduate Education in Chemistry in 2015, Discover Magazine Technology Innovation Award in 2004, the NSF Career Award in 2004, the Susan B. Komen Breast Cancer Foundation Award in 2003, the Camille Dreyfus Teacher-Scholar Award in 2003, the Baxter Foundation Fellow Award in 2002, the Mac Nevin Award in 1987, and election to the Defense Science Study Group (DSSG) panel from 2008-2010.

20. I am qualified to render an opinion in the field of nucleotide analogues, DNA polymerases, and DNA sequencing techniques based on my experience in these fields. Based on my expertise and qualifications, I am qualified to provide an opinion as to what a person of ordinary skill in the art would have understood, known, or concluded as of 2000. I have been doing research in this field since 1999. In addition, the company Synthorx, which I started in 2014 and where I am now employed, was founded to commercialize the use of nucleotide analogues

that I had developed in my lab during my time at The Scripps Research Institute, and in January this year the company was acquired by Sanofi.

21. Attached as JA0266-282 is a copy of my curriculum vitae setting forth my educational experience, employment history, and publications.

22. I have no financial interest in this proceeding, and my compensation is unaffected by the content of my testimony or the outcome of this proceeding.

B. Previous Testimony

23. I previously provided testimony as an expert witness in:

- *Illumina, Inc. v. Trustees of Columbia University*, IPR2020-00988, -01065, -01125, -01177, regarding U.S. 10,407,458; U.S. 10,407,459; U.S. 10,457,984; and U.S. 10,435,742 (Patents-in-Suit in this case);

- *Illumina, Inc. v. Trustees of Columbia University*, IPR2018-00291, - 00318, -00322, -00385, regarding U.S. 9,718,852; U.S. 9,719,139; U.S. 9,708,358; U.S. 9,725,480; and U.S. 9,868,985;

- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00128, regarding U.S. 7,057,026;

- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00266, regarding U.S. 8,158,346;

- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00517, regarding U.S. 7,566,537;

- *Pacific Oxford Nanopore v. Pacific Biosciences*, ITC-337-TA-1032; and

- *Illumina, Inc. v. Oxford Nanopore Technologies Ltd.*, ITC-337-TA-0991.

III. LEVEL OF ORDINARY SKILL IN THE ART

24. I have previously provided an opinion regarding the definition of a POSITA in the IPRs filed by Illumina against the Patents-in-Suit:

> A person of ordinary skill in the art related to the '458 patent would have been a member of a team of scientists developing nucleotide analogues, researching DNA polymerases, and/or addressing DNA sequencing techniques. Such a person would have held a doctoral degree in chemistry, molecular biology, or a closely related discipline, and had at least five years of practical academic or industrial laboratory experience. Thus, a person of ordinary skill in the art includes a person having a doctoral degree in a field related to chemistry, and at least five years of laboratory experience directed toward the research and development of nucleotide analogues, DNA polymerases, and/or DNA sequencing. My opinions concerning the obviousness, as set forth herein, are from the perspective of a person of ordinary skill in the art, as set forth above.

JA0355 at ¶25. Dr. Kuriyan has provided a slightly different definition of a POSITA. JA0162-163 at ¶15. The opinions in this declaration would not change based on either definition of a POSITA.

IV. DR. KURIYAN'S ANALYSIS OF THE JU DECLARATION

25. The Ju Declaration was submitted in response to a rejection by the examiner that the term "small" was a relative term and therefore indefinite. In response to that rejection, Columbia argued that the term "small" in the context of

the Patents-in-Suit would be understood in reference to the Figure 1 disclosure of the crystal structure of a rat DNA polymerase in complex with a dideoxyribonucleoside triphosphate published by Pelletier. Specifically, Columbia submitted the Ju Declaration which purports to analyze "the space available for a 3'-O capping group" based on the Pelletier structure, and concludes that "the diameter of the available space *in* the active site of the polymerase ternary complex is approximately 3.7 Å." JA0082.

26. The Patents-in-Suit and Dr. Ju may not have randomly chosen the Pelletier rat DNA polymerase structure as their model polymerase. At the time of the invention, there were only a small number of published polymerase structures. A POSITA would understand that without a structure, the only way to determine whether a particular protecting group was "small" would be to test it. A POSITA would be required to make the nucleotide with that specific protecting group, and then run an experiment with it to see if the specific target polymerase was able to recognize it. Using a structure would be the only way to estimate, short of extensive experimentation, if a proposed protecting group is "small." For this reason, when faced with an indefiniteness rejection, Columbia and Dr. Ju chose to specifically tie the "small" limitation to the crystal structure disclosed and cited in the patent, Pelletier's rat DNA polymerase.

27. Dr. Ju did not provide the actual analysis or calculations underlying his conclusions, so it is unclear where the conclusion of a diameter of 3.7 Å came from. Dr. Ju simply noted the distances from the 2' and 3' carbons to the nearest polymerase residue (which ranged from 3.2 to 3.5 Å, and which did not include hydrogen atoms), and then summarily stated:

> The distances given in Pelletier et al. were used to calculate the available space around the 3' carbon of the deoxyribose ring of the

> nucleotide. It was determined that the diameter of the available space in the active site of the polymerase ternary complex is approximately 3.7 Å.

As explained further below, a POSITA would appreciate this meant that a sphere with a diameter of 3.7 Å was being used to represent the available space. Again, Dr. Ju does not disclose how he arrived at that exact number, instead of, for example, 3.5 Å or 4 Å. In view of this lack of disclosure, I have reviewed the Ju Declaration from the perspective of a POSITA at the time of the invention, October 2000.

A. A POSITA Would Understand The Ju Declaration To Be Describing A Three Dimensional Shape That Approximates The "Small" Protecting Group

28. In reviewing the Ju Declaration, a POSITA would be familiar with the common sense concept that molecules exist in three-dimensional space. The patent and Dr. Ju emphasize that space around "the 3' position of the dideoxyribose ring (blue) is very crowded." JA0083; *see also* JA0012 at 5:57-58. A POSITA would understand Dr. Ju to be stating that the protecting group (or "3'-O capping group") must supposedly be "small" in order to fit into the space available within the active site of the rat DNA polymerase without disrupting the ability of the polymerase to function, *i.e.*, to recognize the nucleotide and incorporate it into the growing strand of DNA. By this, he means that the protecting group and the polymerase cannot occupy the same space.

29. As can be seen from Figure A, the space around the 3' carbon is indeed crowded, without much space in any direction for a protecting group to occupy.

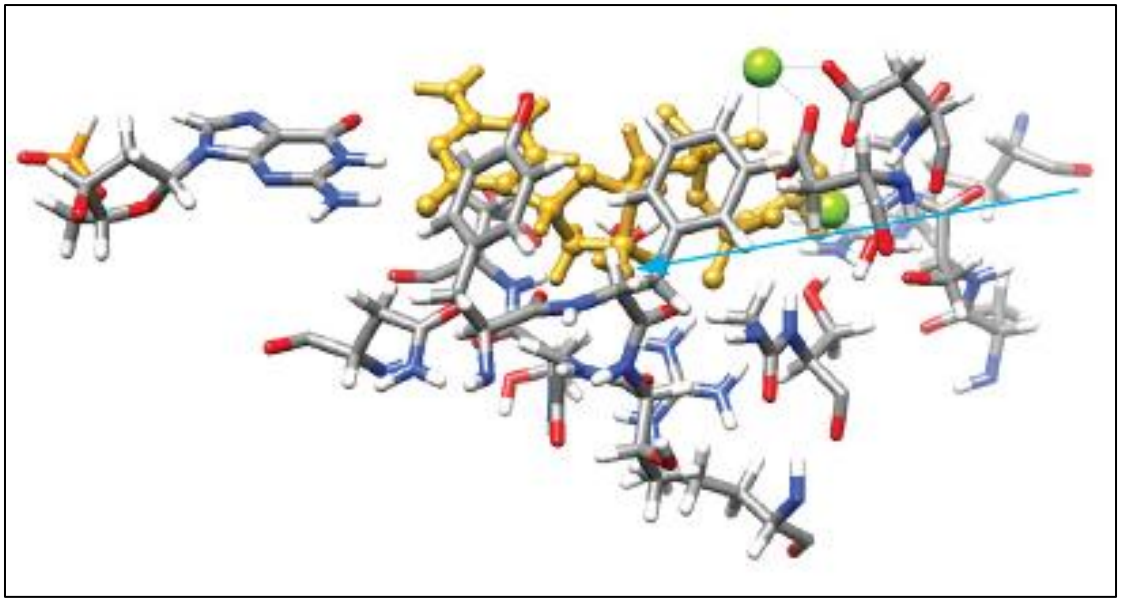

Figure A1

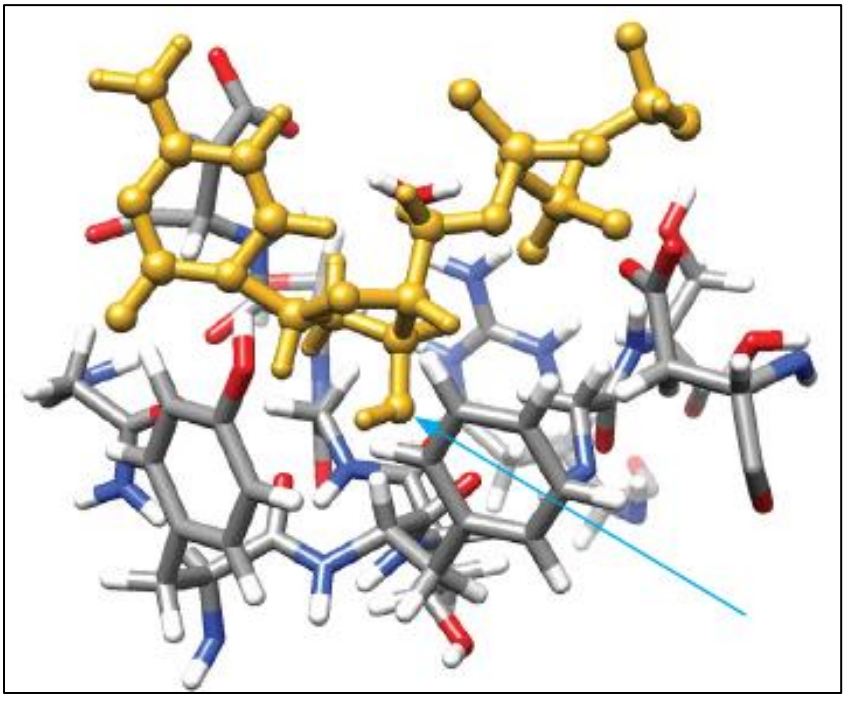

Figure A2

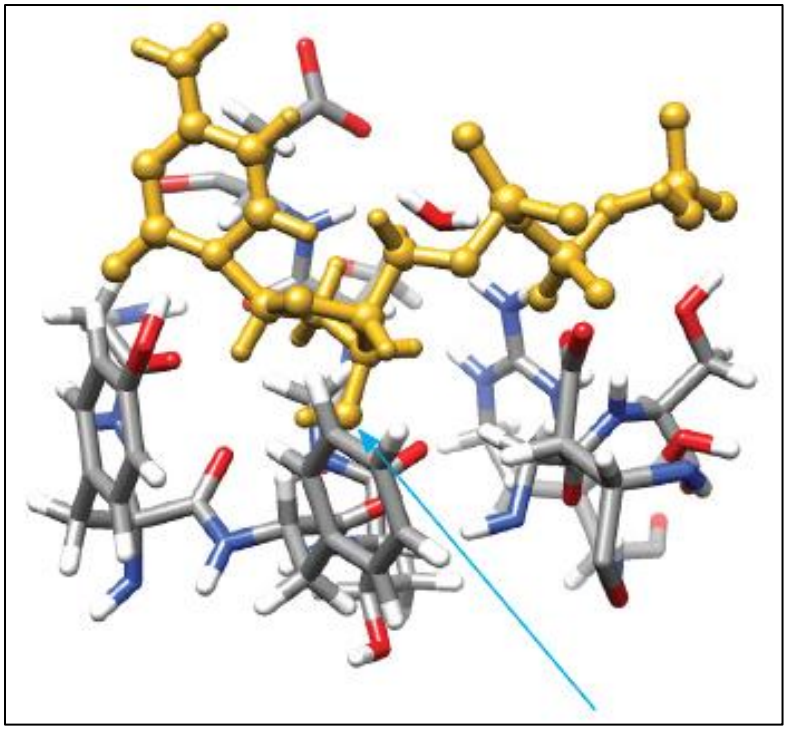

Figure A3

Figure A: Three different viewpoints (A1, A2, A3) of the same Pelletier rat DNA polymerase in complex with a dideoxyribonucleoside triphosphate. The dideoxyribonucleoside triphosphate has been colored gold, and a 3'-OH group has been added, as indicated by the blue arrow. As can be seen, the space around the 3' oxygen is crowded in all directions.

30. A POSITA would understand that the Ju Declaration is attempting to describe the space available for the protecting group, in three dimensions, in order to better understand, in the context of the patent, how "small" the protecting group must be. Thus, a POSITA reviewing the Ju Declaration would understand that whatever space was available, determining whether it would be sufficient to accommodate a protecting group would require that the full dimensions of both the space and the protecting group be specified.

31. A POSITA would further understand the basic geometry of describing specific shapes in three dimensions. For example, a POSITA would understand that to describe a box in three dimensions, one would provide three metrics, namely the length, width, and height. However, to describe a cube, only one metric is needed, because the length, width, and height are by definition understood to be equal (the three conventionally identified as length when only one dimension is mentioned). A POSITA would further understand that to fully describe a cylinder, both the diameter and the length would be needed. In contrast, to fully describe a sphere, only a diameter is needed.

32. With this context, it would be immediately apparent to a POSITA that if a three-dimensional "available space" is only described as having "a diameter of approximately 3.7 Å," without any further metrics, a sphere is being used to approximate the space described. JA0084. As already described, a sphere is the only three-dimensional object that is fully described by just a diameter. A POSITA would not understand the Ju Declaration to disclose a cylinder with "a diameter of approximately 3.7 Å" because without further information, it is impossible to determine the length of the cylinder. If the Ju Declaration were to disclose a cylinder, it would have both specified the diameter of that cylinder, and further provided the length of that cylinder. The only logical conclusion from the Ju Declaration, in fact the only conclusion possible, is that he is describing a sphere with diameter of 3.7 Å.

33. This is further evidenced by the fact that a POSITA would be most concerned with the accommodation of the longest dimension of the object. As can be seen in Figure A, the space around the 3' carbon is constricted in every direction. A POSITA would understand that occasionally, a crystal structure reveals a "tunnel-like" structure through which an object can extend in an unrestricted fashion, and

which thus removes concerns about the accommodation of the length of the object, leaving only restrictions on the object's width and height. However, in most scenarios, such as the crystal structure disclosed in Figure 1 of the Patents-in-Suit, a POSITA would understand that the space available around the 3' position forms a pocket that blocks infinite extension in any direction. In these cases, the length, width, and height of the object (protecting group) are all restricted. When this is the case, the POSITA would be most concerned with whether the longest dimension of the protecting group would be too great to be accommodated within the available space, since the longest dimension is most likely to interfere (the greater the distance in any direction, the greater the likelihood of encountering a restriction).

34. With this understanding, a POSITA would not understand the Ju Declaration to be describing a model that explicitly ignores the longest dimension. Dr. Kuriyan explains that "each chemical group has a length dimension, which is the group's longest dimension, and a dimension substantially perpendicular to the length dimension, which is the chemical group's widest, (i.e., width) dimension." JA0168-169 at ¶31. Dr. Kuriyan's model is based on explicitly ignoring the longest dimension, and focusing on the second longest dimension, *i.e.*, the width perpendicular to the longest dimension. A POSITA would understand that this model would be counterintuitive to the goal of the Ju Declaration, which is to fully describe how large a protecting group can be while still being "small" enough to fit into the active site of the rat DNA polymerase. It is nonsensical to think that the Ju Declaration describes a three-dimensional shape without having an upper bound on its longest dimension, which is the distance that is most likely to matter.

B. Dr. Kuriyan's Model Is Reverse Engineered And Likely Does Not Reflect The Calculations In The Ju Declaration

35. Dr. Kuriyan's model appears reverse engineered in order to obtain similar results to those in the Ju Declaration, but his analysis and calculations are divorced from what would actually fit in the space available in the rat DNA polymerase. Regardless of whatever model is used to approximate the space available, a POSITA would understand that protecting group would only be considered "small" if it actually fits into the active site shown by the Pelletier structure. If an interpretation of the Ju Declaration conflicted with this reality and predicted protecting groups were "small" when they would not actually act small (*i.e.*, fit within the polymerase active site), a POSITA would reject that interpretation.

36. To this end, Figures B and C depict a model of an allyl group and an azidomethyl group, respectively, attached to the 3'-O of the deoxyribonucleoside triphosphate in the context of the Pelletier structure cited in Figure 1 of the Patents-in-Suit. Because many chemical bonds are flexible and allow rotation, the orientation of the protecting group is variable. In reality, instead of a static picture, both the polymerase and the nucleotide/protecting group would adjust to any extent possible to accommodate each other in order to minimize overlap and deleterious interactions. Nevertheless, using crystal structures to visualize the potentially available space and determine whether an object was likely to "fit" was a typical practice for a POSITA at the time of invention.

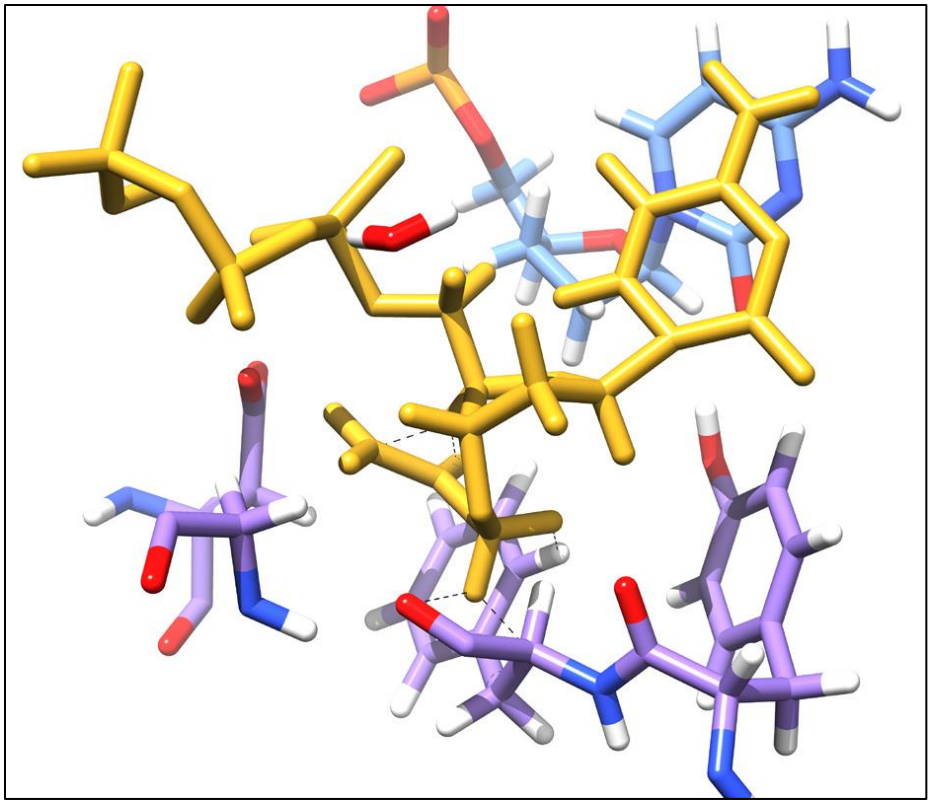

Figure B1

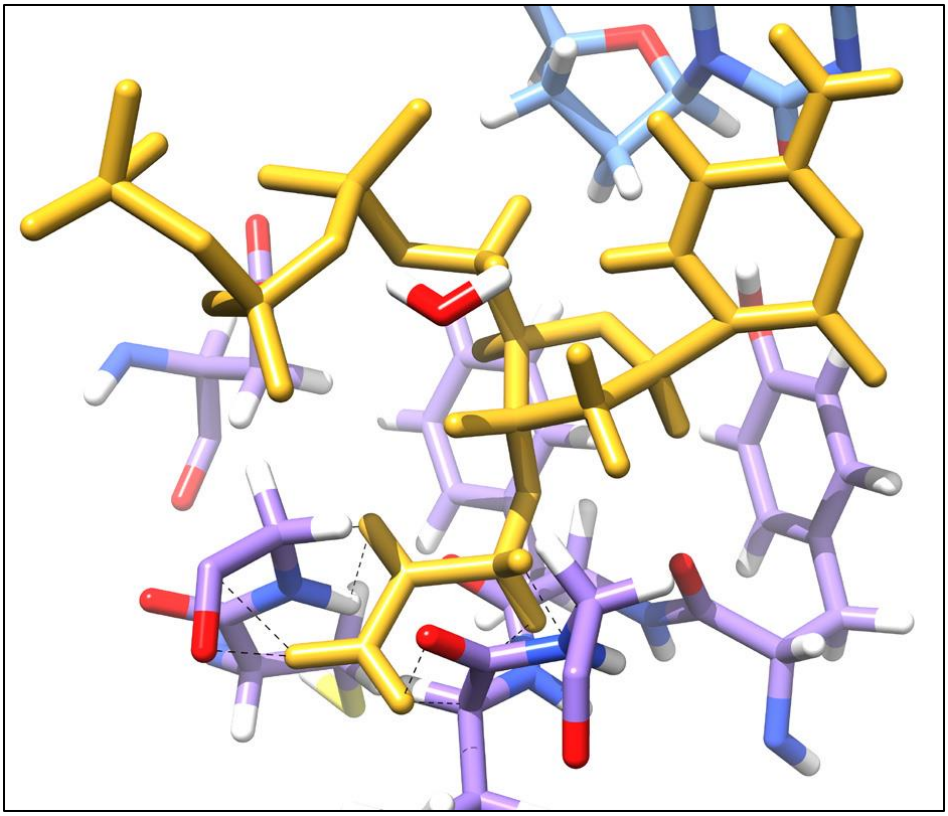

Figure B2

Figure B: Three-dimensional depiction of the Pelletier rat DNA polymerase in complex with a dideoxyribonucleoside triphosphate, modified to include an O-allyl protecting group attached to the 3' carbon. B1 and B2 are two different orientations the protecting group might take without interfering with the surrounding polymerase. In total I found five different potential orientations for the allyl protecting group.

37. Using the published structure, I created models of nucleotides with different 3'-O protecting groups positioned within the polymerase. By manually adjusting the rotatable bonds in the protecting group, I was able to find five orientations of the allyl protecting group attached to a 3'-O that would appear to "fit" into the space available around the 3' carbon and which would appear not interfere with the polymerase in any substantial way. Importantly, the conformations of the

protecting group seem reasonable and in fact typical of those observed in the complexes of other small molecules bound to a protein. This confirms the Patents-in-Suit's disclosure that an allyl protecting group would be considered "small." I performed a similar exercise with the MOM group, and was also able to determine that it too would likely "fit" in several conformations.

