otides in length. In vitro "evolution" was done on this region at 30% mutagenesis, and four more rounds of in vitro selection followed before this second population was cloned. From these sequences, a consensus region was discovered. Certainly though this work is a pioneering achievement in the field, it is an example of how the conventional protocol is significantly more involved than that presented here.

In summary, a novel in vitro selection protocol has been designed to take advantage of a combinatorial library of small size that has multiple copies of every distinct sequence. The method condensed the many days of a typical screening strategy to less than two days. This was a proof-of-concept experiment that showed that the new method succeeds by creating a large number of copies of individual sequences in the initial random pool, consistently reducing the level of nonspecific binding sequences per selection round, and effectively amplifying the few surviving sequences.

Since only the original synthesized sequences were used for all the screenings, the technique should allow for the iterative in vitro selection of modified oligonucleotides that previously could not undergo this powerful process.[10] Hence, this method should significantly increase the power of the in vitro selection method and is the direction that we are currently investigating.

Experimental Section

The DNA library[4] (31 mg) was labeled at the 5′-end with [γ-^{32}P]ATP, purified by gel chromatography, and suspended in 300 mL of folding buffer (300 mM KCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.5). After cooling down to room temperature following denaturation at 75 °C, the ^{32}P-labeled DNA was loaded onto an acetate-agarose precolumn (300 µL), which was attached directly to a 2.5 mM ATP-agarose column (800 µL, Sigma). The precolumn was washed with 600 µL of buffer, and the eluted DNA was allowed to equilibrate on the ATP-agarose column for 10 min. The precolumn was discarded after a single use as were all subsequent columns. After equilibration, the ATP-agarose column was washed with 4 mL of folding buffer to elute unbound or weakly bound oligonucleotides. The retained DNA was eluted with 3 mL of the ATP elution buffer (5 mM ATP in folding buffer) and collected in 500 µL fractions.

In order to perform another round of selection, the ATP had to be removed. Hence, the eluted fractions were collected directly into Microcon-3 microcentrifuge devices (3000 D cutoff, Amicon). After membrane diafiltration, about 98% of the total ATP was removed. The filtered fractions were then pooled, and folding buffer was added until a final volume of 10 mL was attained. The concentration of contaminating ATP concentration was 30 µM for the DNA sample, which was over 80 times more dilute than that of the 2.5 mM ATP-agarose column. Each cycle of selection started with a new set of stacked affinity columns, i.e. a precolumn attached to a ligand column. The screening cycles for the ATP aptamers are summarized in Table 1.

The rare-DNA PCR was performed as follows: On the last cycle the DNA was eluted from the ATP-agarose column with 3 mL of 10 mM ATP in 20 mM Tris, pH 7.5. This last fraction was precipitated twice from ethanol, and the PCR reagents (50 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 20 mM Tris, pH 8.8 at 25 °C, 200 µM dNTPs, 0.1% Triton X-100, 20 units of Deep Vent (exo-) DNA polymerase, 0.5 µg 5′-primer, 0.5 µg 3′-primer) were added. Thermal cycling (94 °C for 45 s; 42 °C for 90 s; 60 °C for 45 s; 45 cycles) was done in a microcentrifuge tube that had first been irradiated with UV light. A positive control containing a dilute solution (~20000 molecules) of a 52-mer, and a negative control containing no DNA also underwent the same amplification protocol. Gel electrophoresis after amplification showed DNA in all lanes except the negative control.

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The Synthesis of Enantiopure ω-Methanoprolines and ω-Methanopipecolic Acids by a Novel Cyclopropanation Reaction: The "Flattening" of Proline**

Stephen Hanessian,* Ulrich Reinhold, and Gabriella Gentile

Proline occupies a prominent position in the hierarchy of natural amino acid constituents of mammalian proteins.[1] As part of a peptidic motif, its unique structure results in secondary amide bonds, leading to important conformational and functional consequences.[2] For example, the well-known *cis–trans* isomerism in prolylamides is associated with vitally important biological phenomena and functions, such as protein folding,[3] hormone regulation,[4] recognition,[5] and transmembrane signaling[6] to mention a few. The importance of *cis–trans* conformational changes is manifested by the role that peptidyl prolyl isomerases such as the immunophilins play in immunoregulation.[7] Proline has also figured prominently as a component of therapeutic agents,[8] in drug design,[9] and in probing enzyme activity.[10]