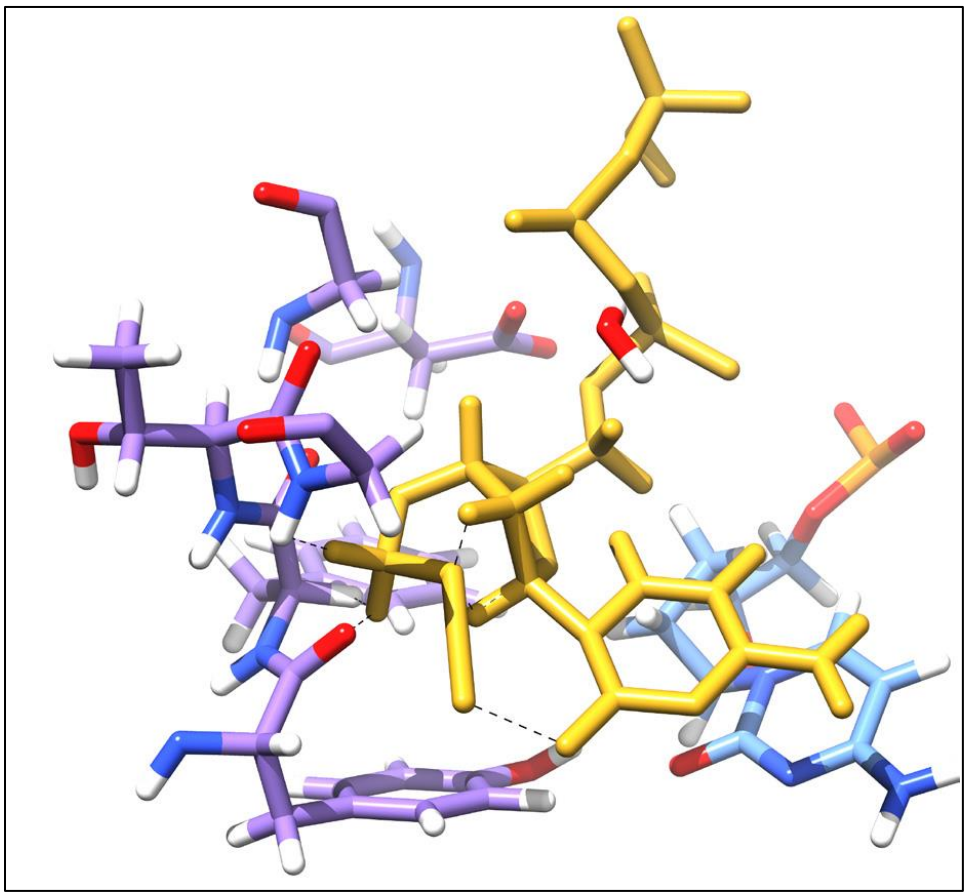

Figure C

Figure C: Three-dimensional depiction of the Pelletier rat DNA polymerase in complex with a dideoxyribonucleoside triphosphate, modified to include an O-azidomethyl protecting group attached to the 3' carbon. This is the orientation of the protecting group that represents the "best" orientation I could find in that it minimizes interactions with the surrounding polymerase. However, it still does not appear to be well accommodated and would not be predicted to bind, as reflected by the short contact distances.

38. In contrast, I was unable to find any orientations of the azidomethyl protecting group that "fit" into the space available around the 3' carbon. The orientation shown in Figure C is the only one I was able to find that at least somewhat minimized the interactions between the protecting group and the surrounding polymerase, although clearly not as well as the allyl group. Indeed, a POSITA would reject this orientation as not feasible because in order to avoid the deleterious interactions with the polymerase, the azido group is forced to bend back on the sugar of the nucleotide. This causes significantly deleterious sugar-azide interactions that in reality would prevent the nucleotide from adopting that conformation.

39. The reason it is more difficult to find an orientation that fits for the azidomethyl group, when compared to, for example, the allyl or MOM group, originates from the protecting group itself. An allyl or MOM group possess critical rotatable bonds that allow them to adopt many different shapes, in particular, shapes that allow them to be accommodated with the polymerase active site. In contrast, the three nitrogen atoms of the azido group are locked by their type of bonding into a linear arrangement, and thus are not free to adjust to the polymerase active site. For this reason, the azidomethyl group requires more space compared to the flexible and more accommodating allyl and MOM groups.

40. This exercise is exactly what a POSITA would actually do if provided with the Pelletier structure and asked to estimate whether allyl, MOM, and azidomethyl groups would be "small" enough to function as protecting groups. Any model must accurately reflect these estimations, or its usefulness as a model is extremely limited. Dr. Kuriyan's model would allow the claims of the Patents-in-Suit to encompass azidomethyl protecting groups, when those groups would clearly not fit within the rat DNA polymerase that Columbia argued was the basis for the definition of "small" in the Patents-in-Suit. A POSITA faced with this contradiction

would reject Dr. Kuriyan's model as unreliable, and rely on the full published Pelletier structure instead. This alone is sufficient reason to reject Dr. Kuriyan's model and construction.

41. In addition, Dr. Kuriyan's analysis differs from Dr. Ju's in several other ways. For example, Dr. Kuriyan measures the lengths of the protecting groups by measuring the distance from the oxygen used to attach the protecting group to the 3' carbon to the end of the molecule. However, Dr. Ju is clear that the distances he contemplates are relative to the 3' carbon itself, not the intervening oxygen. In addition, Dr. Kuriyan explicitly includes hydrogen atoms in his calculations, while Dr. Ju appears not to have (while he is unclear on exactly how he arrived at a diameter of 3.7 Å for his sphere, it is clear that it was based on measurements reported by Pelletier, which do not include hydrogen atoms).

C. A Sphere With Diameter 3.7 Å Correctly Reflects The Space Available In The Rat DNA Polymerase Active Site

42. In contrast to Dr. Kuriyan's model, which both does not provide enough information for actual use and does not reflect the active site environment of the rat DNA polymerase, a spherical model is able to fully and accurately describe the space available. In order to visualize this, Figure D depicts a sphere with diameter 3.7 Å, as taught by the Ju Declaration, positioned within the polymerase active site.

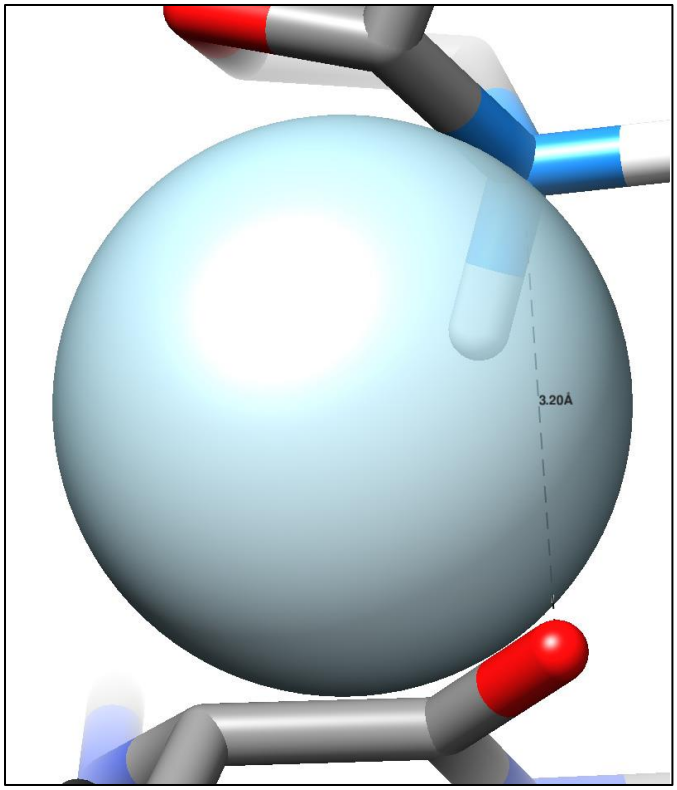

Figure D: Three-dimensional depiction of the Pelletier rat DNA polymerase in complex with a dideoxyribonucleoside triphosphate, with a 3.7 Å diameter sphere abutting the 3' carbon.

43. As can be seen, even with a distance of "3.2 Å between the 3' carbon of the deoxyribose ring and Phe272" as disclosed in the Ju Declaration, a sphere with a 3.7 Å diameter fits nicely within the space available. In fact, the fit seems so perfect that one suspects that this is exactly how Ju arrived at his 3.7 Å diameter. JA0082. A POSITA reviewing the Ju Declaration would understand that this model is what Ju intended when he "determined that the diameter of the available space in the active site of the polymerase ternary complex is approximately 3.7 Å." *Id.*

44. The spherical model is further confirmed by how it accurately reflects which protecting groups would and would not fit into the actual rat DNA polymerase

active site. Figure E shows that MOM and allyl protecting groups fit into the 3.7 Å diameter sphere, while the azido group does not.

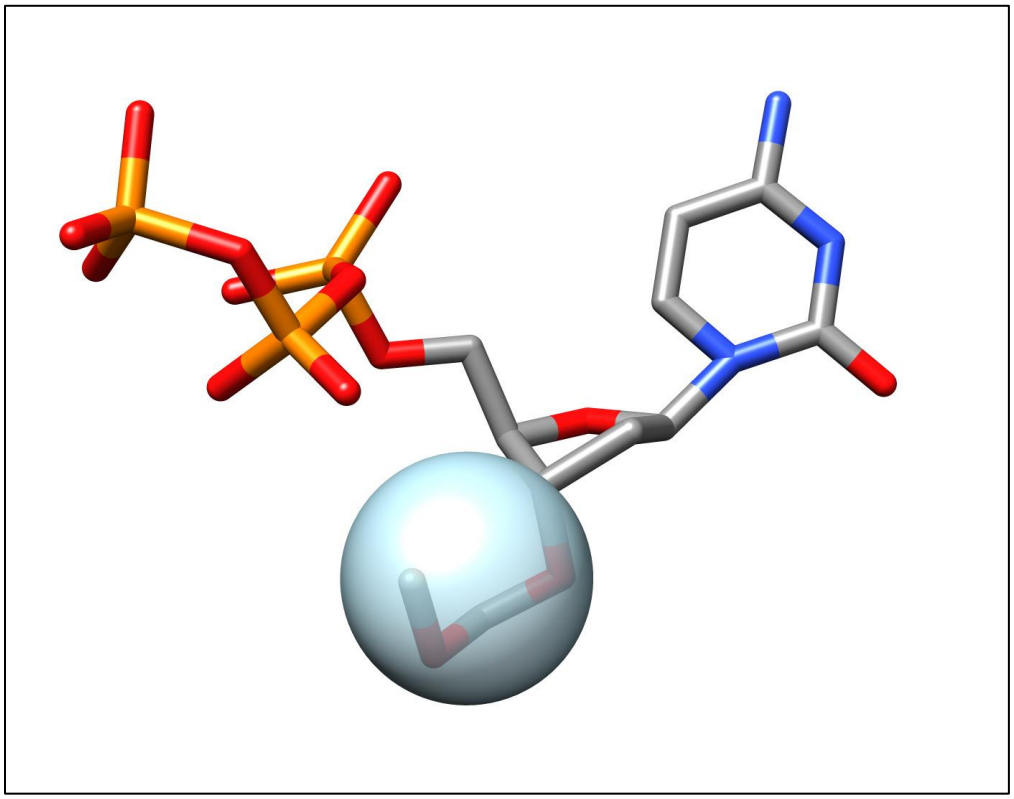

Figure E1

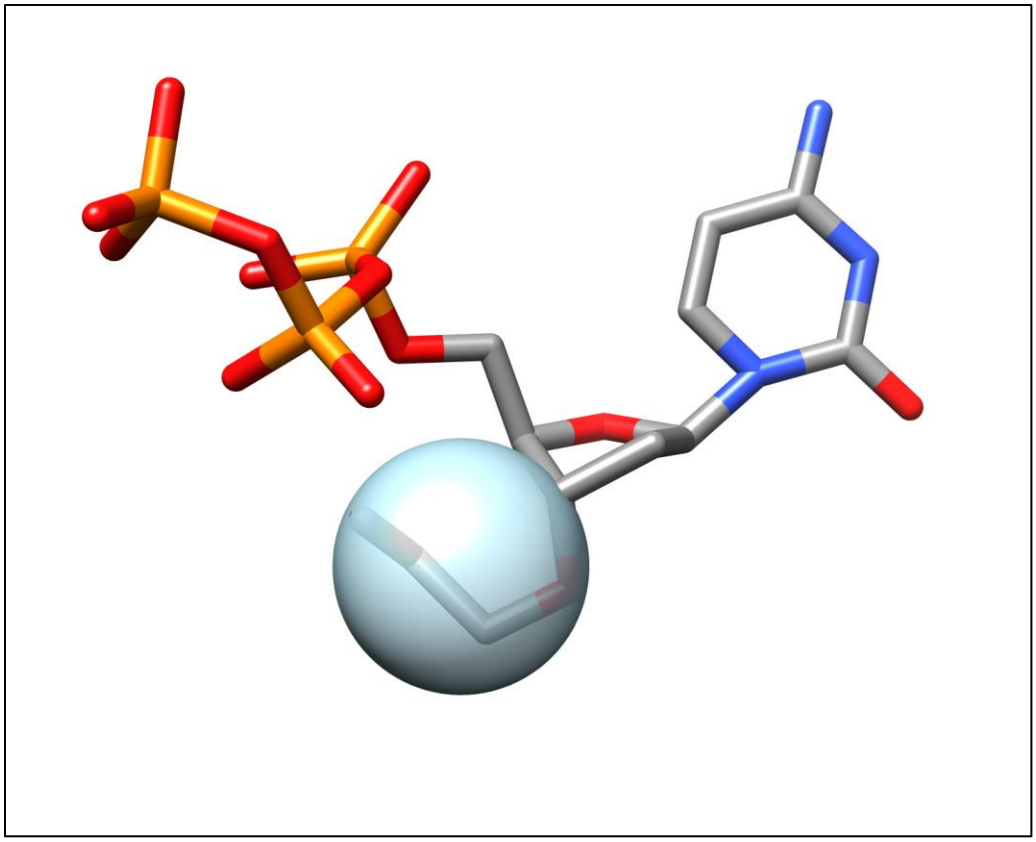

Figure E2

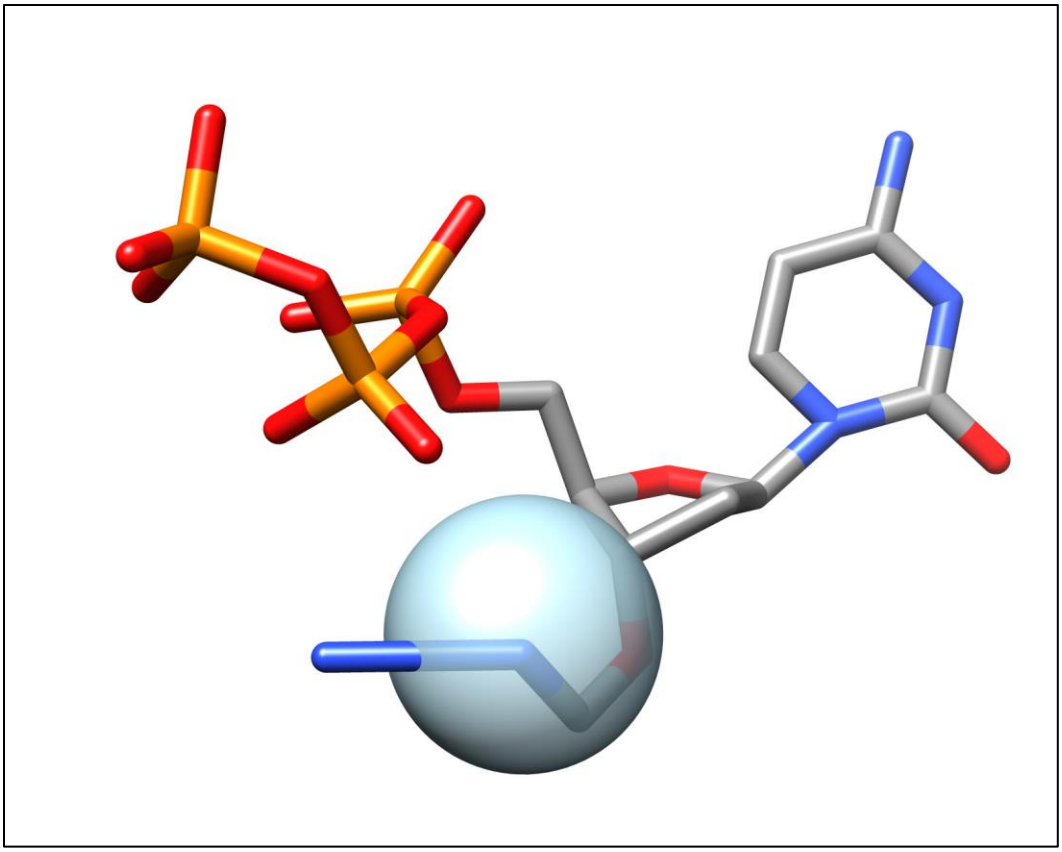

Figure E3

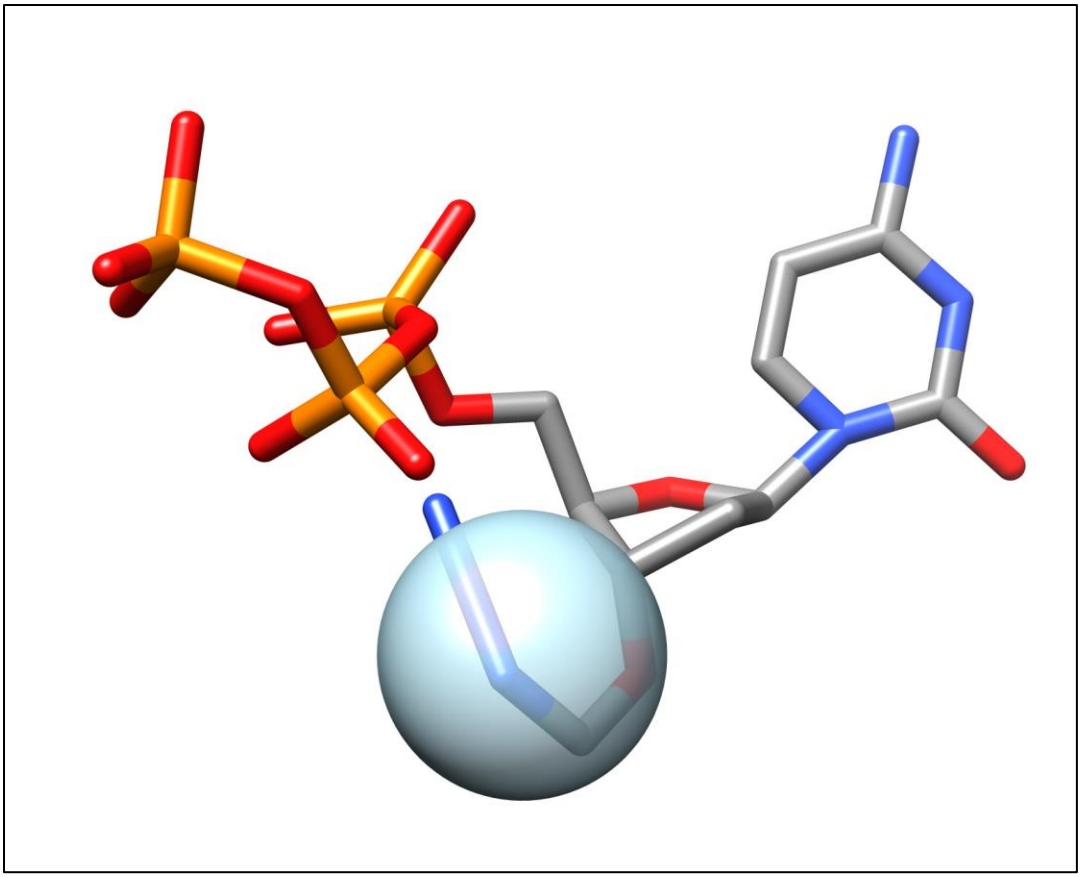

Figure E4

Figure E: Three-dimensional depiction of a deoxyribonucleoside triphosphate with a MOM (E1), allyl (E2), or azidomethyl (E3 and E4) protecting group, showing that the MOM and allyl protecting groups fit to a 3.7 Å sphere whose surface abuts the 3' carbon of the nucleotide. E3 and E4 are two alternate orientations modeled for the azidomethyl group, clearly illustrating that they are not able to be accommodated within the evoked sphere. Note that the hydrogens are not depicted in this Figure.

45. As can be seen in Figure E, the MOM and allyl groups fit within a sphere with diameter 3.7 Å, but the azido does not, regardless of orientation. This is because, as explained above, the MOM and allyl groups bonds are generally more free to rotate than are the bonds in the azidomethyl group. This allows the MOM and allyl groups to bend and twist into conformations that fit into the sphere (and the polymerase active site, as previously demonstrated in Figure B). In contrast, the

rigidity of the azido group force it to remain linear and thus preclude its accommodation within the sphere. This confirms the accuracy of the model to reflect the actual space available in the polymerase.

46. As noted in the Figure caption above, these images do not depict the hydrogens (shown typically as white endcaps in the other Figures). If they were depicted, they would slightly protrude outside of the sphere with both the allyl and MOM groups. However, while for visual convenience the program used (and most used for the same purpose) artificially depicts all atoms as the same size, they are not, and hydrogen atoms are significantly smaller than nitrogen atoms. Thus, a slight protrusion out of hydrogen atoms out of the sphere would have likely been considered incidental by a POSITA, especially considering the crudeness of the model and the ability of the polymerase to adjust to accommodate small perturbations. In contrast, the more significant protrusion by a much larger nitrogen atom would have been considered problematic. Regardless, and most importantly, hydrogen atoms were omitted because they also appear to have been omitted by Dr. Ju when each created their models.

D. Dr. Kuriyan's Model Does Not Actually Limit The Claims

47. In contrast to the spherical model, Dr. Kuriyan's cylindrical model essentially reduces the Ju Declaration's definition of "small" to exclude only cyclic and branching protecting groups, but include essentially all linear protecting groups, regardless of their overall size – even ones that would normally be considered quite large, and larger than many cyclic and branching protecting groups. This is because, since length is not bounded, any linear protecting group (*i.e.* one comprised of atoms connected without branches), would have approximately the same "width." This is true both for the MOM and allyl protecting groups described in the patent, which are each linear chains of three atoms, and a group that is a linear chain of 100 or even

1000 atoms. Each can be represented as a linear chain of connected atoms with a width of approximately 3.1 Å, even though they would have vastly different lengths.

48. In order to test Dr. Kuriyan's model, I tried to determine the best orientation for the proposed cylinder. I took the Pelletier structure, and added a 3'-OH group to the dideoxyribonucleoside triphosphate (its positioning is unambiguous). I placed one end of a 3.7 Å diameter cylinder on that oxygen. I used a cylinder with a length of 4.6 Å, which corresponds to the calculated length of the allyl protecting group that the Ju Declaration discloses should be considered "small." In addition to not specifying the length of the cylinder, it is also not clear the angle at which the cylinder should extend. I fixed the long axis of the cylinder as would be required for its representing a protecting group attached to the oxygen, and examined the possible orientations, and was able to find the position that appeared to minimize deleterious interactions with the polymerase. Other orientations would face similar (but worse) issues as those discussed below, and would not alter the analysis. All orientations suffered from at least some interference from the surrounding polymerase, and that is an independent reason Dr. Kuriyan's model should be rejected.

49. Even in this optimized orientation, I concluded that the cylinder would not fit. This is because the space within the cylinder is penetrated by several parts of the polymerase and because the end of the cylinder abuts against a tyrosine residue with a distance that is well within van der Waal radii. This means that atoms will approach each other to closely to be stable (note that this type of analysis of distances between atoms is well accepted and pervasive in the scientific literature). In addition, it is unlikely that the polymerase could adjust to accommodate the protecting group, as the position of this particular tyrosine residue is critical for polymerase function (it must be precisely positioned to interact with the

deoxyribonucloside triphosphate). A protecting group comprised of a chain longer than three carbon atoms will be even less well accommodated. For example, if the allyl group was extended by even a single carbon atom, its atoms would virtually overlap with those of the tyrosine residue, thus its accommodation would require significant and unfavorable structural rearrangement of the polymerase. It is unlikely that nucleotides bearing such protecting groups would even bind to the polymerase, and even if they did, they would likely interfere with its function. However, under Dr. Kuriyan's model, such a protecting group is just as "small" as the allyl group, since both would have essentially the same width. This model does not aid a POSITA in determining whether a given group is "small" and thus falls within the scope of the claims.

50. Furthermore, a cylinder with the Kuriyan's azido group length does not fit into the space available either. When I preformed the same analysis as above with the azidomethyl protecting group, its atoms virtually overlap with those of the functionally critical tyrosine residue. Under his view, in order to determine whether the azido group fit into the space available in the polymerase, a POSITA would simply draw the molecule in its extended conformation, and measure the width. Doing so, Dr. Kuriyan arrived at the measurement of 2.1 Å, which he compared to the 3.7 Å disclosed in the Ju Declaration, and concluded the azido protecting group would fit. However, as shown in Figure C and this cylinder analysis, regardless of a cylindrical 3.7 Å diameter, an azido group will not fit. This highlights the incompleteness of Dr. Kuriyan's model. By ignoring length entirely, his model does not provide the necessary information to inform a POSITA of the scope of the claim.

51. Indeed, if the Ju Declaration was intended to define the space as unbounded in one dimension, the term "small" would not be appropriate to describe it. That the space available is described as "crowded," and "small" implies that there

is not a lot of room in any direction. Under Dr. Kuriyan's model focused only on the width of the protecting group, both the three carbon allyl and the four, 100, or 1000 carbon chain would be considered "small." However, a more accurate description of the 1000 carbon chain would be "narrow" or "thin," and no POSITA would consider such a large chain "small" in the context of a 3' protecting group, let alone within the context of most other areas of chemistry or biochemistry. In this manner, Dr. Kuriyan's model provides the broadest scope available for "small" by including all linear protecting groups.

E. Dr. Kuriyan's Assumptions Minimize "Width" And Maximize "Length"

52. Dr. Kuriyan further stacks the deck in favor of broadening the scope of which protecting groups would be considered "small" by assuming that the correct measurement of the protecting groups' dimensions is in their extended conformations. As already discussed, most bonds in protecting groups are at least somewhat free to rotate, which means that they can be arranged in a wide variety of conformations. Dr. Kuriyan provides the extended conformation for his models, which aligns the molecules linearly – the conformation which minimizes width, but at the expense of expanding the length. This conformation would be reasonable if the molecule was in the gas phase or free in solution, but almost certainly not within the highly restrictive space available within the active site of a DNA polymerase. Instead, the bonds would rotate to produce a conformation that minimizes conflicting interactions with the rat DNA polymerase. These changes in conformation would change the measured "length" and "width" dimensions as defined by Dr. Kuriyan. Thus, these changes cannot be accounted for within a description that approximates the protecting group as a cylinder (because each dimension would change), but can at least approximately be accounted for by describing the protecting group as a

sphere, as a linear molecule will by definition have space within the sphere along the dimension perpendicular to its longest length.

53. Similarly, Dr. Kuriyan's extended conformations necessarily maximize the "length" of the protecting groups, and in turn minimizes their "width." This acts to allow the claims to cover more "small" protecting groups because virtually any group is measured to be "small" based only on its "width" in its longest conformation. This compounds the errors in Dr. Kuriyan's model, because not only is he ignoring the "length" dimension entirely, he also minimizes the only dimension he purports to matter, the "width." This in fact, renders "width" essentially meaningless as a metric to describe the "smallness" of any protecting group.

V. COMPENSATION

54. My compensation for consulting on this matter is $550 per hour. My compensation does not depend on the outcome of this dispute.

VI. RESERVATION OF RIGHTS

55. I reserve all rights to modify or supplement this declaration if I become aware of any errors or misstatements. I also reserve all rights to respond to any statements made by Plaintiffs or its declarants or expert declarants to which a response is appropriate. I further reserve all rights to correct, modify, or supplement opinions I offer in this declaration if any claim terms are construed in a manner different from the constructions herein. I may also modify or supplement my opinions in view of opinions or arguments made by any person, including Plaintiffs' counsel and anyone engaged by Plaintiffs to provide opinions. I may also modify or supplement my opinions if the Court provides the litigants with any pertinent additional rulings.

56. I declare under penalty of perjury that the foregoing is true and correct.

Dated: 7/1/2020

By: 
Floyd Romesberg, Ph.D.

APPENDIX A

Updated July 2020

Curriculum Vitae
Floyd Eric Romesberg

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Education

Description	*Date*	*Location*	*Advisor*
Postdoctoral Rsch.	1994–1998	UC Berkeley, Berkeley, CA	Professor Peter G. Schultz
Ph.D. in Chemistry	1994	Cornell University, Ithaca, NY	Professor David B. Collum
M.S. in Chemistry	1990	Cornell University, Ithaca, NY	Professor David B. Collum
B.S. in Chemistry	1988	Ohio State University, Columbus, OH	Professor Matthew S. Platz

Appointments

2019 – present	Distinguished Fellow, Synthorx, a Sanofi Company
2014 – 2019	Professor, Department of Chemistry, The Scripps Research Institute
2006 – 2014	Associate Professor, Department of Chemistry, The Scripps Research Institute
1998 – 2006	Assistant Professor, Department of Chemistry, The Scripps Research Institute

Professional Experience

2014 – 2019	Synthorx, La Jolla, CA (Scientific Founder, Consultant, Board of Directors);
2010 – 2013	RQx Pharmaceuticals, Inc., La Jolla, CA (Scientific Founder, Consultant, SAB member); Acquired by Genentech in 2013
2005 – 2011	Achaogen Inc., South San Francisco, CA (Scientific Founder, Consultant, SAB member); NASDAQ: AKAO

Honors

2020	Fellow, National Academy of Inventors
2018	ACS San Diego Section Distinguished Scientist Award
2018	Royal Society of Chemistry Bioorganic Chemistry Award
2015	ACS Nobel Laureate Signature Award for Graduate Education in Chemistry
2008 – 2009	Member, Institute for Defense Analysis, Defense Science Study Group
2005	World Technology Award Nominee in Biotechnology
2004	Discover Magazine Technology Innovation Award
2004	NSF CAREER Award
2003	Camille Dreyfus Teacher Scholar Award
2003	Susan B. Komen Breast Cancer Foundation Award
2002	The Baxter Foundation Award
1994 –1996	NIH National Research Service Award Postdoctoral Fellowship
1987	The Mac Nevin Award

Publications, *electronic list* via PubMed: http://bit.ly/2uEWtff

Publications: Principal Investigator, Primary Research Articles

1. YX Tan, DS Peters, SI Walsh, M Holcomb, D Santos-Martins, S Forli, **FE Romesberg** (2020) Initial analysis of the arylomycin D antibiotics, *J Nat Prod* accepted 12 June, doi: 10.1021/acs.jnatprod.9b01174.
2. EC Fischer, K Hashimoto, Y Zhang, AW Feldman, VT Dien, RJ Karadeema, R Adhikary, MP Ledbetter, R Krishnamurthy, **FE Romesberg** (2020) New codons for efficient production of unnatural proteins in a semi-synthetic organism, *Nat Chem Biol* 16:570–576.
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11. AW Feldman, EC Fischer, MP Ledbetter, J-Y Liao, JC Chaput, **FE Romesberg** (2018) A tool for the import of natural and unnatural nucleoside triphosphates into bacteria, *J Am Chem Soc* 140:1447-1454
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14. MP Ledbetter, RJ Karadeema, **FE Romesberg** (2018) Reprogramming the replisome of a semisynthetic organism for the expansion of the genetic alphabet, *J Am Chem Soc* 140:758-765
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155. A Craney, **FE Romesberg** (2016) Discovery of novel antibacterials, *Bioorg Med Chem* 24:6225-6226
156. SI Walsh, A Craney, **FE Romesberg** (2016) Not just an antibiotic target: Exploring the role of type I signal peptidase in bacterial virulence, *Bioorg Med Chem* 24:6370-6378
157. T Chen, N Hongdilokkul, Z Liu, D Thirunavukarasu, **FE Romesberg** (2016) The Expanding World of DNA and RNA, *Curr Op Chem Biol* 34:80-87
158. A Craney, **FE Romesberg** (2015) The inhibition of type I bacterial signal peptidase: Biological consequences and therapeutic potential, *Bioorg. Med. Chem. Lett.* 25:4761-4766
159. DA Malyshev, **FE Romesberg** (2015) An expanded genetic alphabet, *Chem Int. Ed.* 54:11930-11944
160. T Chen and **FE Romesberg** (2014) Directed polymerase evolution, *FEBS Lett* 588:219-229
161. J Zimmermann and **FE Romesberg** (2014) Carbon-deuterium bonds as non-perturbative infrared probes of protein dynamics, electrostatics, heterogeneity, and folding, *Methods Mol Biol* 1084:101-119
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165. AM Leconte and **FE Romesberg** (2009) Engineering nucleobases and polymerases for an expanded genetic alphabet, Protein Engineering, C Köhrer and U L TajBhandary, eds, Springer-Berlag, 291-314
166. A Mapp and **FE Romesberg** (2008) Editorial overview: Next generation therapeutics, *Curr Opin Chem Biol* 12:387-388
167. J Heideker, ET Lis, **FE Romesberg** (2007) Phosphatases, DNA damage checkpoints and checkpoint deactivation, *Cell Cycle* 6:3058-3064
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169. RT Cirz and **FE Romesberg** (2007) Opening the door for the intervention in evolution as a therapeutic strategy: controlling induced mutagenesis, *Crit Rev Biochem Mol Biol* 42:341-354
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171. AM Leconte and **FE Romesberg** (2006) Chemical Biology: a broader take on DNA, *Nature* 444:553-555
172. AM Leconte and **FE Romesberg** (2006) Amplify this! DNA and RNA get a third base pair, *Nat Methods* 3:667-6689
173. RT Cirz, N Gingles, **FE Romesberg** (2006) Side effects may include evolution, *Nat Med* 12:890-891
174. AA Henry and **FE Romesberg** (2005) The evolution of DNA polymerases with novel activities, *Curr Opin Biotech* 16:370-377
175. RC Holmberg, AA Henry, **FE Romesberg** (2005) Directed evolution of novel polymerases *Biomol Eng.* 22:39-49
176. R L Schowen and **FE Romesberg** (2004) Isotope effects and quantum tunneling in enzyme-catalyzed hydrogen transfer Part I The experimental basis, *Adv Phy Org Chem* 39:27-77
177. AA Henry and **FE Romesberg** (2003) Beyond A, C, G & T: augmenting nature's alphabet, *Curr Opin Chem Biol* 7:727-733
178. **FE Romesberg** (2003) Multidisciplinary Experimental approaches to characterizing biomolecular dynamics, *ChemBioChem* 4:563-571
179. **FE Romesberg**, C Yu, S Matsuda, AA Henry (2002) UNIT 1.5 Development of a universal nucleobase and nucleobases for expanding the genetic code, *Curr Prot Nucleic Acid Chem*

Publications: Postdoctoral work

180. **FE Romesberg** and PG Schultz (1999) A mutational study of a Diels-Alder catalytic antibody, *Bioorg Med Chem Lett* 9:1741-1744
181. **FE Romesberg**, BD Santarsiero, B Spiller, PG Schultz, RC Stevens (1998) Structural and kinetic evidence for strain in biological catalysis, *Biochemistry* 37:14404-14409
182. **FE Romesberg**, ME Flanagan, T Uno, PG Schultz (1998) Mechanistic studies of an antibody-catalyzed elimination reaction, *J Am Chem Soc* 120:5160-5167
183. **FE Romesberg**, BS Spiller, PG Schultz, RC Stevens (1998) Immunological origins of binding and catalysis in a Diels-Alderase antibody *Science*, 279:1929-1933
184. ME Blackwood Jr, TS Rush III, **FE Romesberg**, PG Schultz and TG Spiro (1998) Alternative modes of substrate distortion in enzyme and antibody catalyzed ferrochelation reactions, *Biochemistry* 37:779-782
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186. D Ulrich, PL Yang, PA Patten, **FE Romesberg**, PG Schultz (1995) Expression studies of catalytic antibodies, *Proc Natl Acad Sci USA* 92:11907-11911

Publications: Ph.D. work

187. F Remenar, BL Lucht, D Kruglyak, **FE Romesberg**, JH Gilchrist, DB Collum (1997) Lithium 2,2,6,6-tetramethylpiperidide and lithium 2,2,4,6,6-pentamethylpiperidide: Influence of TMEDA and related chelating ligands on the solution structures. Characterization of higher cyclic oligomers, cyclic dimers, open dimers, and monomers, *J Org Chem* 62:5748-5754
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194. **FE Romesberg** and DB Collum (1992) Determination of the structures of solvated lithium dialkylamides by semiempirical (MNDO) methods comparison of theory and experiment, *J Am Chem Soc* 114:2112-2121
195. **FE Romesberg**, JH Gilchrist, AT Harrison, DJ Fuller, DB Collum (1991) The structure of lithium tetramethylpiperdide and lithium diisopropylamide in the presence of hexamethylphosphoramide: Structure-dependent distribution of cyclic dimers, open dimers, ion triplets, and monomers, *J Am Chem Soc* 113:5751-575
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Patents

1. **F. E. Romesberg**, M. Ledbetter, R. Karadeema. Unnatural Base Pair Compositions and Methods of Use. Provisional Patent Application 62/612,062, 29 December 2017.

2. **F. E. Romesberg**, Y. Zhang, E.C. Fischer, A.W. Feldman, V.T. Dien. Incorporation of Unnatural Nucleotides and Methods Thereof. Provisional Patent Application 62/531,325, 11 July 2017.
3. T. Chen, **F.E. Romesberg**. Polymerase Chain Transcription (PCT): Exponential Synthesis of RNA and Modified RNA. Provisional Patent Application 62/531,603, 12 July 2017.
4. **F.E. Romesberg**, Y. Zhang. Novel Nucleoside Triphosphate Transporter and Uses Thereof. PCT Patent Application US2017039133, 23 June 2017.
5. **F.E. Romesberg**, P. Baran, D. Peters. Synthesis of the Arylomycin Macrocyclic Core. PCT Patent Application US2017036797, 9 June 2017.
6. **F.E. Romesberg**, B. Lamb, Y. Zhang. Production of Unnatural Nucleotides Using a Crispr/CAS9 System. PCT Patent Application US2016067353, 16 December 2016.
7. **F.E. Romesberg**, A. Craney. Compositions and Methods for Identifying Type I Signal Peptidase Inhibitors. PCT Patent Application US2016045796, 5 August 2016.
8. **F.E. Romesberg**, D.A. Malyshev. Import of Unnatural or Modified Nucleoside Triphosphates into Cells via Nucleic Acid Triphosphate Transporters. Patent Application US2017029829 (A1), 2 February 2017.
9. **F.E. Romesberg**, D.A. Malyshev, L. Li, T. Lavergne, Z. Li. A Method for the Site-Specific Enzymatic Labelling of Nucleic Acids In Vitro by Incorporation of Unnatural Nucleotides. Patent Application US20160168187 (A1), 16 June 2016.
10. **F.E. Romesberg**, P.A. Smith, T.C. Roberts. Broad Spectrum Antibiotic Arylomycin Analogs. U.S. Patent 9,187,524 (B2), 17 November 2015.
11. **F. E. Romesberg**, D.A. Bachovchin. Genomic Mutation Inhibitors that Inhibit Y Family DNA Polymerases. Patent Application US20100130439 (A1), 27 May 2010.
12. **F. E. Romesberg**, R.T. Cirz, P.A. Patten. Compositions and Methods for Enhancing Drug Sensitivity and Treating Drug Resistant Infections and Diseases. Patent Application US2006286574 (A1), 21 December 2006.
13. **F. E. Romesberg**, N. David, R.T. Cirz. Compositions and Methods to Reduce Mutagenesis. US Patent 7,455,840 (B2), 25 November 2008.

Invited Seminars

2020

- Pacifichem (*scheduled*)
- Biosystems Design 6.0 Symposium, Biopolis, Singapore (*postponed*)
- San Diego BioPharma Conference (*postponed)*
- Bollum Symposium, University of Minnesota (*postponed*)
- Schulich Chemical Biology Meeting, Haifa, Israel (*postponed*)
- Experimental Biology/FASEB Meeting, San Diego, CA (*webinar*)
- International Symposium in Chemical Biology, Geneva, Switzerland

2019

- IUPAC International Symposium on Bioorganic Chemistry, Shenzhen, China
- Technion, Haifa, Israel
- EMBO Workshop, Heidelberg, Germany
- 2019 Chinese Medicinal Chemistry Symposiu (CMCS) & 2019 CPF-EFMC Joint International Symposium on Medicinal Chemistry, Chengdu, China
- 9th International Conference of Nucleic Acid-Protein Chemisty and Structural Biology for Novel Drug Discovery, Chengdu, China
- 2018 Royal Society of Chemistry Award Lecture Tour; University of Brighton, University of Bristol, Lancaster University, Imperial College of London, United Kingdom
- 16th Workshop/Systems Biology 2019: From Ancient to Synthetic Genomes, Melbourne, Australia
- Yale Chemical Biology Symposium, New Haven, Connecticut
- Advances in Chemical Biology Conference, Frankfurt, Germany
- UNESCO, Paris, France
- Congreso Futuro, Santiago, Chile

2018

- South China University of Technology, Guangzhou, China
- University of Hong Kong, Chemical Biology Symposium Series, Sai Wan, Hong Kong
- Hebrew University of Jerusalem, Jerusalem, Israel
- Ben-Gurion University, Beer-Sheva, Israel
- Tel-Aviv University, Tel-Aviv, Israel
- Synthetic Genomes Conference, Sydney, Australia
- La Ciudad de las Ideas, Puebla, Mexico
- Merck Research Laboratories, Kenilworth, NJ
- New England Biolabs, Ipswich, MA
- SCIX Conference, Atlanta, GA
- Emory University, Atlanta, GA
- 15th Biennial Symposium on Frontiers in Organic Chemistry, University of Illinois, Champaign, IL
- 15th Horizons in Molecular Biology, Gottingen, Germany
- 14th Annual Meeting of the Oligonucleotide Therapeutics Society, Seattle, WA
- 44th Future Lecture Series, Beijing, China
- 256th ACS National Meeting, Boston, MA
- Genentech, Inc., San Francisco, CA
- Institute Gulbenkian of Science, Lisboa, Portugal
- Synthetic Biology, Engineering, Evolution and Design Conference (SEED 2018), Scottsdale, AZ
- 31st Annual Conference of the Chinese Chemical Society, Hangzhou, China
- Foundations of Nanoscience (FNANO 2018) Conference, Snowbird, Utah
- TED2018 Conference, Vancouver, British Columbia, Canada
- National Intelligence University, Bethesda, MD
- 255th ACS National Meeting, New Orleans, LA
- The Bright Futures in Biology Symposium, Alexandria, VA
- Cellular and Molecular Genetics Training Program, University of California, San Diego, CA
- Air Force Research Lab, Wright Patterson AFB, Ohio
- PepTalk The Protein Science Week, San Diego, CA

2017

- ChemBio Seminar Series, University of Southern California, Los Angeles, CA
- International Symposium of Bioorganic Chemistry, Konstanz, Germany
- CBI Annual Research Symposium, University of California, San Diego
- Chemical Biology Program Retreat, University of California, Davis
- San Diego State University, San Diego, CA
- BIO International Convention, San Diego, CA
- 15th Annual Genomics Forum, Vancouver, British Columbia
- World Molecular Engineering Meeting, Los Cabos, Mexico
- Frontiers in Chemical Biology, Henan Normal University, Xinxiang, China
- FNANO 17 Self-Assembled Architectures and Devices, Snowbird, UT
- Purdue University, West Lafayette, IN
- China Society of Biotechnology Young Scientist Forum II, South China University of Technology, Guangdong, China
- University of Miami, Miller School of Medicine, Miami, FL
- Department of Chemistry Colloquium, University of California, Riverside
- Scripps Florida External Seminar Series, Jupiter, FL
- Pathogenesis Affinity Group, Rancho Santa Fe, CA
- PepTalk The Protein Science Week, San Diego, CA

2016

- Nucleic Acid Workshop, Telluride Science Research Center, Telluride, CO
- XXII International Roundtable on Nucleosides, Nucleotides and Nucleic Acids, Paris, France
- Medical University of Vienna, 12th PhD Symposium, Vienna, Austria
- XB2 The Second Conference on Xenobiology, Berline, Germany
- Princeton University, Chemistry Department Lecture Series, Princeton, NJ
- FNANO16 Foundations of Nanoscience, Snowbird, UT
- Antibody Biology & Engineering Gordon Research Conference, Galveston, TX
- UT Southwestern Medical Center, Dallas, TX
- Genome Engineering and Synthetic Biology Conference, Gent, Belgium
- Salk Institute IPSEN Science Symposium on Biological Complexity, La Jolla, CA

2015

- Pacifichem 2015, Honolulu, HI
- Cellular and Molecular Biotechnology Conference, Institut des Hautes Études Scientifiques, Paris, France
- BMB 2015 Biochemistry and Molecular Biology, Kobe, Japan
- TEDMED 2015, Palm Springs, CA
- Molecular Biology Program Retreat, University of Colorado, School of Medicine, Denver, CO
- Denison University, Chemistry Department Lecture Series, Granville, OH
- Chemical and Structural Biology and Biophysics RFG Seminar, The Ohio State University, Columbus, OH
- The Past, Present, and Future of DNA, Radcliffe Institute for Advanced Study, Harvard University, Cambridge, MA
- The Scripps Research Institute Graduate Student Symposium, Lake Arrowhead, CA
- Protein Dynamics 2015, Telluride Science Research Center, Telluride, CO
- Synthetic Biology Gordon Research Conference, Newry, ME
- CAS Conference Synthetic Biology, Munich, Germany
- Bioorganic Chemistry Gordon Research Conference, Andover, New Hampshire
- University of California, Chemical Biology and Biophysics Seminar, San Diego, CA
- Berkeley City College, Science Seminar Program, Berkeley, CA
- PEGS: The Protein and Antibody Engineering Summit, Boston, MA
- World Molecular Engineering Meeting, Los Cabos, Mexico
- University of Wisconsin, Microbiology Doctoral Training Program, Madison, WI
- 12th Annual Foundations of Nanoscience Conference, Snowbird, UT
- Boston College, Chemistry Seminar Series, Boston, MA
- Indiana University, Chemical Biology Seminar Series, Bloomington, IN
- 249th ACS National Meeting, Chemistry & Biology of Non-Natural Nucleic Acids Session, Denver, CO
- Carnegie Mellon University, Chemistry Department Lecture Series, Pittsburgh, PA
- Frontiers in Biomedical Research Symposium, Indian Wells, CA
- RNA Consortium Workshop, The Scripps Research Institute, La Jolla, CA
- 7th Annual Engineering Genes, Vectors, Constructs and Clones PepTalk 2015, San Diego, CA

2014

- Techonomy TE14, Half Moon Bay, CA
- New England Biolabs, Ipswich, MA
- Yale University, Organic Chemistry Seminar Series, New Haven, CT
- Colloquium Series, Origins Institute, McMaster University, Ontario, Canada
- University of Pennsylvania, Department of Biochemistry and Biophysics Seminar Series, Philadelphia, PA
- National Academies CSTL Forum on Synthetic Biology, Washington, D.C.
- Texas A&M University, College Station, TX
- 17th Annual San Diego Medchem Symposium, San Diego, CA
- GTCbio Nucleic Acids Summit, San Diego, CA
- Techonomy Bio, Mountain View, CA
- Plenary Speaker, XVIth Symposium on Chemistry of Nucleic Acid Components, Czech Republic
- North Dakota State Frontiers in Biomedical Research Symposium, Fargo, ND
- World Molecular Engineering Meeting, Los Cabos, Mexico
- SUNY Buffalo Department of Chemistry Foster Colloquium, Buffalo, NY
- 247th ACS National Meeting, Frontiers of Nucleic Acid Chemistry Session, Dallas, TX

2013

- Molecular Basis of Disease Area of Focus, George State University, Atlanta, GA
- University of Montréal, Québec
- 246th ACS National Meeting, Optical Spectroscopy of Proteins Session, Indianapolis, IN
- Georgia Tech College of Sciences, Atlanta, GA
- Biomolecular Interaction Technologies Center Symposium on Antibody Flexibility, Durham, NH
- Chemistry Biology Interface Training Grant Symposium, Univ. of Minnesota Dept. of Chemistry
- Claremont Colleges Keck Science Department, Chemistry Seminar Series, Claremont, CA
- Department of Chemistry Seminar, The Scripps Research Institute, Jupiter, FL
- 65th Fujihara Seminar and Symposium, Hokkaido, Japan
- Tsing Hua University, Hsinchu, Taiwan
- Institute of Chemistry Symposium, Academia Sinica, Taipei, Taiwan
- Department of Chemistry Colloquium, University of Chicago
- World Molecular Engineering Meeting, Los Cabos, Mexico
- PEGS: The Protein and Antibody Engineering Summit, Boston, MA
- California State University - Long Beach, Chemistry and Biochemistry Seminar Series

2012
- 244th American Chemical Society National Meeting and Exposition, Philadelphia, PA
- World Molecular Engineering Meeting, Los Cabos, Mexico
- Georgia Institute of Technology, Atlanta, GA
- 243rd American Chemical Society National Meeting and Exposition, San Diego, CA

2011
- McMaster University Biochemistry and Biomedical Sciences Seminar Series, Ontario, Canada
- University of Maryland, Baltimore County Chemistry-Biology Interface Program, Baltimore, MD
- Society for Industrial Microbiology Annual Meeting, New Orleans, LA
- Gordon Research Conference on Tuberculosis Drug Development, Lucca, Italy
- 22nd American Peptide Society Symposium, San Diego, CA
- Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China
- Shanghai Institute of Meteria Medica, Chinese Academy of Sciences, Shanghai, China
- Chemical and Structural Biology of Nucleic Acids and Proteins for Novel Drug Discovery, Beijing, China
- CLIO Proposers Day Workshop, DARPA

2010
- University of Victoria Lecture Series, Victoria, BC
- Lectures in Modern Chemistry Series, University of British Columbia, Vancouver, BC
- Simon Fraser University Lecture Series, Vancouver, BC
- Nucleic Acid Chemistry Workshop, Telluride, CO
- Center for Workshops in the Chemical Sciences: Nucleic Acids Workshop, Atlanta, GA
- Evolving DNA Polymerases: Chemistry Meets Biology, Ascona, Switzerland
- World Molecular Engineering Meeting, Los Cabos, Mexico
- Johns Hopkins Chemistry-Biology Interface Program, Baltimore, MD
- Highs in Chemistry and Biology Conference, Dead Sea, Israel

2009
- Wayne State University Lecture Series, Detroit, MI
- Michigan State University Lecture Series, East Lansing, MI
- 3éme Cycle Lecture Tour in Chemistry, Switzerland
- Functional Nucleic Acids Conference, Regensburg, Germany
- Gordon Research Conference on Proteins, Holderness, NH
- New York University, New York, New York
- Society for General Microbiology, Hastings, England
- Case Western Reserve University, Cleveland, OH

2008
- Mesilla Chemistry Workshop, Mesilla, New Mexico
- 43rd Congress of the Mexican Chemical Society, Tijuana, Mexico
- Workshop on Evolution in Health and Disease, Oeiras, Portugal
- 236th American Chemistry Society National Meeting, Philadelphia, PA
- Mutagenesis Gordon Conference, Oxford, UK
- Broad Institute, Cambridge, MA
- Reuben H. Fleet Science Center, San Diego, CA

2007
- Nucleic Acids Gordon Conference, Salve Regina University
- UCSD Medical Center, San Diego, CA
- San Diego State University, San Diego, CA
- 223rd American Chemical Society National Meeting, Chicago, IL
- American Society of Biochemistry and Molecular Biology 2007 Annual Meeting, Washington DC

2006
- Frontiers in Biological Chemistry Session, 20th Annual Symposium of the Protein Society, San Diego, CA
- Second International Symposium on Biomolecular Chemistry, Kobe, Japan
- Osaka Prefecture University, Osaka, Japan
- Beckman Symposium on Evolution, Palo Alto, CA
- Sixth Osaka University Forum, San Diego, CA
- Department of Biological Sciences, University of Southern California
- Department of Chemistry, University of Utah
- Department of Pharmacology and Molecular Sciences, Johns Hopkins University
- Gordon Research Conference on Chemistry and Biology of Peptides, Ventura, CA

2005
- Symposium on Chemically Modified DNA: Synthesis, Photochemistry, and Applications, Pacifichem, Honolulu, HI

- 17th Annual Frontiers of Science Symposium of the National Academy of Sciences, Frontiers in Synthetic Chemistry and Biology Session, Irvine, CA
- Ambassador Speaker, Gordon Research Conference on Epigenetics, Plymouth, NH
- Plenary Speaker, 32nd Symposium on Nucleic Acids Chemistry, Fukuoka, Japan
- Plenary/Novartis Speaker, XIIIth Symposium on the Chemistry of Nucleic Acid Components, Prague, Czech Republic
- Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Prague, Czech Republic
- Department of Chemistry, Uppsala University, Uppsala, Sweden
- Biomimetic Polymers symposium, 229th American Chemical Society National Meeting, San Diego, CA
- Gordon Research Conference on Nucleosides, Nucleotides and Oligonucleotides, Newport, RI
- Telluride Science Research Center Workshop on Vibrational Dynamics of Biological Molecules, Telluride, CO
- Department of Defense Era of Hope Conference, Philadelphia, PA
- Instituto Tecnologico de Tijuana, Tijuana, Mexico
- BioAgenda2005, Palm Springs, CA

2004

- Department of Pharmaceutical Chemistry, University of California, San Francisco, CA
- Department of Chemistry, University of Iowa, Iowa City, IA
- Department of Chemistry, University of Texas, Dallas, TX
- Department of Chemistry & Biology, University of Maryland, College Park, MD
- Department of Chemistry, Emory University, Atlanta, GA
- Department of Chemistry & Chemical Engineering, California Institute of Technology, Pasadena, CA
- Department of Biochemistry, Tufts University, Boston, MA
- Department of Chemistry, Ohio State University, Columbus, OH
- Department of Chemistry & Biochemistry, Denison University, Granville, OH
- Department of Chemistry & Biochemistry, University of Colorado, Boulder, CO
- Department of Chemistry, Michigan State University, Ann Arbor, MI
- Department of Chemistry, Wayne State University, Detroit, MI
- Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA

2003

- International Symposium on Bio-Inspired Engineering, Tel Aviv, Israel
- Department of Chemistry, Stanford University, Stanford, CA
- Department of Chemistry & Biochemistry Physical Chemistry Lecture, University of California, San Diego, CA
- Department of Chemistry, University of Rochester, Rochester, NY
- Conference on Lasers and Electro Optics/Quantum Electronics & Laser Science Conference, Baltimore, MD
- Department of Chemistry, Cornell University, Ithaca, NY

2002

- Department of Biochemistry & Biophysics, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY
- Department of Chemistry, Yale University, New Haven, CT
- Department of Chemistry, Bowdoin College, Brunswick, ME
- Archemix Corp., Cambridge, MA
- Reaction Mechanisms Conference, Bartlett Session, Columbus, OH

2001

- Gordon Research Conference on Bioorganic Chemistry, Andover, NH
- New Frontiers in Chemical Biology, Seoul National University, Seoul, Korea
- Biomolecular Engineering Research Institute, Osaka, Japan
- Department of Chemistry, Pohang University of Science and Technology, Pohang, Korea

2000

- Department of Chemistry, University of Southern California, Los Angeles, CA
- Department of Chemistry, University of California, Irvine, C

1999

- International Conference on Phosphorus Chemistry, Cincinnati, OH

1995

- Gordon Research Conference on Isotopes in the Biological and Chemical Sciences, Oxnard, CA

1994

- Department of Chemistry, Yale University, New Haven, CT
- Department of Chemistry, Columbia University, Columbia, NY
- Department of Chemistry, University of Rochester, Rochester, NY
- Department of Chemistry, University of Wisconsin, Madison, WI
- Department of Chemistry, Ohio State University, Columbus, OH
- Department of Chemistry, University of Colorado, Boulder, CO

Extracurricular Professional Service

Grant Review	Synthetic and Biological Chemistry A Study Section, Center for Scientific Review, NIH, Ad Hoc review for NIGMS Bioorganic and Natural Products, American Chemical Society Petroleum Research Fund, Defense Threat Reduction Agency, Chemical and Biological Technologies, National Human Genome Research Institute, National Institutes of Health, National Science Foundation, Samsung Corporation
Manuscript Review	*Accounts of Chemical Research, Angewandte Chemie, Biochemistry, Bioorganic and Medicinal Chemistry Letters, Cellular and Molecular Life Sciences, Chemistry & Biology, ChemPhysChem, Chemical Reviews, Journal of the American Chemical Society, Journal of Molecular Biology, Journal of Organic Chemistry, Journal of Physical Chemistry, Nucleic Acids Research, Nature, Nature Biotechnology, Nucleosides, Nucleotides, and Nucleic Acids, Proceedings of the National Academy of Sciences, Science, Tetrahedron, Tetrahedron Letters*
Editorial Boards	Current Opinion in Chemical Biology 2019–present Bioorganic and Medicinal Chemistry 2018–present Current Protocols in Chemical Biology, 2010 – present *PLoS ONE*, 2006 – present
Professional Affiliations	American Chemical Society (1998 – present) American Society for Microbiology (2005 – present) Biophysical Society (2014 – present)

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

----------x

ILLUMINA, INC.,

Petitioner,

vs.

PATENT OF THE TRUSTEES
OF COLUMBIA UNIVERSITY IN
THE CITY OF NEW YORK

Patent Owner.

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VOLUME I

CONFIDENTIAL VIDEOTAPED DEPOSITION

OF DR. GEORGE L. TRAINOR

Wednesday, September 4, 2013

New York, New York

2:00 p.m.

Reported by:
Maureen Ratto, RPR, CCR, CLR
Job No: 31222-A

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

----------x

ILLUMINA, INC.,

Petitioner,

vs.

PATENT OF THE TRUSTEES
OF COLUMBIA UNIVERSITY IN
THE CITY OF NEW YORK

Patent Owner.

----------x

VOLUME III

VIDEOTAPED DEPOSITION OF DR. GEORGE TRAINOR

Thursday, September 5, 2013

New York, New York

9:00 a.m.

Reported by:
Maureen Ratto, RPR, CCR, CLR
Job No: 31224

* * *

Deposition of DR. GEORGE L. TRAINOR, held at the offices of Fitzpatrick, Cella, Harper & Scinto, LLP, 1290 Avenue of the Americas New York, New York, 10104-3800, pursuant to notice, before Maureen Ratto, Registered Professional Reporter, License No. 817125, and Notary Public.

* * *

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Illumina Senior Patent Counsel
Manuel Abreu, Videographer
Kevin Burgess, Texas A&M University

TRAINOR

THE WITNESS: -- it does not interfere with the recognition of the polymerase.

Q. Is that the definition of "small"?

A. I have -- "small" can have many definitions, obviously, and I look to the specification of what his intention is and I read the claim. And the -- the intention is that it be sufficiently small that it is now accepted by the polymerase.

Q. Okay. So it's sort of a functional definition, the way you interpret it. Is -- is that fair to say?

A. Well, I think you could ask a chemist: Is this small? And they'd say, Well, relative to what?

And so there's a -- there is a functional definition here. But if you read the claim it simply says, "Does not interfere with recognition by the polymerase." It's a small group, so it

does not interfere. So I think it's understood that -- that the invention involves a 3'-cap that is removable that is -- is accepted by the polymerase.

Q. Okay. And so that's sort of the cutoff between something that is small and something that is not small as you understand it in the context of the Ju invention?

A. Yes.

Q. As a structural matter, do you have in mind any cutoff between something that is small and not small, in terms of either size or weight or however you'd -- you would measure smallness?

A. No, I -- I don't have a cutoff because I think it's always context dependent.

Q. Okay.

A. So I -- so I can't give you a single -- single criteria to that.

Q. So as long as it works, it --

it's going to be small, in the context of the Ju invention?

A. I think it's -- I can't give you a precise cutoff, I imagine if you give me something with a molecular weight of 1,000 and that was accepted, I would say that wouldn't be small, but I think most chemists would say I would never call that small but it was accepted and perhaps surprising. But I think the notion in this invention is that, is -- is that the insight that Dr. Ju had was that you were going to have to do something relatively small to be accepted by the polymerase. And that was the motivation to look at all the other ways to label -- to look at the base label.

Q. Now, the way you just testified, and I understand that your testimony was somewhat hypothetical, but what you suggested there was that there was some circumstances in which you could envision a molecule that would be

accepted and -- and would not interfere with recognition by the polymerase but would still not be small?

A. Yeah. It was -- it was a hypothetical saying if someone were to show me that, I would -- I guess I would have a hard time saying that was small. If it was a molecular weight of 1,000 than the rest of the nucleotide, for example.

I've never seen an example of such a species and, in fact, I mean the -- what my understanding is certain literature leading up to 2000 that -- that there was relatively little data on groups that any chemist would consider very large, or accepted and the question of where the cutoff is something that has to be experimentally determined and a function of which polymerase you're talking about.

Q. There was literature prior to 2000 that would characterize certain blocking groups as being -- as not

TRAINOR

MR. COSTAKOS: Well, then, I don't have any questions about it. So that solves everyone a lot of heartache, doesn't it?

I have no further questions of you today. Thank you very much for your time.

THE WITNESS: Thank you.

VIDEOGRAPHER: The time is 4:53 p.m., and we're going off the record.

(Whereupon, the proceedings were adjourned at 4:53 p.m.)

J U R A T

I do hereby certify that I have read the foregoing transcript of my deposition.

GEORGE L. TRAINOR

Sworn and subscribed
before me
this 21 day of
Sept, 2013

a Notary Public of
the State of Delaware

JOHN M. DENNIS
NOTARY PUBLIC • DELAWARE
My Commission Expires 3-7-16

C E R T I F I C A T E

I, MAUREEN M. RATTO, a Registered Professional Reporter, do hereby certify that prior to the commencement of the examination, GEORGE L. TRAINOR was sworn by me to testify the truth, the whole truth and nothing but the truth.

I DO FURTHER CERTIFY that the foregoing is a true and accurate transcript of the proceedings as taken stenographically by and before me at the time, place and on the date hereinbefore set forth.

I DO FURTHER CERTIFY that I am neither a relative nor employee nor attorney nor counsel of any of the parties to this action, and that I am neither a relative nor employee of such attorney or counsel, and that I am not financially interested in this action.

_____

MAUREEN M. RATTO, RPR

License No. 817125

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK and QIAGEN SCIENCES, LLC,	)	
Plaintiffs,	)	Civil Action No. 19-1681-CFC
v.	)	
ILLUMINA, INC.,	)	
Defendant.	)	

PLAINTIFFS' PRELIMINARY PROPOSED CONSTRUCTIONS

Pursuant to the Court's Scheduling Order, Plaintiffs The Trustees of Columbia University in the City of New York and QIAGEN Sciences, LLC hereby provide Defendant Illumina, Inc. with preliminary proposed constructions for the terms identified by Plaintiffs and Defendant. The proposed constructions relate to language found in the claims of U.S. Patent Nos. 10,407,458 ("the '458 Patent"); 10,407,459 ("the '459 Patent"); 10,428,380 ("the '380 Patent"); 10,435,742 ("the '742 Patent"); and 10,457,984 ("the '984 Patent") (collectively "the Patents-in-Suit").

This exchange is meant to facilitate narrowing the issues and preparing the parties' joint claim chart for submission to the Court. Plaintiffs specifically reserve the right to modify the proposed constructions as discovery continues, and in view of the Defendant's proposed constructions. Below are Plaintiffs' preliminary proposed constructions:

Terms	Preliminary Proposed Construction
"R"	Represents a part of the nucleotide analogue, attached to the oxygen at the 3′ position of the deoxyribose of the deoxyribonucleotide analogue, as depicted in the illustration of the nucleotide analogue in the claim

Terms	Preliminary Proposed Construction
"Y" "chemical linker"	Represents a part of the nucleotide analogue, attaching the base of the nucleotide analogue to a tag, as depicted in the illustration of the nucleotide analogue in the claim
"small"	A chemical group that has a diameter less than 3.7Å
"chemically cleavable"	Plain and ordinary meaning
"does not interfere with recognition of the analogue as a substrate by a DNA polymerase"	Plain and ordinary meaning
"stable during a DNA polymerase reaction"	R remains bonded to 3′ oxygen during a DNA polymerase reaction Y remains bonded to base and tag during a DNA polymerase reaction
"is recognized as a substrate"	Plain and ordinary meaning
"is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction"	Plain and ordinary meaning
"produces a 3'-OH group on the deoxyribose upon cleavage of R"	Plain and ordinary meaning
"A method for sequencing a nucleic acid"	A method for detecting the identity and sequence of a strand of nucleotides

Dated: April 3, 2020

MORRIS, NICHOLS, ARSHT & TUNNELL LLP

/s/ Jack B. Blumenfeld
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Attorneys for Plaintiff QIAGEN Sciences, LLC

OF COUNSEL:

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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK AND QIAGEN SCIENCES, LLC, Plaintiffs, v. ILLUMINA, INC., Defendant.	)	C.A. No. 19-1681-CFC

DEFENDANT ILLUMINA, INC.'S PRELIMINARY DISCLOSURE OF PROPOSED CLAIM CONSTRUCTIONS FOR DISPUTED CLAIM TERMS/PHRASES

Pursuant to Paragraph 15 of the Court's Scheduling Order (D.I. 17) and the Stipulation and Order Extending Claim Construction Deadlines (D.I. 31 & 32), Defendant Illumina, Inc. hereby provides its disclosure of proposed claim constructions for the claim terms/phrases for U.S. Patent Nos. 10,407,458; 10,407,459; 10,428,380; 10,435,742; and 10,457,984:

Claim Term/Phrase	Nominating Party (P/D)	Relevant Patents/Claims	Proposed Construction
"chemical linker"	P	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	No construction necessary, but if construed "a linker cleavable by chemical means but excluding other means, such as physical, physical chemical, heat, or light"
"A method for sequencing a nucleic acid"	P	Claims 1 and 3 of the '380 Patent	Preamble is not limiting.

"R"	D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	"a chemical group used to cap the 3'–OH group"
"Y"	D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	"a single linker that directly connects the base to the label"
"does not interfere with recognition of the analogue as a substrate by a DNA polymerase"	D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	"does not prevent use of the analogue as a polymerase substrate during a sequencing-by-synthesis reaction"
"stable during a DNA polymerase reaction"	D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	[Indefinite under 35 U.S.C. § 112]
"small"	P/D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	[Indefinite under 35 U.S.C. § 112]
"chemically cleavable"	D	Claims 1 and 2 of the '458, '459, '742, and '984 Patents Claims 1 and 3 of the '380 Patent	"a linker cleavable by chemical means but excluding other means, such as physical, physical chemical, heat, or light"

Illumina reserves the right to modify, supplement, amend, or otherwise alter this disclosure.

ASHBY & GEDDES

/s/ Andrew C. Mayo

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Andrew C. Mayo (#5207)
500 Delaware Avenue, 8th Floor
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Attorneys for Illumina, Inc.

Of Counsel:

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Derek C. Walter
WEIL, GOTSHAL & MANGES, LLP
201 Redwood Shores Parkway
Redwood Shores, CA 94065
(650) 802-3000

Dated: April 3, 2020

Thakore, Chittam U. (Atlanta)

From: Lavin, Christopher <Christopher.Lavin@weil.com>
Sent: Tuesday, April 14, 2020 10:19 PM
To: Baron, Robert (Phila)
Cc: Murnane, John D.; Giusini, Brittany M. (Del); Reines, Edward; Walter, Derek; Steven J.; amayo@ashby-geddes.com; Schwartz, Robert S.; Oliver, Justin J.; Garrett, Zachary L.; JBlumenfeld@MNAT.com; dfahnestock@mnat.com; Illumina Columbia; Segal, Marc S. (Phila); Moskow-Schnoll, Beth (Del); Harmon, Whitney (Del)
Subject: RE: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681

⚠ EXTERNAL

Following up as to the claim terms/phrases for which we are revising our proposed constructions:

- "stable during a DNA polymerase reaction" (as to R): "to have at least the stability of a MOM ether (-CH_2OCH_3) or allyl (-$CH_2CH=CH_2$) group"

- "small": "A chemical group that has a longest dimension less than 3.7Å, including the 3'–O"

Please provide Plaintiffs' revised proposed constructions and the initial draft joint claim construction chart.

At this point, anticipating we may not receive the materials tonight given the ET hour, it probably makes sense to push Wed.'s originally-scheduled 11 AM ET / 8 AM PT call back - please let us know what time works for Plaintiffs.

Thanks,
Chris

-----Original Message-----
From: Baron, Robert <Baron@ballardspahr.com>
Sent: Tuesday, April 14, 2020 12:53 PM
To: Lavin, Christopher <Christopher.Lavin@weil.com>
Cc: Murnane, John D. <JDMurnane@venable.com>; Giusini, Brittany M. <GiusiniB@ballardspahr.com>; Reines, Edward <edward.reines@weil.com>; Walter, Derek <Derek.Walter@weil.com>; Steven J. <SBalick@ashbygeddes.com>; amayo@ashby-geddes.com; Schwartz, Robert S. <RSchwartz@venable.com>; Oliver, Justin J. <JOliver@venable.com>; Garrett, Zachary L. <ZGarrett@venable.com>; JBlumenfeld@MNAT.com; dfahnestock@mnat.com; Illumina Columbia <Illumina.Columbia@weil.com>; Segal, Marc S. <segalm@ballardspahr.com>; Moskow-Schnoll, Beth <moskowb@ballardspahr.com>; Harmon, Whitney <HarmonW@ballardspahr.com>
Subject: Re: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681

Chris,
We also would like the new constructions you proposed as soon as you can. Thanks.
Rob

Robert Baron
Ballard Spahr LLP
1735 Market Street, 51st Floor
Philadelphia, PA 19103
baron@ballardspahr.com
Office: 215-864-8335

Cell: 215-847-3999

> On Apr 14, 2020, at 2:58 PM, Baron, Robert (Phila) <baron@ballardspahr.com> wrote:
>
> Chris,
> Shall we use this call in for tomorrow at 11a/8a?
>
> Robert Baron
> Ballard Spahr LLP
> 1735 Market Street, 51st Floor
> Philadelphia, PA 19103
> baron@ballardspahr.com
> Office: 215-864-8335
> Cell: 215-847-3999
>
>
>> On Apr 13, 2020, at 9:04 PM, Lavin, Christopher <Christopher.Lavin@weil.com> wrote:
>>
>> ⚠ EXTERNAL
>>
>> Got it. We rejuggled here and can start tomorrow at 1:00 PM EST / 10 AM PST.
>>
>> Again, we can use the following dial-in:
>>
>> Dial-In: (888) 235-7501
>> Conf. Code: 6508023074
>>
>> Thanks.
>>
>>
>> -----Original Message-----
>> From: Baron, Robert <Baron@ballardspahr.com>
>> Sent: Monday, April 13, 2020 5:30 PM
>> To: Lavin, Christopher <Christopher.Lavin@weil.com>; Murnane, John D.
>> <JDMurnane@Venable.com>
>> Cc: Giusini, Brittany M. <GiusiniB@ballardspahr.com>; Reines, Edward
>> <edward.reines@weil.com>; Walter, Derek <Derek.Walter@weil.com>;
>> Steven J. <SBalick@ashbygeddes.com>; amayo@ashby-geddes.com;
>> Schwartz, Robert S. <RSchwartz@Venable.com>; Oliver, Justin J.
>> <JOliver@Venable.com>; Garrett, Zachary L. <ZGarrett@Venable.com>;
>> JBlumenfeld@MNAT.com; dfahnestock@mnat.com; Illumina Columbia
>> <Illumina.Columbia@weil.com>; Segal, Marc S.
>> <segalm@ballardspahr.com>; Moskow-Schnoll, Beth
>> <moskowb@ballardspahr.com>; Harmon, Whitney
>> <HarmonW@ballardspahr.com>
>> Subject: RE: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681
>>
>> A lot of people have a 2pET, which is why we proposed 1pET.
>>
>> -----Original Message-----
>> From: Lavin, Christopher <Christopher.Lavin@weil.com>

>> Sent: Monday, April 13, 2020 8:14 PM
>> To: Murnane, John D. <JDMurnane@Venable.com>; Baron, Robert (Phila)
>> <Baron@ballardspahr.com>
>> Cc: Giusini, Brittany M. (Del) <GiusiniB@ballardspahr.com>; Reines,
>> Edward <edward.reines@weil.com>; Walter, Derek
>> <Derek.Walter@weil.com>; Steven J. <SBalick@ashbygeddes.com>;
>> amayo@ashby-geddes.com; Schwartz, Robert S. <RSchwartz@Venable.com>;
>> Oliver, Justin J. <JOliver@Venable.com>; Garrett, Zachary L.
>> <ZGarrett@Venable.com>; JBlumenfeld@MNAT.com; dfahnestock@mnat.com;
>> Illumina Columbia <Illumina.Columbia@weil.com>; Segal, Marc S.
>> (Phila) <segalm@ballardspahr.com>; Moskow-Schnoll, Beth (Del)
>> <moskowb@ballardspahr.com>; Harmon, Whitney (Del)
>> <HarmonW@ballardspahr.com>
>> Subject: RE: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681
>>
>> ⚠ EXTERNAL
>>
>> John,
>>
>> How about a 1:30 PM EST / 10:30 AM PST start?
>>
>> Thanks,
>> Chris
>>
>>
>> -----Original Message-----
>> From: Murnane, John D. <JDMurnane@Venable.com>
>> Sent: Monday, April 13, 2020 2:23 PM
>> To: Baron, Robert <Baron@ballardspahr.com>; Lavin, Christopher
>> <Christopher.Lavin@weil.com>
>> Cc: Giusini, Brittany M. <GiusiniB@ballardspahr.com>; Reines, Edward
>> <edward.reines@weil.com>; Walter, Derek <Derek.Walter@weil.com>;
>> Steven J. <SBalick@ashbygeddes.com>; amayo@ashby-geddes.com;
>> Schwartz, Robert S. <RSchwartz@Venable.com>; Oliver, Justin J.
>> <JOliver@Venable.com>; Garrett, Zachary L. <ZGarrett@Venable.com>;
>> JBlumenfeld@MNAT.com; dfahnestock@mnat.com; Illumina Columbia
>> <Illumina.Columbia@weil.com>; Segal, Marc S.
>> <segalm@ballardspahr.com>; Moskow-Schnoll, Beth
>> <moskowb@ballardspahr.com>; Harmon, Whitney
>> <HarmonW@ballardspahr.com>
>> Subject: RE: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681
>>
>> Chris,
>>
>> Plaintiffs' counsel are available tomorrow between 1:00 and 2:00 pm EDT. Would that be convenient for you folks?
>>
>> Thanks,
>>
>> John
>>
>> John D. Murnane, Esq.
>> Venable | Fitzpatrick

>> t 212.218.2527
>> Venable LLP, 1290 Avenue of the Americas, 20th Floor New York, NY
>> 10104
>>
>> JDMurnane@Venable.com | www.Venable.com -----Original Message-----
>> From: Baron, Robert <Baron@ballardspahr.com>
>> Sent: Monday, April 13, 2020 1:40 PM
>> To: Lavin, Christopher <Christopher.Lavin@weil.com>
>> Cc: Giusini, Brittany M. <GiusiniB@ballardspahr.com>; Reines, Edward
>> <edward.reines@weil.com>; Walter, Derek <Derek.Walter@weil.com>;
>> Steven J. <SBalick@ashbygeddes.com>; amayo@ashby-geddes.com; Murnane,
>> John D. <JDMurnane@Venable.com>; Schwartz, Robert S.
>> <RSchwartz@Venable.com>; Oliver, Justin J. <JOliver@Venable.com>;
>> Garrett, Zachary L. <ZGarrett@Venable.com>; JBlumenfeld@MNAT.com;
>> dfahnestock@mnat.com; Illumina Columbia <Illumina.Columbia@weil.com>;
>> Segal, Marc S. <segalm@ballardspahr.com>; Moskow-Schnoll, Beth
>> <moskowb@ballardspahr.com>; Harmon, Whitney
>> <HarmonW@ballardspahr.com>
>> Subject: Re: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681
>>
>> Caution: External Email
>>
>> Can we start it 30 minutes later?
>>
>> Robert Baron
>> Ballard Spahr LLP
>> 1735 Market Street, 51st Floor
>> Philadelphia, PA 19103
>> baron@ballardspahr.com
>> Office: 215-864-8335
>> Cell: 215-847-3999
>>
>>
>> On Apr 13, 2020, at 1:36 PM, Lavin, Christopher <Christopher.Lavin@weil.com> wrote:
>>
>>
>> ⚠ EXTERNAL
>> Counsel,
>>
>> We propose a meet and confer to discuss preparation of the Joint Claim Construction Chart and the parties' proposed claim constructions tomorrow, Tue., Apr. 14th at 1:30 PM EST / 10:30 AM PST.
>>
>> Please let us know if that works for your side.
>>
>> Regards,
>> Chris
>>
>> <image001.jpg>
>>
>> Chris Lavin
>>
>> Weil, Gotshal & Manges LLP

>> 201 Redwood Shores Parkway
>> Redwood Shores, CA 94065-1134
>> Christopher.lavin@weil.com<mailto:Christopher.lavin@weil.com>
>> +1 650 802 3074 Direct
>> +1 650 802 3100 Fax
>> From: Giusini, Brittany M. <GiusiniB@ballardspahr.com>
>> Sent: Friday, April 3, 2020 2:50 PM
>> To: Reines, Edward <edward.reines@weil.com>; Walter, Derek
>> <Derek.Walter@weil.com>; 'Steven J.'' <SBalick@ashbygeddes.com>;
>> 'amayo@ashby-geddes.com' <amayo@ashby-geddes.com>; 'John D.''
>> <JDMurnane@Venable.com>; 'Robert S.'' <RSchwartz@Venable.com>;
>> 'Justin J.' <JOliver@Venable.com>; 'Zachary L.''
>> <ZGarrett@Venable.com>; JBlumenfeld@MNAT.com; 'dfahnestock@mnat.com'
>> <dfahnestock@mnat.com>; Illumina Columbia
>> <Illumina.Columbia@weil.com>
>> Cc: Segal, Marc S. <segalm@ballardspahr.com>; Baron, Robert
>> <Baron@ballardspahr.com>; Moskow-Schnoll, Beth
>> <moskowb@ballardspahr.com>; Harmon, Whitney
>> <HarmonW@ballardspahr.com>
>> Subject: Columbia/QIAGEN v. Illumina (D. Del.) No. 19-1681
>>
>> Counsel:
>>
>> Please see attached.
>>
>> Regards,
>> Brittany
>>
>> Brittany M. Giusini, Esquire
>>
>> [http://images.ballardspahr.com/EmailTemplate/Ballard-llp-x2.gif]<htt
>> p://www.ballardspahr.com/>
>>
>>
>>
>>
>>
>>
>>
>> 919 N. Market Street, 11th Floor
>> Wilmington, DE
>> 19801-3034<http://maps.apple.com/?address=919%20N.%20Market%20Street,
>> 11th%20Floor,Wilmington,DE,19801-3034>
>> 302.252.4430<tel:302.252.4430> direct 302.252.4466<tel:302.252.4466>
>> fax
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>>
>> <http://maps.apple.com/?address=,,,,>
>>
>>

>>
>> giusinib@ballardspahr.com<mailto:giusinib@ballardspahr.com>
>> vcard<https://vcard.ballardspahr.com/Api/Signature/?vkey=AC4C0C5D6B6B
>> 20CE69643FF104689DB0&format=vcard>
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>> www.ballardspahr.com<http://www.ballardspahr.com/>
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>> privileged information. If you believe you have received this message in error, please notify the sender by reply transmission and delete the message without copying or disclosing it.
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>> ***

UNITED STATES DISTRICT COURT
DISTRICT OF DELAWARE

Case No. 19-1681-CFC

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK AND QIAGEN SCIENCES, LLC,
Plaintiff,
v.
ILLUMINA, INC.,
Defendants.

REMOTE VIDEOCONFERENCED AND VIDEOTAPED
DEPOSITION OF
FLOYD ROMESBERG, PhD

DATE TAKEN: JULY 15, 2020

REPORTED BY:
PAUL J. FREDERICKSON, CSR

UNITED STATES DISTRICT COURT

DISTRICT OF DELAWARE

Case No. 19-1681-CFC

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK AND QIAGEN SCIENCES, LLC,

Plaintiff,

v.

ILLUMINA, INC.,

Defendants.

Remote Videoconferenced and Videotaped Deposition of FLOYD ROMESBERG, PhD, the witness herein, at 8:31 a.m. Pacific Time, pursuant to notice, reported by certified court reporter Paul J. Frederickson, CSR. All parties appeared remotely and the witness was sworn remotely.

A P P E A R A N C E S

FOR THE PLAINTIFF:

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1290 Avenue of the Americas
New York, NY 10104-3800
212.218.2100
BY: ROBERT S. SCHWARTZ, ESQ.
BY: ZACHARY GARRETT, ESQ.
BY: JOHN MURNANE, ESQ.

FOR CO-PLAINTIFF QIAGEN:

BALLARD SPAHR LLP
1735 Market Street
51st Floor
Philadelphia, PA 19103-7599
215.665.8500
BY: ROBERT R. BARON, JR., ESQ.
BY: SCOTT MARTY, ESQ.
BY: CHITTAM THAKORE, ESQ.

FOR DEFENDANT ILLUMINA:
WEIL, GOTSHAL & MANGES
201 Redwood Shores Parkway
Redwood Shores, CA 94065-1192
DEREK WALTER, ESQ.
ANDREW GESIOR, ESQ.

ALSO PRESENT:
MARCUS, BURCH, ESQ.
Illumina

ALSO PRESENT:
BRADEN BATES
Videographer

was sent to me.

Q. Okay.

So you received the materials that you refer to in paragraph 2 from your attorneys?

A. I did, yes.

Q. If you were to testify concerning the opinions expressed in your declaration, will you only rely on the materials that you list in paragraph 2 to support your opinions?

A. No. So I -- your -- just so I can be clear, you're including Pelletier --

Q. Yes.

A. -- in the -- okay.

Q. Yeah, yes.

A. No, I used a program called Chimera to form some of the opinions that I articulate in my declaration.

And I also just used -- you know, I guess chemical intuition. Frankly, it's sort of sophomore structural intuition.

And also geometry, sort of just concepts from geometry.

Q. Okay.

I'm specifically talking about materials, you know. That includes, like, written materials, not concepts. You're going to be relying on what you point to in paragraph 2 in which includes the Pelletier paper; is that correct?

A. Right now I think that's correct. I mean, I can't -- I can't -- I mean, it's possible that I relied on something else at some spot, but right now I don't remember relying on something else.

Q. Okay. Okay.

And you reviewed Dr. Ju's declaration that was submitted during the prosecution of the patents in suit; correct?

A. I did.

Q. And you attached Dr. Ju's declaration as Appendix B to your declaration?

A. That's correct.

Q. And I'm going to ask Zach to turn to Appendix B. And I'm going to ask Zach to turn to paragraph 17 of Dr. Ju's declaration.

Do you see that on the screen,

Doctor?

A. I do.

Q. And take a moment to read that to yourself and let me know when you're done.

A. I'm done.

Q. In this paragraph, Dr. Ju concluded that azidomethyl was small; correct?

A. He states that.

Q. And in your declaration you conclude that the azidomethyl is not small; is that right?

A. Using the definition provided by -- by Ju in this -- Professor Ju in this declaration, yes, I concluded that azidomethyl was not small.

Q. And so while Dr. Ju concluded that the azidomethyl group is small, you reached the opposite conclusion; correct?

A. To the extent that not small is the opposite of small, sure, yes.

Q. So your conclusion is inconsistent with Dr. Ju's conclusion that the azidomethyl group is small; is that

right?

A. That is correct, yes.

Q. Okay.

Now let's look at Dr. Ju's Exhibit C, which is also part of the materials that you reviewed in this analysis. And specifically I'm going to ask you to go to the -- or ask Zach to turn to the third page. And I want you to focus on the conclusion. You see that?

A. I do.

Q. And take a moment to read that to yourself and let me know when you're done.

A. I'm done.

Q. Dr. Ju concludes that the azidomethyl group will fit into the available space of the active site; right?

A. He concludes that it will fit into the active space of -- of rat polymerase beta, yes.

Q. And is it your position that the azidomethyl group will not fit into the available space of that active site?

A. No, it is my position -- it is

my opinion that using the methods that Ju describes in his declaration, I believe this declaration I think is 2015, that you would have concluded that it would not fit. It is not small by the definition provided here.

Q. Well, what is your -- what is your opinion about whether or not the azidomethyl group will fit?

A. My opinion?

Q. Yes.

A. In -- here in 2020?

Q. Yeah, sure.

A. Well, I believe that azidomethyl has been shown to be incorporated. That's why we're all here. So it would be -- it obviously is accommodated by a polymerase. So if you're describing it as being accommodated by a polymerase, then it is by definition fits. It by definition fits.

Q. All right.

And in reference to the Pelletier polymerase, do you have a position on whether or not the azidomethyl will fit in the Pelletier polymerase?

A. I do not have any data on that.

Q. Well, if we could go back to Dr. Ju's declaration, actually the analysis, the Exhibit C.

MR. SCHWARTZ: Zach, if you wouldn't mine go back there.

Q. And we looked at Exhibit C previously. Dr. Ju provide -- I would like to go to page 3 where Dr. Ju provides these calculated diameters for each of those five groups. And my question is did you attempt to match up Dr. Ju's calculated diameters for any of those five groups with your models?

A. I don't know what Dr. Ju did. There's not enough detail to understand how he generated these numbers.

Q. So you didn't try to match them up with your models because you didn't understand what he did?

A. Well, what -- I spent a fair bit of time playing with these models on a computer screen and measuring distances. There were certainly cases where numbers like this came up. I didn't write them down, I didn't record them, because they

would have been in the context of still penetrating outside of the sphere. One part of the molecule, may be a different part, didn't penetrate out. I don't know how Dr. Ju defined these diameters of these molecules. And I certainly don't know how he then got values for them.

So, you know, it was in the back of my mind all the time. I was very curious as to what Dr. Ju had done. And I was unable to find anything that -- in any sort of a simple sense was both chemically reasonable and fit these numbers.

Q. Again, so you didn't attempt when you did your modeling to try to match your models to Dr. Ju's calculated diameters that he reports here; correct?

A. Again, I did not know what these diameters are. I did not know how he measured them. But I did know the values. And so I was very interested in knowing -- I was curious in -- as to knowing what Dr. Ju precisely did, how he arrived at these numbers, because that would have perhaps illustrated to me how he defined the

diameter for these molecules.

And I was actually kind of interested in that. I was curious. So I did sort of keep an eye on those numbers and I was looking at measurements, but nothing ever came out that was chemically sensible and -- and satisfied these numbers.

So in the end I didn't come up with an obvious explanation. But I -- I don't think it would be accurate to say that I didn't keep this in my mind, keep his values in my mind as I was looking, because if there would have been numbers that started to look consistent that I understood, then I would have understood what he did, and I was unable to do that.

Q. Well, I was just asking you for a yes-or-no answer, Doctor. You know, did -- did you -- did you attempt to match up Dr. Ju's numbers on this page with your models?

A. That was implicit during a lot of what I was doing, but I explicitly did not set out to only do that, no.

Q. Let's turn to paragraph 35 of

record.

EXAMINATION CONTINUING

BY MR. SCHWARTZ:

Q. Dr. Romesberg, in my previous questions when I was asking you about Dr. Ju's diameter measurements, if we assume that Dr. Ju's diameter measurements were meant to indicate width, did you take into account Dr. Ju's measured widths in any of your models for any of those capping groups?

A. Well, they would have been included by definition because I included the molecule. So it has all its dimensions. If you would like to call one a width, then it would be by definition there, so sure.

Q. But did you attempt to match up Dr. Ju's widths that he reports in his declaration with your models?

A. I don't believe Dr. Ju uses the word "width." I believe he uses the word "diameter."

Q. But I'm asking you to assume that -- let's assume that's what he meant. Did you -- did you -- in that case did you attempt to match up any of Dr. Ju's

diameters where he meant "width" in any of your models?

A. Are you asking --

MR. WALTER: Hold on. Objection, vague, incomplete hypothetical, lacks foundation.

Q. You can answer that question, Doctor, if you --

A. So if you're asking if I used Kuriyan's definition of the Ju deck, because Dr. Kuriyan does use width -- that's what you're asking?

Q. Yes.

A. I certainly looked at Dr. Kuriyan's proposal. I wouldn't know how to incorporate it into a model to predict -- I endeavored to ask the question by Ju's declaration and the patent: Would these variation groups be considered small?

I wasn't quite sure how to use the Kuriyan model in addressing that question. I don't debate the metrics that Kuriyan produced. If that answers the question. I did reproduce them just for fun. They're quite standard. They're quite

obvious. So it didn't take a lot of computational power.

Q. Okay. Okay.

So I guess that's sort of my next line of questions here. In paragraph 35 you actually say that:

"Dr. Kuriyan's model appears reverse engineered in order to obtain similar results in the Ju declaration...."

And you said that you were able to do that yourself; correct?

A. I was able it look at what Kuriyan had provided and measure those distances. They're obvious. And they -- and Kuriyan -- I -- I agree that those are the measured distances as Kuriyan describes them.

Q. And so you agree that Dr. Kuriyan measured the width of the protecting groups; correct? Right?

MR. WALTER: Hold on.

Objection, vague, lacks foundation.

A. It's also a little bit difficult to use the word "width" here because

Dr. Kuriyan is a little bit vague in some spots about how he defines "diameter." Because he describes it as being perpendic- -- I think he said "largely perpendicular" to the -- to the plane of the -- the length of the long molecule, the longest length. Which I find a very odd use. And -- just because perpendicular has a very specific meaning. So I don't know what "largely" or "substantially" or "mostly perpendicular" means. I assume that means that he allowed for some wiggle room of some sort. But I measured the distances between the atoms in three dimensions and found the values that he -- that he recorded.

Q. And you agree that the measurements that Dr. Kuriyan identified as the diameters of the capping groups are the same as reported by Dr. Ju in his declaration; correct?

MR. WALTER: Objection, lacks foundation --

Hold on, hold on.

Objection, lacks foundation, incomplete hypothetical, vague.

MR. SCHWARTZ: You can answer, Doctor.

A. I -- I do not disagree with -- I do not debate Dr. Kuriyan's measurement of those numbers, nor do I debate that they match -- that they match Dr. Ju's numbers that he published as diameters.

Q. And you would agree that Dr. Kuriyan's measurements that he provided for the width are not the longest dimension of the protecting groups; correct?

A. They are not. That is correct.

Q. Did you conduct any measurements of the protecting groups yourself?

A. A lot, yes.

Q. And some of them -- I'm sorry.

A. I'm sorry.

And I tried to describe this in my declaration.

MR. SCHWARTZ: I would like to put up Dr. Kuriyan's declaration.

MR. GARRETT: I have -- this is Zach.

I have emailed this exhibit to everybody just like I emailed the

first exhibit. But it's also on the screen here.

MR. SCHWARTZ: Yes. This will be Romesberg Exhibit 2.

[Deposition Exhibit 2 marked for identification.]

BY MR. SCHWARTZ:

Q. Let's turn to page 12, please. And I want to focus on page 32. Dr. Romesberg, take a moment it read this paragraph to yourself and let me know when you're done.

A. I'm done.

Q. So Dr. Kuriyan measured the width of the allyl capping group by measuring the distance from atom C to atom D, both of which are hydrogens; correct?

A. I believe that's correct, yes. That's what he says.

Q. And, again, that measured distance closely aligns with Dr. Ju's reported diameter of the allyl in Dr. Ju's declaration; correct?

A. I believe so.

MR. WALTER: Hold, on hold.

Objection, lacks foundation, incomplete hypothetical, vague, calls for speculation.

Q. So Dr. Ju -- Dr. Kuriyan showed that Dr. Ju's measured diameter for the allyl included the hydrogens?

MR. WALTER: Hold on.

Objection, lacks foundation, calls for speculation.

A. That would be speculative because in my reading Dr. Ju's model would appear not to call for hydrogens.

Q. I'm asking about Dr. Kuriyan.

A. Dr. Kuriyan is using hydrogens, yes.

Q. And his measured diameters that he's reporting included those hydrogens?

A. Yes, that's what he describes. That's if you equate width with diameter as he suggests.

Q. So if I could turn to your declaration, paragraph 41. And if you focus -- you can read the whole thing, but if you just focus on the last sentence, it starts with:

"In addition...."

It's your position -- is it your position that Dr. -- strike that.

When you say Dr. Ju appears not to have considered hydrogens, you're referring to his analysis of Pelletier and the active site; is that right?

A. Yes because I believe that's where he generated his model from.

Q. You're not referring specifically to Dr. Ju's measurements of the capping groups he reports in the declaration?

A. I'm referring to, as I say, the 3.7 angstrom diameter that he reports.

Q. Okay.

Let's go back to Dr. Ju's declaration. And Exhibit C. That we were looking at before.

And let's focus on this paragraph about eight lines down there's a sentence that begins with:

"This space can only...."

It's toward the end. Do you see eight lines down:

"This space can only...."

Do you see where that sentence is, Doctor?

A. Yes, I do.

Q. It says:

"This space can only accommodate a capping group of limited diameter on the 3 prime position of the deoxyribose of the nucleotide.

Dr. Ju was using the term "diameter" to describe the available space in the active site; right?

A. He appears to be, yes. That's how I read that.

Q. And is it your opinion that Dr. Ju was describing a sphere when he uses the term "diameter"?

A. He doesn't -- he doesn't use the word "sphere." He doesn't provide any geometrical description. The only -- the only teaching he provides, the only -- the only hint that he provides is he provides a diameter. And like -- like I mentioned earlier, there's only -- in three dimensions there's only one geometrical object that has

the diameter that only needs a diameter for a full description. And when I read this, I -- as I think a lot of -- most people would have, I immediately considered a sphere because it was then fully described. And the sphere would be a sort of first order approximation as to what you might consider when you were thinking about space.

Q. And Dr. Kuriyan did an analysis of what Dr. Ju meant by "diameter," and he was able to reproduce Dr. Ju's diameter measurements to mean the width of the capping group; correct?

A. Well, I think we're talking about two different things.

What I'm looking at here is a description of the space within rat polymerase data. What Dr. Kuriyan is opining about is the diameter of the protecting groups. And so those are two different things.

Q. So you're suggesting that Dr. Ju was using the word "diameter" in different ways in this declaration?

A. No, I'm not necessarily

suggesting that at all. I think that -- I think that he -- he -- it's very dif- -- I don't want to too negatively critique him because I don't actually understand the model. The only -- the most significant critique I would have is that he just doesn't provide sufficient data to really understand what he means. You know, you see it right there. He says the space has a diameter. Okay. I'll take that as a sphere. I'll give him that.

And then I think that later when he's talking about the diameter of a molecule that's where I begin to have a significant issue in really understanding what he means.

I can make a good guess as to what he means with the diameter to describe the space. That's easy. You can -- there's a geometrical object that is described by diameter. And so I -- I assume that's what he's talking about.

And, again, I apologize but I -- it would not be typical to describe a molecule as having a diameter unless it --

unless you're describing it as in sort of a spherical thing. And so I just don't know how to describe the diameter.

Now, I'll -- Kuriyan provides a description of diameter. And if you wish for me to discuss Kuriyan's interpretation of the use of that term, I'm happy to. But I -- I just don't have enough to offer what -- what Ju is describing in comparing the two diameters that he uses, if that's clear.

Q. Let's go back to Dr. Kuriyan's declaration, please, Romesberg Exhibit 2, and let's look at the allyl on page 22. I think we've got it up on the page.

Using this illustration, Dr. Kuriyan was able to determine that the diameter of the allyl that matched Dr. Ju's calculated diameter for allyl -- strike that. I think I left a word out. Let's start over.

Using this illustration Dr. Kuriyan was able to determine --

MR. SCHWARTZ: I have an echo here. You hear me okay, everybody?

about is the carbon not the oxygen. So you have to extend it out by one so the length will increase somewhat. So that would make it -- even in the particular conformation that Dr. Kuriyan is drawing, that would make the length even longer relative to the -- to the width, as he's describing it. So that would seem to be even increasingly less well approximated by a sphere and -- and leaving really only a cylinder if you're endeavoring to describe this molecule in this particular conformation with a three-dimensional object.

Q. Let's look at Dr. Kuriyan's illustration of MOM on page 13. And using that structure, Dr. Kuriyan was able to determine that the diameter of MOM matched Dr. Ju's calculated diameter for MOM that he reported in his declaration; correct?

A. That is what --

MR. WALTER: Objection, lacks foundation, calls for speculation.

A. That is what Dr. Kuriyan reports.

Q. And in Dr. Kuriyan's structure,

MOM is not a sphere?

A. It would look difficult -- it would -- same answer as -- as previously. Especially if you attach it to a carbon, it is looking to me if you wish to -- if you wish to ascribe a geometrical object, if you wish to use a geometrical object to describe these protecting groups, as you get longer, as your length gets longer, as one dimension gets increasingly long relative to the other two, I think it's common sense that that becomes a better -- that would be better described by a cylinder as opposed to a sphere by definition.

Q. And let's look at Dr. Kuriyan's illustration of methylthiomethyl. And using that structure, Dr. Kuriyan was able to determine that the diameter of the methylthiomethyl matched Dr. Ju's calculated diameter for methylthiomethyl that he reported in his declaration; correct?

A. Using the specific --

MR. WALTER: Objection, lacks foundation, calls for speculation.

A. Using the measurement from C to

A. There's nothing inconsistent.

THE COURT REPORTER: I'm sorry, was there an objection.

MR. SCHWARTZ: Okay. I'm ready to move on.

MR. WALTER: Yes, there was an objection.

THE COURT REPORTER: Repeat the objection.

MR. WALTER: Actually, Dr. Romesberg, make sure you give me a moment to object.

THE COURT REPORTER: And I still didn't get the objection.

MR. WALTER: It was an objection, vague.

THE COURT REPORTER: Thank you.

BY MR. SCHWARTZ:

Q. Let's talk a little bit about a cylinder, Dr. Romesberg. A cylinder has a diameter; right?

A. Yes.

Q. And the diameter of a cylinder is the cylinder's width; is that right?

A. At least as commonly used, yeah,

that's right.

Q. And a cylinder also has a length?

A. That's right.

Q. So it's not wrong to say that a diameter is a partial description of a cylinder; correct?

A. It's a partial or incomplete -- yeah, sure, that's correct.

Q. Let's turn to your declaration, paragraph 31. That's Romesberg 1. And, again, you know, if you have it in front of you, feel free. We'll put it up on the screen.

And please go to the last sentence that starts with:

"A POSITA...."

MR. SCHWARTZ: Zach, can you fit that all within the --

BY MR. SCHWARTZ:

Q. Do you see that last sentence that starts with:

"A POSITA..."?

A. No, the last sentence starts with:

THE WITNESS: Whatever you guys want. I'm okay.

MR. SCHWARTZ: Okay.

Let's turn to page 12 of your declaration.

BY MR. SCHWARTZ:

Q. And I would like to start with paragraph 33, which we've put up on the screen. And if you want to go down line -- to line 4, there's a sentence that starts with:

"A POSITA would understand...."

Do you see that?

A. Yes, I do.

Q. And you write:

"A POSITA would understand that occasionally, a crystal structure reveals a 'tunnel-like' structure through which an object can extend in an unrestricted fashion, and...thus removes concerns about the accommodation of the length of the object, leaving only restrictions on the object's width and height."

You wrote that; right?

A. Yes, I did.

Q. You agree that polymerases can have tunnel-like structures through which an object can extend in an unrestricted fashion?

A. I believe that it would be rare. In general there have been polymerase structures solved, including rat polymerase beta, and there are no such tunnels. It is possible. But there's not one in rat pol beta, and it's certainly possible there could be.

Q. And tunnels are generally cylindrical; is that right?

MR. WALTER: Objection, vague.

A. You can approximate a tunnel as a cylinder.

Q. And the width of the tunnel would be its diameter; correct?

A. The width of the tunnel --

MR. WALTER: Objection, vague.

A. If you're describing the tunnel as a cylinder, then width could be used to describe its diameter, yes.

Q. And in those instances whether an object would fit into the active site

would be determined by the width of the object and not the length; correct?

A. As I state, such a tunnel would eliminate the restriction of length. So in that case that would leave only the width as the determining factor for these shape arguments. So yes.

Q. You say that you've read the Pelletier paper, if I remember correctly; right?

A. I have.

Q. And the Pelletier paper reports the crystal structure of a DNA polymerase; correct?

A. Rat polymerase beta. That is correct.

Q. Would a POSA -- you know what I been by POSA; right? A person of ordinary skill in the art? -- as of 2000 know that the active site of a polymerase is not infinite in size?

A. That is correct.

Q. Are polymerase active sites perfect spheres?

A. No.

protruding.

I'm going to mark another exhibit. It will be Romesberg 5.

[Deposition Exhibit 5 marked for identification.]

MR. GARRETT: I am sending Romesberg 5 by email now, and I will also share it on the screen.

BY MR. SCHWARTZ:

Q. Dr. Romesberg, in Romesberg 5 we took your figure E2 except that we added the hydrogens. Do you have any reason to doubt that this is an accurate re-creation of the model you presented in figure E2?

MR. WALTER: Objection, lacks foundation, calls for speculation, incomplete hypothetical.

A. I have -- if you tell me that's what you did, then I have no doubt that's what you did.

Q. Okay.

But I'm asking you whether or not you have any reason to doubt that this is an accurate re-creation of your model with the hydrogens added.

A. It's a straightforward thing to do, and if that's what you did then I'd believe you. Then I think it must be accurate, sure.

Q. And you see the hydrogens of the allyl protrude outside the sphere; correct?

A. Yes. And only the hydrogens. And only two of them.

Q. And it's almost the entirety of the hydrogens that protrude. In fact in one case it is the entirety of the hydrogens. Do you agree?

A. So I believe -- so in this particular case there appears to be a hydrogen that is substantially out of the sphere. That is right.

Q. When you made your model E2, did you make a model with the hydrogens?

A. I did.

Q. And do you have -- do you have that? Do you have any files or documents or notes showing that model, having that model?

A. No, I do not.

Q. And you don't provide any details regarding that model with the

Q. How far does the nitrogen protrude from the sphere in this figure?

A. Well, that's the same problem that I described earlier when I was looking at the hydrogens. This program doesn't give you that sort of resolution.

What's clear is that in this case there's a significant amount of the -- of the nitrogen sticking out. And --

Q. How far?

A. I can't -- that's not available in the program. You can't measure that.

Visually I had an intuitive feel, and I believe in my deck I said "significant perturbation," and I -- I believe I described the hydrogen penetrations as minor.

Q. I'm asking you to put some meat on the bones there. Can you tell me what -- what's the difference between significant and minor in terms of a metric?

A. So the vast majority of the hydrogens, even I think in the representation even that you showed, just showed sort of the tip of the stick sticking

out.

Now, this is a horribly inaccurate mechanism. I'll give you some meat but I can't speak to the quality of it. Because these programs just don't -- aren't intended to do this.

The majority of the hydrogens were -- the bulb was just sort of sticking out a little bit with the majority. And the nitrogen here, in all of the conformations, seemed to me to be significantly more. That's what -- this is why I showed illustrations.

Q. But we looked at -- we looked at my representation of the allyl, your figure with the hydrogens, and you agreed that in one of them the entire hydrogen was sticking out; right?

A. Yeah. So I said that the majority of the hydrogens were just peeking out. And the one conformation that you showed -- which I think might have been the most exposed hydrogen of all of them, although I'm not sure. But -- but yeah --

Q. And how much -- I'm sorry. I

top of your sphere is restricted by the nucleotide; correct?

A. Yes.

Q. And the bottom of the sphere is restricted by an amino acid of the polymerase; is that right?

A. Yeah, I guess that would be one way to describe it.

Q. And in your figure D you don't show any structure restricting the available space to the left of your sphere, do you?

A. There was nothing that impinged into the sphere. I chose to show what I chose because, as Pelletier describes in the rat polymerase beta structure which Ju was using, the shortest contact was 3.2 angstroms, and I wanted to show that contact. And so that's the only contact I showed.

But as Pelletier points out and is implicit in this representation, there aren't closer amino acids or parts of the polymerase. And none of them -- none that I found impinged within that sphere, I believe.

Q. So if I understand your testimony correctly, there is additional available space both to the right and left of that sphere?

A. The space is not perfectly described by a sphere. So yes.

My point was -- and I believe what I said was that a sphere fits into this space and describes it fairly well. If you're going to use a sphere, 3.7 is about the diameter that you would have chosen.

MR. SCHWARTZ: I'm going to mark another exhibit. It's going to be Romesberg -- what am I up to, Romesberg 6? It's going to be an illustration of the polymerase active site.

For -- for Zach's -- for, you know, Zach's understanding of what to put up, the next illustration is going to be -- exhibit is going to be Romesberg 6, I believe it is 6 of -- Zach, you can tell me if I'm wrong with the numbering, and it's going to be the Pelletier active site and where

break.

MR. WALTER: Okay. Thanks, guys.

MR. SCHWARTZ: All right.

THE VIDEOGRAPHER: It is 11:25. We're off the record.

[Recess at 11:25 a.m.]

[Resuming at 11:53 a.m.]

THE VIDEOGRAPHER: All right. It is 11:53 a.m., and we're on the record.

EXAMINATION CONTINUING

BY MR. SCHWARTZ:

Q. Dr. Romesberg, I'm going to mark as my next exhibit Romesberg 9. Do you recognize Romesberg 9 as a paper from your laboratory or from your group?

A. I do.

Q. And essentially this is -- this paper is about DNA in the broadest sense; right?

A. In a very broad sense, yes. Yes.

Q. And DNA usually occurs in a double helix; correct?

A. Very often, yes.
Q. Okay.
Can we turn to page 4 of the paper? And there's a paragraph, the first one that starts with:
"The structures...."
Now, if you go down about, I don't know, ten lines to where it says "Supporting Information"?
MR. SCHWARTZ: Zach, can you just make that a little bigger for my old eyes?
Q. Do you see where it says "Supporting Information," Doctor?
A. I do.
Q. And then -- and then it's written here:
"Analysis of the four ordered duplexes using the 3DNA package" -- and it has a reference number -- "revealed a right-handed B-form DNA conformation with a mean helix diameter of 19.9 angstroms, consistent with standard Watson-Crick base pairing (except at the 5 prime and 3 prime ends of the duplex, where the nucleobases

intercalate into neighboring duplexes in the crystal to form two sets of semi-continuous helices)."

Do you see that sentence?

A. I do.

Q. And you use the term "diameter" in this sentence; correct?

A. I do.

Well, so to be clear, I wrote the majority of this paper, but there are collaborators on this paper. And this particular part that we're looking at is referring largely to their data. But I probably wrote this or was involved in writing this at least, and I do see where the word "diameter" is there.

Q. And "diameter" as used here is in reference to the width of the DNA duplex; correct?

A. It appears -- it says a helix diameter.

Q. That refers to the width of the helix; right?

A. Well, it's the diameter.

Q. Which means the width. It's not

its length you're referring to. You're referring to its width; correct?

A. We -- we say -- we say -- sorry, where is this? We say "diameter" but I think that in the common use of the word "width" that you're describing, I think that would -- I don't think I would object to calling it that.

MR. SCHWARTZ: Okay. Dr. Romesberg, thank you very much. I have no further questions.

THE WITNESS: Okay.

THE COURT REPORTER: Derek, you're muted.

You're still muted.

MR. WALTER: All right. Am I unmuted now?

THE COURT REPORTER: Yes.

MR. WALTER: Sorry.

Why don't we go to the breakout rooms and we can talk for a second. And then we'll come back. We won't need too much time I don't think.

THE COURT REPORTER: Okay.

THE VIDEOGRAPHER: All right.

paper that I published in -- that I am corresponding author on in 2007?

Q. Yes.

A. That paper? Yes.

Q. Yeah.

Did your reference to diameter in that article have anything to do with the space available in the active site of rat polymerase B?

A. No, there was no polymerase at all there.

MR. WALTER: Okay. I don't have any further questions.

MR. SCHWARTZ: Neither do I.

Thank you very much for your time, Dr. Romesberg. You're a free man now. You have the rest of your day.

THE WITNESS: Thank you.

MR. SCHWARTZ: Nice to see you again.

THE WITNESS: All right. It's nice to see you, Bob.

MR. SCHWARTZ: Okay. Bye-bye.

[Deposition concluded at

p.m.]

[Deposition Exhibits 1 through 9 marked for identification and attached to the original deposition transcript.]

[Signature reserved.]

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C E R T I F I C A T E

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I, PAUL J. FREDERICKSON, California Certified Shorthand Reporter No. 13164, do hereby certify:

That prior to being examined, the witness named in the foregoing deposition was by me remotely sworn or affirmed to testify to the truth, the whole truth and nothing but the truth;

That said deposition was taken down by me remotely in shorthand at, and thereafter reduced to print by means of computer-aided transcription; and the same is a true, correct and complete transcript of said proceedings.

I further certify that I am not interested in the outcome of the action.

Witness my hand this 16th day of July 2020.

PAUL J. FREDERICKSON, CSR

CA CSR 13164

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Efforts Toward Expansion of the Genetic Alphabet: Structure and Replication of Unnatural Base Pairs

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Abstract

Expansion of the genetic alphabet has been a long time goal of chemical biology. A third DNA base pair that is stable and replicable would have a great number of practical applications and would also lay the foundation for a semi-synthetic organism. We have reported that DNA base pairs formed between deoxyribonucleotides with large aromatic, predominantly hydrophobic nucleobase analogs, such as propinyl isocarbostyril (d**PICS**), are stable and efficiently synthesized by DNA polymerases. However, once incorporated into the primer, these analogs inhibit continued primer elongation. More recently, we have found that DNA base pairs formed between nucleobase analogs that have minimal aromatic surface area in addition to little or no hydrogen-bonding potential, such as 3-fluoro benzene (d**3FB**), are synthesized and extended by DNA polymerases with greatly increased efficiency. Here we show that the rate of synthesis and extension of the self pair formed between two d**3FB** analogs is sufficient for *in vitro* DNA replication. To better understand the origins of efficient replication, we examined the structure of DNA duplexes containing either the d**3FB** or d**PICS** self pairs. We find that the large aromatic rings of d**PICS** pair in an intercalative manner within duplex DNA, while the d**3FB** nucleobases interact in an edge-on manner, much closer in structure to natural base pairs. We also synthesized duplexes containing the 5-methyl substituted derivatives of d**3FB** (d**5Me3FB**) paired opposite d**3FB** or the unsubstituted analog (d**BEN**). In all, the data suggest that structure, electrostatics and dynamics can all contribute to the extension of unnatural primer termini. The results also help explain the replication properties of many previously examined unnatural base pairs and should help design unnatural base pairs that are better replicated.

Introduction

Expansion of the genetic alphabet to include a third base pair would be a fundamental accomplishment that would not only have immediate utility for a number of applications, such as site-specific oligonucleotide labeling, but would also lay the foundation for an organism with an expanded genetic code. Efforts toward this goal were first reported by Benner and coworkers[1], who designed nucleobase analogs to pair based on hydrogen-bonding (H-bonding) patterns that are complementary to each other, but not to any of the natural nucleobases. While these analogs have found practical applications and improvements continue to be reported, work from the Kool group has shown that H-bonds are not absolutely essential for polymerase-mediated base pair synthesis[2–6]. This work demonstrated that forces other than H-bonding

optimized within the purine-rich strand than the pyrimidine-rich strand, which is distorted by the buckling at the self pair.

The structures of two different duplexes containing the d**3FB** self pair were determined, one using X-ray crystallography and the other using NMR spectroscopy (Figure 4). Figure 4A shows the central section of the DNA duplex d($C_1G_2{}^{Br}C_3G_4A_5A_6$**3FB**$_7T_8T_9C_{10}G_{11}C_{12}G_{13})_2$ containing a single d**3FB** self pair as determined by X-ray crystallography with a resolution of 2.8 Å (Table 1 and Supporting Information). Six copies of the duplex are present in the crystallographic asymmetric unit. Four copies are well ordered (chains A-H) and well defined by the electron density. The remaining two copies are less well ordered (chains I-L), and characterized by diffuse electron density; however the density was successfully fit using the bromine atoms in the Patterson maps (Supporting Information). Analysis of the four ordered duplexes using the 3DNA package[36] revealed a right-handed B-form DNA conformation with a mean helix diameter of 19.9 Å, consistent with standard Watson-Crick base pairing (except at the 5′ and 3′ ends of the duplex, where the nucleobases intercalate into neighboring duplexes in the crystal to form two sets of semi-continuous helices). The root mean square deviation between the duplexes is 1.50 Å for backbone atoms and 0.84 Å for nucleobase atoms. The root mean square deviation between the average duplex and an ideal B-form duplex is 1.26 Å and 0.58 Å for sugar-phosphate backbone and nucleobase atoms, respectively. The d**3FB** nucleobases are oriented so that their fluorine atoms are positioned in the major groove of the duplex, separated by 9.8 Å (Figure 4B). At their closest approach, the nucleobase analogs are separated by an average carbon to carbon distance of 3.75 Å. This is slightly greater than the sum of the van der Waals radii (3.4 Å), which suggests that the nucleobase analogs are not optimally edge-to-edge packed. The unnatural base pairs adopt an average propeller twist of −12°, which is virtually identical to that of canonical B-form DNA. The mean distance between the d**3FB** nucleobase analog and the flanking natural nucleobases is 3.2 Å, which suggests that the analogs pack favorably with their flanking natural nucleobases. In fact, the only significant deviation from an ideal duplex geometry appears to be due to these stacking interactions, as the flanking natural nucleobases tilt in order to achieve optimal co-planarity with the unnatural nucleobases (Figure 4B).

Characterization of d($C_1G_2C_3$**3FB**$_4A_5A_6T_7T_8$**3FB**$_9G_{10}C_{11}G_{12})_2$ by NMR spectroscopy and NOE restrained MD simulations (Supporting Information) also indicates a canonical B-form DNA duplex as demonstrated by characteristic NOE connectivities and intensities (Figure 4C). The base-sugar connectivities along each strand are not interrupted at the d**3FB** self pair (Figure 4C), unlike the connectivities observed for the d**PICS** self pair which show clear breaks between the d**PICS** sugars and their 3′ neighbor base protons. In addition, imino to imino and imino to adenine H2 NOE connectivities are observed throughout the DNA helix except at the terminal base pair (data not shown). The similarity of all proton chemical shifts to those reported for the fully natural DNA duplex (containing a dG_4 and dC_9, Table S1 and Figure S1)[37] further demonstrates that both d**3FB** self pairs are accommodated within the double helix without substantial structural distortions.

Interestingly, the NMR data indicate that the d**3FB** self pair adopts multiple conformations that are related by simple ring flips about each C-glycosidic linkage (Figure 4C & D). This heterogeneity is demonstrated by NOE cross peaks from d**3FB**$_4$ H2 and H6 to dC_3 H2′/H2″, and from d**3FB**$_9$ H2 and H6 to T_8 H2′/H2″, respectively (Figure 4C), as well as by heteronuclear NOEs between the fluorine of d**3FB**$_4$ and both dA_5 H2 and dC_3 H6, which are mutually exclusive for a single ring orientation (Figure 4D). An NOE between the d**3FB**$_9$ fluorine and both dT_8 CH_3 and dT_8 H6, as well as between the H4 and H5 protons of the d**3FB**$_4$ further demonstrate that both nucleobase analogs undergo rapid ring flipping. Since single resonance lines are observed for the two fluorine atoms and for each aromatic proton, the rate of base flipping must be fast on the chemical shift time scale for these resonances, *i.e.* exchange

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner.

Case IPR2020-00988
Patent 10,407,458

DECLARATION OF FLOYD ROMESBERG, PH.D., IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 10,407,458

Illumina Ex. 1038
IPR Petition - USP 10,407,458

the same function it had been known to perform and yields no more than one would expect from such an arrangement, the combination is obvious.

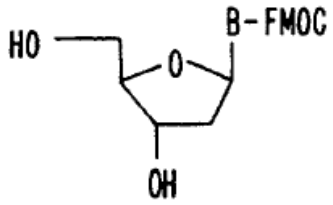
IV. THE PERSON OF ORDINARY SKILL IN THE ART

25. I understand that obviousness is analyzed from the perspective of a hypothetical person of ordinary skill in the art. I understand that October 2000 is the relevant time frame for analyzing the obviousness of the '458 patent. A person of ordinary skill in the art related to the '458 patent would have been a member of a team of scientists developing nucleotide analogues, researching DNA polymerases, and/or addressing DNA sequencing techniques. Such a person would have held a doctoral degree in chemistry, molecular biology, or a closely related discipline, and had at least five years of practical academic or industrial laboratory experience. Thus, a person of ordinary skill in the art includes a person having a doctoral degree in a field related to chemistry, and at least five years of laboratory experience directed toward the research and development of nucleotide analogues, DNA polymerases, and/or DNA sequencing. My opinions concerning the obviousness, as set forth herein, are from the perspective of a person of ordinary skill in the art, as set forth above.

V. THE '458 PATENT

26. The '458 patent (Ex. 1001) is directed to a "massive parallel method for decoding DNA and RNA." Ex. 1001 at Title.

27. The '458 patent has two claims:



a. <u>It was known that the 3′-capping group should be small</u>

57. Dower disclosed nucleotides having "small blocking groups" on the 3′-OH. Ex. 1030 (Dower) at 25:48-51 ("A second, unlabeled and reversible, set of terminators is also required. Examples of these compounds are deoxynucleoside triphosphates with <u>small blocking groups</u> such as acetyl, tBOC, NBOC and NVOC on the 3'OH. These groups are easily and efficiently removed under conditions of high or low pH, exposure to light or heat, etc.") (emphasis added). Dower also makes an additional reference to size, stating: "The synthetic scheme depends, in part, on the <u>stepwise elongation by small and identifiable units</u>." Ex. 1030 (Dower) at 14:47-48 (emphasis added). A large blocking group would have been less desirable given Dower's statement regarding "small" size.

58. Tsien also suggests the use of small blocking groups on the 3′-OH. Ex. 1031 (Tsien) at 26:17-27:1. In discussing the use of a 3' blocking group that also acts as a detectable label, Tsien "caution[s]" that one needs "to try to select fluorophores which are not so large and bulky that the labeled dNTP can not be incorporated readily into the growing DNA chain by a polymerase or similarly functioning enzyme." *Id.* at 26:31-35. Tsien then suggests using a fluorophore

fragment to "reduce size and minimize steric interference." *Id*. at 26:35-27:1. Thus, Tsien plainly evidences a concern about nucleotide incorporation by polymerase due to the size of the 3′-capping group and suggests avoiding "large and bulky" groups at this position.

59. The crystal structure of a typical DNA polymerase was published in 1994 by Pelletier. Ex. 1044 (Pelletier) at 1897 (Table 3); *id.* at 1903, note 101 ("Full coordinates for both ternary complex structures are available from the Brookhaven Protein Data Bank and are designated 1bpf and 1bpg for the $P6_1$ and $P2_1$ structures, respectively."). Pelletier's publication would have been relevant to the methods of Dower and Tsien.

60. Before the year 2000, skilled artisans routinely considered the three-dimensional structure of enzymes in order to better understand the type of substrates that might be used. For example, when I carried out work designing non-natural nucleotides in the late 1990s, I examined three-dimensional structures of DNA polymerases, and from those structures I understood that the space in the active site was limited. This was understood to be at least partially responsible for the specificity of polymerases that use deoxyribose substrates over ribose substrates. The difference between these two substrates (dNTPs and NTPs) is a single oxygen atom at the 2′ position. Based on this ability to discriminate between very similar

substrates, a skilled artisan would have understood that there was limited space at the polymerase active site.

61. This understanding is confirmed by a variety of publications that directly addressed the possibility of using 3′ hydroxyl modifications. For example, Dower (Ex. 1030) recognized that the 3′ hydroxyl must be protected with small chemical structures. Ex. 1030 (Dower) at 25:48-51. Welch and Burgess (Ex. 1033) evaluated the crystal structure of the T7 polymerase and determined that large chemical structures at the 3′ position cannot fit within the active site of the polymerase. Ex. 1033 (Welch) at Abstract. Likewise, Stemple (Ex. 1099) explained that the space available at the 3' hydroxyl may limit the groups that could be attached to the 3' hydroxyl. Ex. 1099 (Stemple) at 22:64-67.

62. DNA polymerases are at the heart of SBS, and, in my opinion, a skilled artisan in the year 2000 would have wanted to take advantage of any three-dimensional crystal structure regardless of who published it. Three-dimensional structures determined using x-ray crystallography have been reported since the 1950s. A three-dimensional crystal structure provides a clear picture of the arrangement of non-hydrogen atoms in a protein. Sometimes three-dimensional protein structures are obtained with a substrate bound, which helps to further define the active site where substrate binding and enzymatic catalysis takes place. Such three-dimensional structures help a skilled artisan to understand how the substrate is

XI. CONCLUSION

283. For the reasons described above, a person of ordinary skill in the art would have found Claims 1 and 2 of the '458 patent obvious over (1) Tsien in view of Prober and Hiatt and (2) Dower in view of Prober and in further view of Hiatt. Additionally, my opinions and prior testimony concerning the obviousness of the claim in U.S. 9,725,480 have not changed.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: 5-26-20 By: _____
Floyd Romesberg, Ph.D.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK and QIAGEN SCIENCES, LLC, Plaintiffs,	)	Civil Action No. 19-1681-CFC
v.	)	
ILLUMINA, INC., Defendant.	)	

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REMOTE ORAL AND VIDEOTAPED DEPOSITION OF

JOHN KURIYAN, PH.D.

JULY 10, 2020

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REMOTE ORAL AND VIDEOTAPED DEPOSITION OF JOHN KURIYAN, PH.D., produced as a witness at the instance of the Defendant, and duly sworn, was taken remotely in the above-styled and numbered cause on the 10th day of July, 2020, from 8:16 a.m. Pacific Time to 12:40 p.m. Pacific Time, via Zoom, before Julie C. Brandt, RMR, CRR, and CSR in and for the State of Texas, reported by machine shorthand, with the witness located in Berkeley, California, pursuant to the Federal Rules of Civil Procedure and the provisions stated on the record or attached hereto.

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ALSO PRESENT:

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Madison Bower - Weil summer associate

VIDEOGRAPHER:

Daniel Burke - Lexitas

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P R O C E E D I N G S

THE VIDEOGRAPHER: We are now on the record. My name is Daniel Burke. I am a videographer retained by Lexitas Reporting. This is a video deposition for the United States District Court, District of Delaware. Today's date is July 10, 2020, and the video time is 8:16 Pacific a.m.

This deposition is taken in the matter of The Trustees of Columbia University, et al. versus Illumina, Inc.

The deponent is John Kuriyan, Ph.D. All counsel will be noted on the stenographic record.

The court reporter is Julie Brandt and will now swear in the witness.

JOHN KURIYAN, PH.D.,

having been first duly sworn and having confirmed that he is John Kuriyan, Ph.D., testified remotely as follows:

EXAMINATION

BY MR. REINES:

Q. Please state your name and address for the record.

A. Are you asking me?

Q. It will be directed to you.

A. John Kuriyan, K-U-R-I-Y-A-N, 622 San Luis,

L-U-I-S, Road, Berkeley, California 94707.

Q. And you prepared a declaration in this case. Is that correct?

A. Yes, I have.

Q. And you executed that on June 1 of 2020. Is that correct?

A. I haven't memorized the date. So I can look at it, if you wish.

Q. Okay. Have you ever had your deposition taken before?

A. Yes.

Q. How many times?

A. I couldn't tell you how many times. I have read it many times, both in preparation and after this time.

Q. No, no, no. This question is different.

How --

A. Sorry.

Q. Have you had your deposition taken before?

A. Yes. Not in this case, in other cases.

Q. How many times?

A. Twice.

Q. What were the situations?

A. They were both cases related to intellectual property, and the depositions occurred -- the most

process is?

MR. SCHWARTZ: Objection, calls for legal conclusion.

A. I realize claim construction is a legal -- legal framework that often is -- a legal term that is often important in the early stages of a case such as this, but beyond that, I have no idea what goes into it.

Q. (BY MR. REINES) Do you have any idea what the methodology is for performing claim construction? Or let me start again.

Do you have any idea what the methodology is for opining on claim construction?

MR. SCHWARTZ: Objection, calls for a legal conclusion, outside the scope of Dr. Kuriyan's declaration.

A. I have absolutely no idea. I have not discussed it with anyone.

Q. (BY MR. REINES) Have you made any attempt to understand the claims of the patents-in-suit?

MR. SCHWARTZ: Same objections.

A. No, I have not. I was asked to opine on a specific issue, and I did that without trying to understand the claim construction issues here.

Q. (BY MR. REINES) My question was a little different --

many claim construction terms there are, for example, in this case.

Q. (BY MR. REINES) Yeah, that's not really my question. Let me try my question again, and try to listen to it carefully.

Do you have an understanding of the general subject matter of the claims of the patents at issue in this case?

MR. SCHWARTZ: Same objections, calls for legal conclusion, outside the scope of Dr. Kuriyan's declaration.

A. My analysis was very limited in scope, and in particular, I was not asked to and I did not analyze the claims in this case.

Q. (BY MR. REINES) Now one of the opinions that you have in this case is about the level of ordinary skill in the art. Is that correct?

A. That is correct.

Q. And in terms of the level of ordinary skill in the art, what considerations did you make to define that?

A. I had reviewed the matters that I say that I reviewed, and I -- based on my experience, I formed a conclusion as to what the person of ordinary skill in art -- a person of ordinary skill in the art would look

state biochemistry, which is a field that includes enzymology.

Q. (BY MR. REINES) Do you think one way or the other whether the level of ordinary skill in the art involves knowledge of enzymology?

A. Somebody with the training I have specified would have some knowledge of enzymology, but I didn't consider enzymology particularly or specifically in forming my definition.

Q. Would a person of ordinary skill in the art, with the level of ordinary skill in the art that you've identified, be sufficiently educated that they would understand the Pelletier article?

A. Understand, of course -- understanding and science occur at many levels, but I would expect a person of ordinary skill in the art to be able to read and understand at least parts of the Pelletier article.

Q. Are there parts -- well, which parts of the Pelletier article do you believe a person of ordinary skill in the art may have difficulty understanding?

A. I did not consider the Pelletier article in terms of relying on it to form my opinions, and so I do know from my reading of it that it concerns more than one complex topic in structural biology, biochemistry and chemistry. So if you wish me to identify things

that are more challenging than the other -- than others, I would have to review the article. I haven't relied on it, as I said, so I haven't marked it up in that way.

Q. In preparing your opinions in this case, did you consider the Pelletier article?

A. I had stated earlier the items that I reviewed, and the Pelletier article was -- if I remember correctly, it's in the -- it's either in the Ju declaration or some aspect of the prosecution history. So I reviewed the article, yes, but I did not rely on it for the conclusions I reached in my declaration.

Q. When you read the Pelletier article, did you understand it?

MR. SCHWARTZ: Objection, vague.

A. Here is how I would answer the question. I read the Pelletier article without any impediment to understanding, but I focused on issues that were relevant for the testimony I gave in this matter, and I did not rely on any aspect of the Pelletier article for the testimony that I gave.

Q. (BY MR. REINES) Which parts of the Pelletier article did you consider to be relevant?

A. Relevant to the opinion I gave in my declaration, I did not consider the -- any aspect of the Pelletier article to be relevant to the specific items

that I opined on in my declaration.

Q. Would a person of ordinary skill in the art be familiar with protecting groups?

A. A person of ordinary skill in the art, as I have defined it, would understand what a protecting group meant, yes.

Q. Would a person with the level of ordinary skill in the art that you've opined on in this case be familiar with polymerases?

A. Yes, a person of ordinary skill in the art as I have defined it would understand what a polymerase meant.

Q. And in 2000, at the time that the application was submitted, October 6th, what would be the polymerases that a person of ordinary skill in the art would be familiar with?

MR. SCHWARTZ: Objection, outside the scope of Dr. Kuriyan's declaration.

A. I understood that I could form my opinion on the matter as asked to study and render an opinion on without considering polymerases as a group, and I did not carry out a reasoned exercise in understanding knowledge 20 years ago concerning polymerases. That is, I didn't attempt to achieve a clear understanding of time with respect to research advances in that field.

Q. (BY MR. REINES) As part of your opinions in this case, did you consider what a person with the level of ordinary skill in the art would know about sequencing by synthesis?

A. Once again, I determined that to render an opinion, I did not need to consider the process and methodologies underlying sequencing by synthesis. So I did not position myself in the year 2000 and survey the relevant literature to understand what they would know.

Q. Do you have any idea at all whether any of the protecting groups referenced in your declaration would actually fit so -- such that they could successfully serve as protecting groups in a sequencing by synthesis process?

MR. SCHWARTZ: Objection, outside the scope. When I make my scope objections, I am including reference to the order.

A. I made no analysis of whether a protecting group of any kind would fit within the polymerase, and so I did not form an opinion about the ability of a protecting group to function, if that's what you're asking me, in sequencing by synthesis.

Q. (BY MR. REINES) Would a person with the definition of level of ordinary skill in the art that's the subject of your opinion understand that protecting

fact, all the capping groups -- sorry -- without exception, the most common confirmation helped me understand the measurements. For some, I looked to see what would happen if I generated alternative confirmations, but I don't remember which ones I analyzed that way.

Q. Do you have any documentation of the different confirmations that you considered for your work in this case?

A. No, because they are generated by computer graphics programs, and the only ones I recorded are the ones that I recorded in my declaration, because they immediately helped me understand what I was seeking to understand and, therefore, the ones that I manipulated, I didn't record.

Q. And in terms of the different confirmations that you used for your analysis of the dimensions of the protecting groups, did you take into account anything relating to sequencing by synthesis in choosing which confirmation you would use?

MR. SCHWARTZ: Objection to scope.

A. No, I did not.

Q. (BY MR. REINES) Before, you referred to the most common confirmations. Do you remember that testimony generally?

A. I don't remember the exact words. It could be read back, but I meant the lowest energy confirmation.

Q. Did you understand that during actual sequencing by synthesis, the confirmations of protecting groups are not necessarily the lowest energy confirmations?

MR. SCHWARTZ: Objection to scope. Ed, this is completely outside the realm of his declaration. I mean, you're just ignoring the Judge's order. You're asking him questions about sequencing by synthesis. There's nothing in there about sequencing by synthesis. I am just making these objections. I am asking you to stay within the scope of the Judge's order.

MR. REINES: If you think that's a serious objection, then we have a serious difference of agreement about the scope of opinions and what's appropriate and what's not. That's all I can say.

MR. SCHWARTZ: I will continue to make my objections.

Q. (BY MR. REINES) Dr. Kuriyan, did you take into account whether in sequencing by synthesis reactions the lowest energy confirmation is what will apply --

A. No, I did not, because I was looking to understand what Dr. Ju meant by his measurements, and I

stopped when I understood that.

Q. For your opinions in this case, did you take into account that bonds can bend and rotate leading to different confirmations?

A. I just want to be clear that -- let me state an opinion because I think -- I am not sure I understand your questions. Bonds do not bend and rotate to an appreciable extent for the kinds of analyses I was doing, so I did not explicitly consider changes in bond length or changes in bond angle.

Q. (BY MR. REINES) Did you consider that implicitly?

A. No.

Q. Okay. Now a couple of times, or at least once, you've stated that chemical bonds don't -- can't involve bending or rotation. Do you believe that to be just in terms of what your analysis was, or do you think in the real world that's true?

A. So what I said is chemical bonds -- by that, I mean the length between nuclei of atoms, and bond angles, the angles between bonds, what I said is they do not change appreciably.

Q. Okay. And so when you described a molecule as involving different confirmations, which you've testified about multiple times, how is it that a

A. I didn't actually understand what the question was to me, so I will answer it if you would just restate it.

Q. (BY MR. REINES) Fair enough.

Based on your work in this case, you don't have any reason to contest defendant's proposed construction of small that includes the requirement that the chemical group fit within the rat DNA polymerase active site shown in figure 1?

MR. SCHWARTZ: Asked and answered.

A. I did not reach an opinion on this matter.

Q. (BY MR. REINES) All right. If you would turn to paragraph 22, please. And do you see it states that the active site of the benchmark polymerase is part of the paragraph? Do you see that phrase?

A. Yes.

Q. Okay. In terms of what the benchmark polymerase is that you refer to there, you understand that to be the rat DNA polymerase shown in figure 1. Correct?

A. Yes, I do.

Q. Whether allyl, MOM or azidomethyl fits within the active site of the benchmark polymerase, that's not something that you've opined on at all, correct, or considered?

A. That's correct.

Q. Do you state in your declaration that you selected the confirmations based on the lowest energy?

A. I don't believe I do. My purpose was to understand what measurements Dr. Ju was referring to. I was able to achieve that with the confirmations that I illustrated.

Q. And in terms of how Dr. Ju did his calculations, do you know whether he was using the confirmations that he would expect would apply in a sequencing by synthesis reaction or whether he was assuming the confirmations of the lowest energy?

A. I have no opinion of what Dr. Ju was attempting to do. I do know what he did.

Q. And when you say you know what Dr. Ju did, did you discuss this with him?

A. No.

Q. Do you understand he works for Columbia?

A. I understand that from material in the patent prosecution history.

Q. Did you --

A. Not -- sorry, not directly.

Q. Sorry about that.

Did you request to discuss with Dr. Ju what he was attempting to do?

A. No.

Q. Now you testified you know exactly -- would you say -- well, let me ask the question.

Do you believe you know exactly what Dr. Ju did in his measurement calculations?

A. I believe I know what Dr. Ju did in his measurement calculations, yes.

Q. Did you evaluate what the available space was in the rat polymerase as part of your work in this case?

A. No.

Q. If we could turn to Exhibit C of the Ju declaration, please. Let me know when you have that in front of you.

MR. SCHWARTZ: Would you be able to put that up on the screen, Ed, for the share?

MR. REINES: Sure. Andrew is the person taking care of that, but I am sure he will do it.

MR. SCHWARTZ: Thank you.

MR. REINES: No problem.

Q. (BY MR. REINES) Now in terms of Dr. Ju's calculations, do the figures in here have the Phe272 or Tyr271?

MR. SCHWARTZ: The document speaks for itself.

Q. (BY MR. REINES) In terms of the residues?

you assume what you're calling the lowest energy, did they take into account the solvent that you have in a sequencing reaction?

MR. SCHWARTZ: Objection to scope, requires foundation.

A. The measurement -- measurements that I made pertained to the molecule alone.

Q. (BY MR. REINES) Gas phase?

MR. SCHWARTZ: Same objections.

A. They're -- they refer to the fundamental properties of molecule no matter what phase it is at, so I don't characterize it as gas phase.

Q. (BY MR. REINES) Did you include the hydrogens in the protecting group when you --

A. Yes, I did.

Q. And how did you include them? Please describe that.

A. All of the molecules, all of the components of the molecules, carbon atoms, oxygen atoms, whatever, hydrogens, their positions of space are described as I have set forth in detail in my declaration. And their positions are calculated from the known interatomic bond lengths, bond angles and what are called dihedral lengths.

Q. As part of Dr. Ju's calculations, do you

believe that he determined the available space in the rat polymerase?

MR. SCHWARTZ: Objection to scope. Objection, calls for speculation.

A. I did not analyze or consider in detail whatever Dr. Ju may have done outside analysis of structures of the cabin groups specified in my declaration.

Q. (BY MR. REINES) Could you tell from Dr. Ju's declaration whether he considered the available space in the rat polymerase as part of his analysis?

MR. SCHWARTZ: Objection, vague. Objection, scope, calls for speculation.

A. Are you asking me -- when you say analysis, are you referring to the interatomic distances of the cabin groups?

Q. (BY MR. REINES) Just the available space. I mean, however you want to understand that from a chemistry perspective.

A. Yeah, I did not focus on the available space and, therefore, what's obvious is what's in the documents that are in front of us or in the materials I considered, but I did not analyze myself the available space or interpret what Dr. Ju might have meant by the available space in the polymerase.

Q. So in the Exhibit 3 to the Ju declaration that we're looking at in the second paragraph where it states the distances given in Pelletier et al. were used to calculate the available space around the 3' carbon of the deoxyribose ring of the nucleotide. It was determined that the diameter of the available space in the active site of the polymerase ternary complex is approximately 3.7 angstrom. Do you see that?

A. Yes.

Q. Did you do anything to -- did you consider at all in this case about how Dr. Ju came to the 3.7 angstrom calculation?

A. No.

Q. Do you have any idea how Dr. Ju reached the 3.7 angstrom calculation for the available space as described in Pelletier?

MR. SCHWARTZ: Objection, calls for speculation. Sorry, John. Calls for speculation.

A. I did not go beyond the statements made at the documents we see before me and associated text.

Q. (BY MR. REINES) In the bottom of the first paragraph, it states that Pelletier shows 3.2 angstroms between the 3' carbon of the deoxyribose ring and Phe272. Do you see that?

A. Yes.

Q. Did you attempt to understand how that calculation was performed?

A. No.

MR. SCHWARTZ: Objection, asked and answered.

MR. REINES: Someone must be in Fort Worth.

MR. SCHWARTZ: Or they just won the jackpot.

MR. REINES: Why don't we take a little bit of a break and, if people are fine, is bottom of the hour okay for resumption?

THE WITNESS: What's that mean, 11 o'clock my time?

MR. REINES: Well, we have different people at different time zones, but yes. So bottom of the hour is usually half an hour. Top of that hour would be the top, but I'll be specific. 10:30 Pacific and 1:30 Eastern.

THE WITNESS: Understood. Thank you.

THE VIDEOGRAPHER: Stand by, please. The time is 10:14 a.m. Pacific, off the video record.

(Break from 10:14 a.m. to 10:31 a.m.)

THE VIDEOGRAPHER: The time is 10:31 a.m. Pacific, back on the video record.

Q. (BY MR. REINES) When Dr. Ju evaluated Pelletier set forth in his declaration, did he consider the hydrogens distance calculations he performed?

MR. SCHWARTZ: Objection, calls for speculation, outside the scope.

A. All molecules, whether they are proteins or nucleic acids or small molecules, have hydrogens, and their positions are defined by the positions of the atoms to which they're attached. So I can't answer the question without knowing what specific aspect of Ju's analysis in the material I considered you're referring to.

Q. (BY MR. REINES) Do you have any idea how Ju came up with the 3.7 angstrom parameter based on his analysis of Pelletier?

MR. SCHWARTZ: Objection, asked and answered, calls for speculation.

A. I read what is stated in the materials I considered, but I didn't look at it beyond that or consider it beyond that.

Q. (BY MR. REINES) Okay. Do you have any opinions that you formed in this case regarding how Dr. Ju arrived at the 3.7 angstrom number based on Pelletier?

A. I don't have an opinion that is independent of

the statements made by Ju.

Q. When you say independent of the statements made by Ju, let's probe into that. One of the statements made by Ju is that he calculated the available space around the 3' carbon. Correct?

A. I am not looking at it, but I believe so, yes.

Q. Do you know how he calculated that?

MR. SCHWARTZ: Objection, asked and answered.

A. I did not analyze how he calculated the available space other than the statements he made about it, which I could look at but they're not in front of me right now.

Q. (BY MR. REINES) Okay. Well, let's put it in front of you.

A. Thank you.

Q. In terms of how Ju calculated the available space around this 3' carbon in Pelletier, do you know what he did based on what's here?

MR. SCHWARTZ: Objection, asked and answered, the document speaks for itself, outside the scope.

A. I didn't verify or check what he meant by the measurements that he records here on this page.

Q. (BY MR. REINES) In forming your opinions in

this case, did you take into account for those opinions how Ju calculated the 3.7 angstrom number based on Pelletier?

A. No.

MR. SCHWARTZ: Asked and answered.

Q. (BY MR. REINES) All right. Let me ask you questions now about your use of width. One of the opinions that you did make in this case was that you opined on -- that the parameter of -- let me state again.

You opined that the term diameter means width. Correct?

A. Not exactly. That's not what I opined.

Q. Okay. Let me get it in front of me.

All right. If you turn to paragraph 19 of your declaration, please. We can put that up for you.

MR. REINES: Andrew, are you putting that up?

MR. SCHWARTZ: Also, Dr. Kuriyan, feel free to look at your hard copy if that's helpful to give you context.

Q. (BY MR. REINES) All right. In paragraph 19, do you see where it says plaintiffs assert that the dimension is diameter, i.e., width?

A. Yes.

synonymous. Is that correct?

MR. SCHWARTZ: Objection, mischaracterizes the patent at issue in this case.

A. I am not sure. I haven't memorized the patents by any means, but I know that the term diameter is used by Dr. Ju. I am sure it's used in his declaration. I am not sure it's used anywhere in the patent. I don't recall.

Q. (BY MR. REINES) Let me do it this way then.

Do you believe the term diameter as used by Dr. Ju in his declaration is ambiguous?

A. After my analysis, no.

Q. When you say not after your analysis, do you believe that the term diameter is ambiguous in any sense?

MR. SCHWARTZ: Objection, vague.

A. I had said in my answer to your previous question that the term diameter requires context to understand what is meant. So I needed context, but I found the context in the Ju declaration.

Q. (BY MR. REINES) Do you have any explanation for why Dr. Ju used the term diameter rather than width?

MR. SCHWARTZ: Objection, calls for speculation.

A. I have no opinion.

A. No, that's not what I said.

Q. Well, do you believe it is clear or not that diameter means width in the context of this matter?

A. I believe that it's clear that diameter means width in the context of the patent prosecution history and the materials I considered, yes.

Q. And you didn't rely on Pelletier. Correct?

A. That's correct, I did not rely on Pelletier.

Q. And let me ask again. Do you have any explanation at all or even a working hypothesis as to why Dr. Ju used the term diameter, whereas you're saying the word width is more precise?

MR. SCHWARTZ: Objection, calls for speculation. Objection, asked and answered several times.

A. I would say that both diameter and width require context, and as to the question of why Dr. Ju used the term diameter, I have no opinion.

Q. (BY MR. REINES) Okay. And in terms of width, can you describe that, what the different meanings are of width as it relates to the protecting group?

A. I don't follow what the different meanings of width are as it relates to the protecting group.

Q. Okay. Width could mean different things in the plaintiffs' proposed construction. Correct?

Q. Withdrawn. I didn't do it right. Thank you for just holding back, because the court reporter can only take down one of us.

For the three or four different width calculations for each of the protecting groups, how did you decide which one you would use for your opinions in this case?

A. I didn't say at any point that there were three or four different width calculations. That was -- if I did, that was not my intention, but I don't believe I did.

I said that there are three or four internuclear distances that are roughly orthogonal, any of which are identical, and I looked to see which of those matched what Dr. Ju reported and would correspond to a widest dimension. So of the internuclear distances that are by distance of three or four, some of them are small and some of them are larger, and it looks like Dr. Ju took the largest dimension, but I reported the measurement that corresponds to what I believe Dr. Ju measured.

Q. So you believe Dr. Ju measured the longest dimension of the protecting group. Is that correct?

MR. SCHWARTZ: Mischaracterizes testimony.

And secondly, the line connecting C to B would not be largely perpendicular to the longest dimension of the molecule.

Q. (BY MR. REINES) And where did you get this parameter of selecting a width by virtue of being orthogonal to the longest dimension of the molecule?

A. I looked at the structures of the molecules and recognized that they had a longest dimension and, therefore, dimensions that were perpendicular to that longest dimension.

Q. Well, I mean according to the perpendicular, how come you're using this concept of a dimension that's orthogonal to the longest dimension? What's your principle basis for using that as a way to determine what diameter means?

A. I have been given the numbers that are in Dr. Ju's declaration as corresponding to the diameters of the molecules, and I can readily see, based on my knowledge of chemical principles, that these numbers are much smaller than the longest internuclear distances in the molecules. And, therefore, I started looking at internuclear distances that were roughly perpendicular to the first set of internuclear distances, and I immediately found the match to Dr. Ju's measurements.

Q. When you went, for example, in the allyl in

paragraph 32 from C to D, I mean, that dimension is not perpendicular or orthogonal to the longest dimension, is it?

A. No. I used the word roughly perpendicular or largely -- I think in the abstract I used the word -- I am not able to find it immediately, but I think I used the word largely perpendicular, roughly perpendicular.

Q. Did you -- in using something perpendicular, largely or roughly, was there any particular tolerance you used?

A. I restricted myself to internuclear distances or interatomic distances, and there are a very small number of interatomic distances in this molecule. So it was a judgment by eye that I made.

Q. Was there any numerical tolerance you used to determine what would be orthogonal from the longest dimension?

A. No. I used visual inspection by eye using the computer program that I used.

(Reporter clarification.)

Q. (BY MR. REINES) And just to be clear, when you're showing, for example, an allyl that is 3.1 angstroms width, is that measuring B to C, you know, as the crow flies, or is this tracing through bonds as illustrated in this figure?

MR. SCHWARTZ: Mischaracterizes testimony.

A. It is the distance, Cartesian distance between XYZ positions of the atom B from the XYZ positions of the atom C.

Q. (BY MR. REINES) When you say Cartesian, is that as the crow flies or is that tracing through the bonds that are depicted in the illustration?

A. I am actually a bird watcher, and so I prefer to just retain Cartesian distance so I don't confuse what I did.

Q. The Cartesian distance, is that the same as tracing through the bonds as illustrated in the figures?

A. No, the Cartesian distance is the straight line distance from atom C to atom D.

Q. So the 3.1A you're -- angstrom dimension you're showing with that arrow that's vertical, that actually isn't the right -- that should actually be tilted to the right if you're referring to C to D, correct, in terms of accurately showing what the length is.

A. This is a three-dimensional projection really of a three-dimensional object. So in a two-dimensional projection -- let me start again because I think I may have -- let me start again.

This is a two-dimensional projection of a three-dimensional object. And in this two-dimensional projection, the vertical arrow denoted 3.1 angstroms accurately runs through the center of the atom C and the center of the atom D, and so we do have to consider that this is a three-dimensional projection. We could have -- I could have also drawn a line from the center of atom C to center of atom D. That would be a different way of showing it. The number wouldn't change.

Q. In the distance from C to B, why did you not conclude that was the valid width in this situation, the allyl in paragraph 32?

A. I believe you asked me that question regarding this molecule, and I will try to give the same answer, which is if you measured the distance from B to C, it would be a distance that deviated greatly from the measurement of 3.1 angstrom reported did I Dr. Ju. And I would also consider that the line connecting atom C to atom B is not largely perpendicular to the longest direction or dimension of the molecule.

MR. REINES: Why don't we take a break.

THE WITNESS: Yes, I was just going to say. Thank you. I am getting a little hungry out in California, and I was just going to suggest that, so

thank you.

MR. REINES: No problem. Do you want to take a lunch break?

THE WITNESS: Is this a lunch break? Could we take a lunch break, or do you just want a short break?

MR. REINES: It's not just for me, but I was thinking we maybe going to noon Pacific, 3:00 p.m. Eastern, and then you should have ample time to have a meal. Does that make sense?

THE WITNESS: That's fine with me.

MR. REINES: Is that enough time for everybody? Speak up, if it's not enough time.

Okay. So we'll reconvene at noon Pacific, 3:00 p.m. Eastern.

THE WITNESS: Thank you.

THE VIDEOGRAPHER: Stand by, please. The time is 11:36 a.m. Pacific, off the video record.

(Break from 11:36 to 12:04 p.m.)

THE VIDEOGRAPHER: The time is 12:04 p.m. Pacific, back on the video record.

Q. (BY MR. REINES) Dr. Kuriyan, is there anything in the patents-in-suit that supports any use of width as the diameter?

MR. SCHWARTZ: Objection, vague.

John, you're muted.

A. Are you referring to the patent specifications or are you including --

Q. (BY MR. REINES) Yes.

A. Oh, okay.

In the patent specifications, I had been asked earlier if the term width occurs and whether I have noticed it. And my answer at that time had been I had not noticed it, and so I assumed that the term width doesn't occur. And I am fairly certain the term diameter also doesn't occur. So I do not believe, based on that, that the patent specifications speak to this matter.

Q. If the -- if you had a new protecting group and you wanted to determine whether it satisfied the concept of small, as set forth in the claims of the patents-in-suit, what's the test that you would use to determine width according to your analysis?

A. I think in paragraph 29 -- we already discussed this -- I described the procedure that I used to understand Dr. Ju's use of the term diameter. I would use the same procedure.

Q. I am sorry. The same procedure as what?

A. I would use the same procedure as I specified Dr. Ju used, which I followed as described in paragraph

along that direction.

Q. (BY MR. REINES) And why did you exclude the length dimension as being potential diameter dimension?

MR. SCHWARTZ: Asked and answered.

A. As I had answered before, Dr. Ju had specified length that he had measured for the diameter, and none of the longest dimensions matched Dr. Ju's measurements of the diameter. In fact, they were significantly larger.

Q. (BY MR. REINES) So if you had a new protecting group and you wanted to determine whether it met the definition of small, you would look at the farthest opposing atoms in the length and the width dimensions, and then check whether the width dimension was greater or smaller than the 3.7 angstroms?

MR. SCHWARTZ: Again, I am going to object to this as outside the scope.

A. So at the beginning of my testimony, I had stated an answer to a question of yours that I understand that the word small and its meaning is a claim construction term and also one under dispute, and I did not concern myself with the determination of whether a group was small or not.

Q. (BY MR. REINES) Okay. In determining a diameter, do you have an opinion as to what the diameter

is for purposes of understanding the claims?

MR. SCHWARTZ: Objection to scope.

A. Again, I had stated at the beginning of my deposition that I did not engage in any consideration of matters relating to claims.

Q. (BY MR. REINES) In -- for a new protecting group, if one were attempting to determine what the diameter is as set forth in the Ju declaration, they would have to select the furthest opposing atoms in the width dimension. Is that correct?

A. That's what Dr. Ju did, yes.

Q. And is that the test that you would apply to determine if something met the description of being less than 3.7 angstroms in diameter?

MR. SCHWARTZ: Objection to scope.

A. Yes, within the context that I did not actually analyze any molecules other than the ones mentioned in the Ju declaration, that's correct.

Q. (BY MR. REINES) Now the way you were analyzing diameter, a protecting group could be 3 feet long and still fall within the definition of being less than 3.7 angstroms in length?

A. That is correct. It's an exaggerated characterization of my testimony, but I will not object to it.

guess, Pacific?

MR. SCHWARTZ: That's 3:40? Yeah, that's fine.

THE VIDEOGRAPHER: Stand by, please. The time is 12:16 p.m. Pacific, off the video record.

(Break from 12:16 p.m. to 12:40 PM.)

THE VIDEOGRAPHER: The time is 12:40 p.m. Pacific. Back on the video record.

MR. SCHWARTZ: Thank you, Dr. Kuriyan. We have no further questions.

MR. REINES: Thank you, everybody. Have a good weekend.

MR. SCHWARTZ: You too, Ed.

THE VIDEOGRAPHER: That concludes this video deposition. The time is 12:40 p.m. Pacific. Off the video record.

(Proceedings ended at 12:40 p.m.)

REPORTER'S CERTIFICATE

The undersigned Certified Shorthand Reporter licensed in the State of Texas does hereby certify:

I am authorized to administer oaths or affirmations, and prior to being examined, the witness was duly administered an oath by me.

I am not a relative or employee or attorney or counsel of any of the parties, nor am I a relative or employee of such attorney or counsel, nor am I financially interested in the outcome of this action.

I am the deposition officer who stenographically recorded the testimony in the foregoing deposition, and the foregoing transcript is a true record of the testimony given by the witness.

Before completion of the deposition, review of the transcript [X] was [ ] was not requested. If requested, any changes made by the deponent (and provided to the reporter) during the period allowed are appended hereto.

In witness whereof, I have subscribed my name this _____ day of _____, 2020.

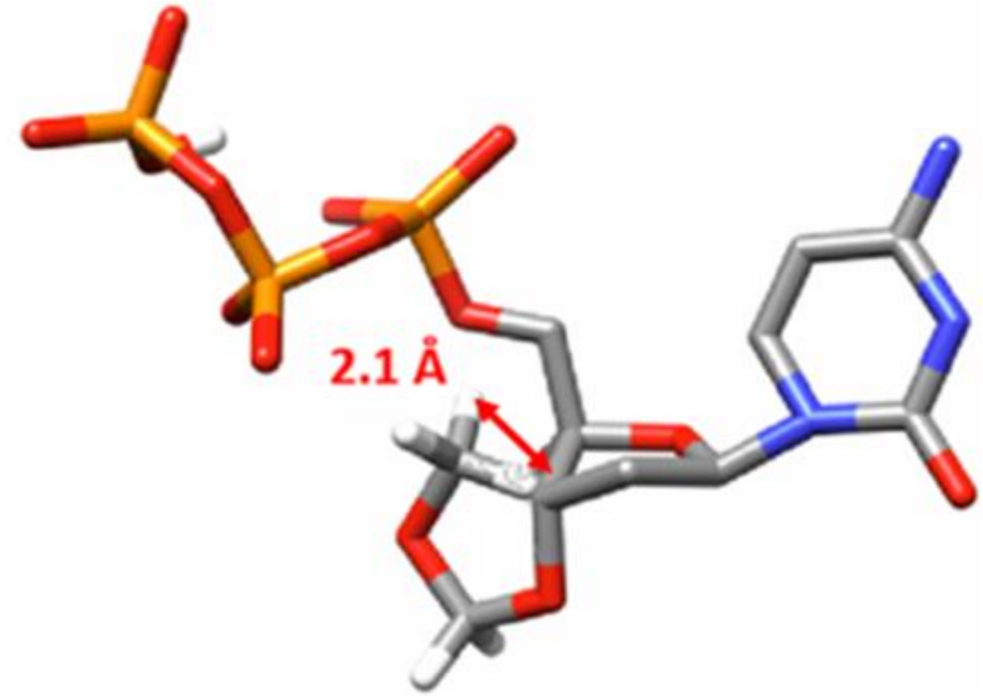
Julie C. Brandt

_____

Julie C. Brandt, CSR, RMR, CRR

TX CSR No. 4018, Exp. 10/31/21

MOM



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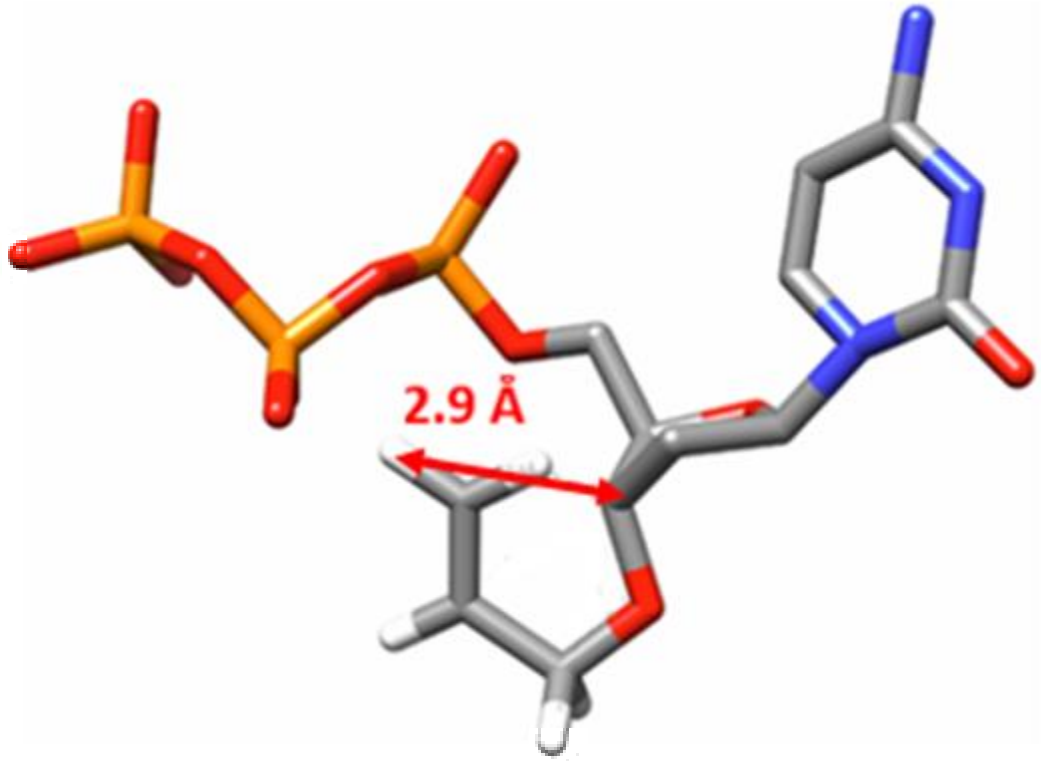
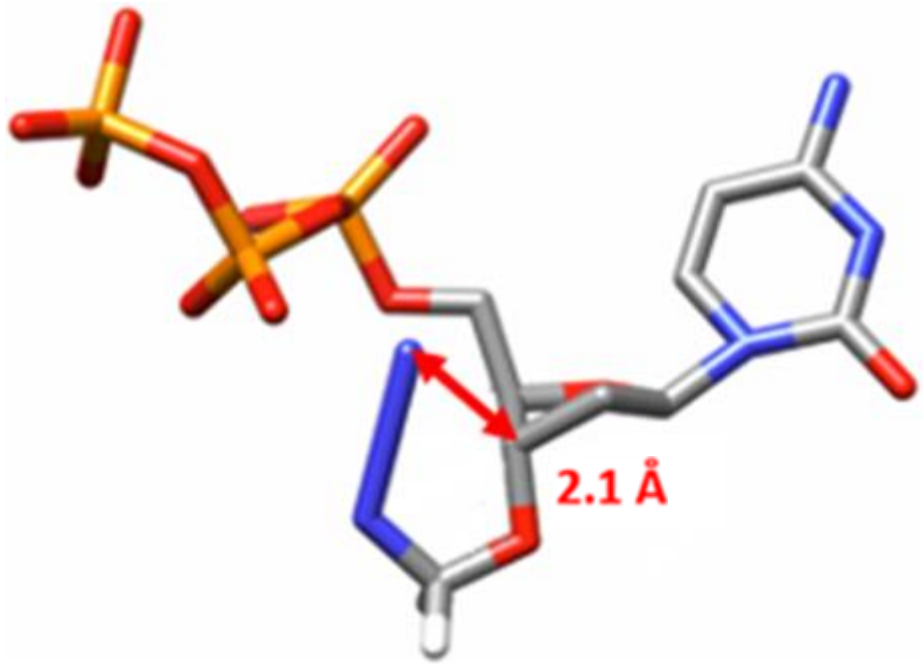
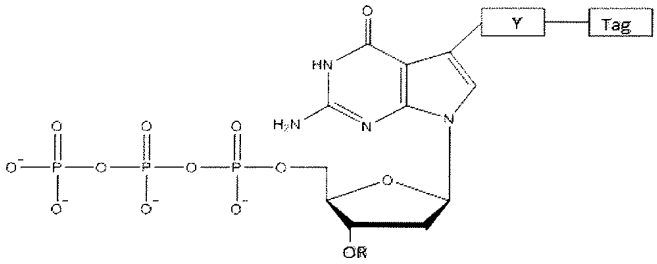


Azidomethyl



Dkt. 62239-BZA6AA

CLAIM SUPPORT TABLE

U.S. Serial No. 16/149,098	Support
1. A guanine deoxyribonucleotide analogue having the structure:  	**[0016]** "each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an -OH group at a 3'-position of the deoxyribose" **[0036]** "FIG. 2A-2B: ...A, C, G, T; nucleotide triphosphates comprising bases adenine, cytosine, guanine, and thymine; d, deoxy; dd, dideoxy; R, cleavable chemical group used to cap the -OH group; Y, cleavable linker" **FIG. 2B**: dG — Y — tag-3 (Link to the base) dG — O — R (Block the 3'-OH group) **FIGS. 7** and **8** **[0093]** "In one embodiment, the unique label is attached through a cleavable linker to...a 7-position of...deazaguanine."
wherein R (a) represents a small, chemically cleavable chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide	**[0006]** "More recent work in the literature exploring DNA sequencing by a synthesis method is mostly focused on designing and synthesizing a photocleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of deoxynucleoside triphosphates (dNTPs) (Welch et al. 1999). Limited success for the incorporation of the 3'-modified nucleotide by DNA polymerase is reported. The reason is that the 3'-position on the deoxyribose is very close to the amino acid residues in the active site of the polymerase, and the polymerase is therefore sensitive to modification in this area of the deoxyribose ring. On the other hand, it is known that modified DNA polymerases (Thermo Sequenase and Taq FS polymerase) are able to recognize nucleotides with extensive modifications with bulky groups such as energy transfer dyes at the 5-position of the pyrimidines (T and C) and at the 7-position of purines (G and A) (Rosenblum et al. 1997, Zhu et al. 1994). The ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP) have been determined (Pelletier et al. 1994) which supports this fact. As shown in FIG. 1, the 3-D structure indicates that the surrounding area of the 3'-position of the deoxyribose ring in ddCTP is very crowded, while there is ample space for modification on the 5-position the cytidine base." **[0007]** "The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye or a mass tag through a cleavable linker to the nucleotide base or an analogue of the nucleotide base, such as to the 5-position of the pyrimidines (T and C) and to the 7-position of the purines (G and A), to use a small

	[0139] "...the R group protecting the 3'-OH is removed chemically to generate free 3`-OH group with high yield (step 4 in FIG. 2A)." **[0142]** "...a small cleavable chemical group (R) to cap the 3'-OH group...The R protecting group is then removed chemically and washed away to generate free 3'-OH group with high yield (step 5 in FIG. 2B)." **[0149]** "...the 3'-OH cap group is chemically cleaved off" **[0159]** "These groups can be removed chemically with high yield as shown in **FIG. 14** (Ireland, et al. 1986; Kamal et al. 1999)."
(b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase	**[0008]** "If small chemical moieties that can be easily cleaved chemically with high yield can be used to cap the 3'-OH group, such nucleotide analogues should also be recognized as substrates for DNA polymerase." **[0089]** "Any chemical group could be used as long as the group...does not interfere with the recognition of the nucleotide analogue by polymerase as a substrate..."
(c) is stable during a DNA polymerase reaction	**[0089]** "Any chemical group could be used as long as the group 1) is stable during the polymerase reaction..."
(d) does not contain a ketone group	**[0008]** "Chemical groups with electrophiles such as ketone groups are not suitable for protecting the 3'-OH of the nucleotide in enzymatic reactions due to the existence of strong nucleophiles in the polymerase."
(e) is not a $-CH_2CH=CH_2$ group	**[0008]** "...allyl ($-CH_2CH=CH_2$) group[] can be used to cap an -OH group..." **[0008]** "...the 3'-OH is capped with a cleavable chemical moiety such as...an allyl group ($-CH_2CH=CH_2$)..." **[0063]** "Other examples include analogues in which a small chemical moiety such as... $-CH_2CH=CH_2$ is used to cap the -OH group at the 3'-position of deoxyribose." **[0089]** "In one embodiment, the cleavable chemical group that caps the -OH group at the 3'-position of deoxyribose in the nucleotide analogue is... $-CH_2CH=CH_2$." This negative limitation is permissible under MPEP 2173.05(i). *See Inphi Corporation v. Netlist, Inc.*, 805 F.3d 1350, 1356-57, 116 USPQ2d 2006, 2010-11 (Fed. Cir. 2015).
wherein OR is not a methoxy group	**[0008]** "...the procedure to chemically cleave the methoxy group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methoxy group to cap the 3'-OH group for sequencing DNA by synthesis."
or an ester group	**[0008]** "An ester group was also explored to cap the 3'-OH group of the nucleotide, but it was shown to be

	cleaved by the nucleophiles in the active site in DNA polymerase (Canard et al. 1995)."
wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction	**[0007]** "...use a small cleavable chemical moiety to cap the 3'-OH group of the deoxyribose to make it nonreactive" **[0089]** "Any chemical group could be used as long as the group 1) is stable during the polymerase reaction..."
wherein tag represents a detectable fluorescent moiety [as part of structure Y–Tag attached to the 7-position of a deaza-guanine nucleotide analogue]	**[0007]** "The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye...through a cleavable linker to the nucleotide base or an analogue of the nucleotide base…to the 7-position of the purines (G and A)." **[0036]** "FIG. 2A-2B...the unique labels are dyes...Y, cleavable linker." **[0041] FIG. 7**...Each nucleotide analogue has a unique fluorescent dye attached to the base through a ...cleavable linker" **[0043] FIG. 9**...The dye is detected and cleaved to test the approach. Dye1=Fam; Dye2=R6G; Dye3=Tam; Dye4=Rox." **[0090]** In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescent moiety..." **[0117]** "In one embodiment, the unique label is a fluorescent moiety or a fluorescent semiconductor crystal."
wherein Y represents a chemically cleavable, chemical linker	**[0036]** "Y, cleavable linker." **[0007]** "linking a unique label such as a fluorescent dye...through a cleavable linker to the nucleotide base or an analogue of the nucleotide base" **[0063]** "Further examples include analogues in which a label is attached through a cleavable linker…to the 7-position of...deaza-guanine." **[0094]** "In one embodiment, the linker is cleaved by a chemical means."
which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase	**[0093]** "In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as...the nucleotide analog can be recognized by polymerase as a substrate."
and (b) is stable during a DNA polymerase reaction ; and	**[0093]** "In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction..."

Paper No. _
Filed: February 5, 2019

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK
Patent Owner.

IPR2018-00797 (Patent 9,868,985)

PATENT OWNER'S SUR-REPLY

III. Claim Construction

The claims are for "[a] method for sequencing a nucleic acid," which requires multiple cycles because a "nucleic acid" is a polymer of nucleotides. Response, 11; Ex. 1075, Abstract, 4:45-48, 8:33-36, 12:22-28 (*e.g.*, "This invention is directed to a method for sequencing a nucleic acid…."). Illumina's argument that the preamble is non-limiting is belated and thus improper. The preamble defines a "fundamental characteristic of the claimed invention," as evidenced by its being repeatedly "highlighted as important by the specification." *Proveris Scientific Corp. v. Innovasystems, Inc.*, 739 F.3d 1367, 1372-73 (Fed. Cir. 2014); *Deere & Co. v. Bush Hog, LLC*, 703 F.3d 1349, 1357-59 (Fed. Cir. 2012). The Board should also construe the terms "small" and "chemical linker." Response, 9-10.

IV. Illumina's Grounds Fail Because There Was No Motivation To Use The Allyl Capping Group For SBS

Illumina's Grounds rise or fall with its argument that the prior art would lead a POSA to select the allyl capping group for use in SBS. As explained below and in Columbia's Response, Illumina failed to meet its burden.

A. Real-World Evidence Shows No Motivation

Prior to 2000, many research groups pursued SBS-compatible nucleotides. Ex. 1119, ¶11. Despite this effort, ***none*** reported interest in the allyl capping group for SBS after Metzker 1994. Response, 23.

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.,
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK
Patent Owner.

IPR2018-00797 (Patent 9,868,985)

PATENT OWNER'S RESPONSE

C. "A Method For Sequencing A Nucleic Acid"

"A method for sequencing a nucleic acid" is a method for sequencing a polymer of nucleotides. Ex. 2114 ¶22. A POSA would have understood that a "nucleic acid" is a polymer of nucleotides. *Id*. Dr. Romesberg agrees. Ex. 2113 at 140. Therefore, a POSA understood that "[a] method for sequencing a nucleic acid" requires multiple cycles of SBS (incorporation, cleavage, reinitiation) to generate the DNA sequence of a nucleic acid polymer. Ex. 2114 ¶22.

The patent-at-issue's specification supports this construction, stating that the "the tag and the group capping the 3'-OH need to be removed with high yield to ***allow the incorporation and detection of the next nucleotide***" and "in the DNA sequencing system disclosed herein, more than 10,000 bases can be identified ***after each cycle*** and after 100 cycles, a million base pairs will be generated from one sequencing chip." Ex. 1075 at 21:8-10, 21:60-64; Ex. 2114 ¶23.

V. Illumina's Ground 1 Challenge For Obviousness Over Tsien In View Of Prober Fails

Illumina's Ground 1 challenge relies on a single premise—that it would have been obvious to make a base-labeled nucleotide with the 3-carbon, 5-hydrogen allyl capping group ($-CH_2CH=CH_2$) ("the allyl capping group"). Illumina's Ground 1 challenge fails because:

capping group referred to as small by Dower contains "nitrobenzyl" ("2-***nitrobenzyl***oxycarbonyl"; Ex. 1015 at 18:54), which as explained above, is not less than 3.7Å in diameter. Similarly, the tBOC and NVOC are not smaller than 3.7Å in diameter and Metzker showed that the acetyl was not incorporated at all by any polymerase. Ex. 1016 at 4263.

E. A POSA Would Not Have Had A Reasonable Expectation Of Success In Achieving The Claimed Invention

As explained below, a POSA would not have had a reasonable expectation of performing the claimed methods using a nucleotide analogue with the allyl capping group and a base label. Ex. 2114 ¶90.

1. There Was No Reasonable Expectation Of Sequencing A Nucleic Acid

In its Institution Decision, the Board invited the parties to address whether the challenged claims require multiple cycles of sequencing. IPR2018-00797, Paper 20 at 26 (September 18, 2018). They do. The claims are directed towards "a method for sequencing a nucleic acid," which as described in Section IV(C), is a method for sequencing a polymer of nucleotides. Ex. 2114 ¶91. Therefore, performing the claimed methods requires multiple cycles of SBS (incorporation, cleavage, reinitiation) in order to generate the DNA sequence of a nucleic acid polymer. *Id.*

More specifically, a POSA would have understood that in order to perform the claimed methods, accurate sequences of 20 base pairs or greater were necessary to permit the assembly of the sequenced fragments. Ex. 2114 ¶92. Illumina's expert, Dr. Romesberg, and Illumina have previously agreed that such lengths are necessary to practice SBS. Ex. 2029 at 6 ("an SBS process should be able to determine the sequence of at least 20 consecutive nucleotides"); Ex. 2126 at 22-24, 60; Ex. 2035 at 179.

A POSA would not have had a reasonable expectation of conducting multiple cycles of DNA sequencing, let alone 20 cycles, using a nucleotide that had been modified to include (i) the allyl capping group and (ii) a base label. Ex. 2114 ¶93. By the Priority Date in October 2000, there were ***no*** reports of successfully using a nucleotide analogue with (i) any capping group, no less the allyl capping group, and (ii) a base label to conduct even ***one*** cycle of SBS (incorporation, cleavage, reinitiation).[13] Ex. 2114 ¶93. In addition, for the reasons explained in Sections V(A)-(C), a POSA would not have had a reasonable expectation that a nucleotide analogue containing the allyl capping group could

[13] In his 1994 publication, Metzker reported incorporation of nucleotide analogues that had capping groups (e.g., 2-nitrobenzyl capping group, methyl capping group) but no fluorescent labels.