Conformationally constrained analogues of proline have been used extensively in connection with peptidomimetic research.[11] Although 2,3- and 3,4-methanoprolines have been

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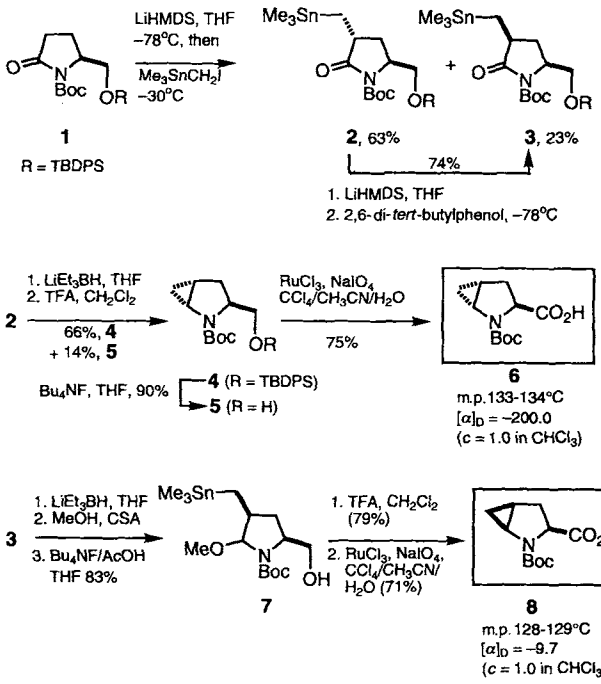
described,[12, 13] the 4,5-methanoprolines are relatively unexplored.[14] Furthermore, structural investigations that study the consequences of introducing strain and its effects on the configuration and stability of amide linkages are not available to compare such systems to their proline counterparts.

We describe herein highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions and the extension of the methodology to other congeners.[15, 16]

Treatment of the readily available lactam **1**[17a] with lithium hexamethyldisilazide (LiHMDS) and Me_3SnCH_2I gave the α-alkylated products **2** ($[\alpha]_D = -15.3$, $c = 0.43$ in $CHCl_3$) and **3** ($[\alpha]_D = -16.0$, $c = 1.23$ in $CHCl_3$) in 63% and 23% yields, respectively (Scheme 1). The *syn*-isomer **3** could be easily obtained

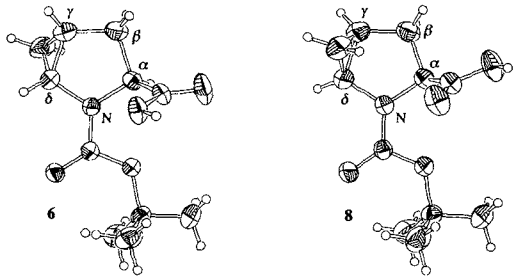


Scheme 1. TBDPS = *t*-BuPh$_2$Si, TFA = trifluoroacetic acid, Boc = *tert*-butoxycarbonyl, CSA = camphor-10-sulfonic acid.

by treatment of the enolate from **2** with the proton source 2,6-di-*tert*-butylphenol.[18, 19] Generation of the hemiaminal from **2** and treatment with TFA led to the (4*R*,5*R*)-methanopyrrolidine derivative **4** ($[\alpha]_D = -69.3$, $c = 1.41$ in $CHCl_3$), which was smoothly deprotected to **5**, and the latter oxidized to give the crystalline (4*R*,5*R*)-methano-*N*-Boc-L-proline in excellent overall yield.

Similar treatment of the *syn*-isomer **3** gave the diastereomeric crystalline acid **8** via its methylaminal derivative **7**. The structures and conformations of **6** and **8** in the solid state were unambiguously confirmed by single-crystal X-ray analysis. Table 1 lists selected torsion angles for compounds **6** and **8**, where considerable "flattening" of the pyrrolidine ring is observed relative to *N*-Boc-L-proline,[20] particularly in the case of **6**. The flattening of the pyrrolidine ring in **6** is also manifested in the root-mean-square value of 0.003 Å for the C_β and N atoms from the plane defined by C_β,C_γ,C_δ, and N (0.013 Å in **8**). The lowest deviation of 0.018 Å in the case of *N*-Boc proline was found for

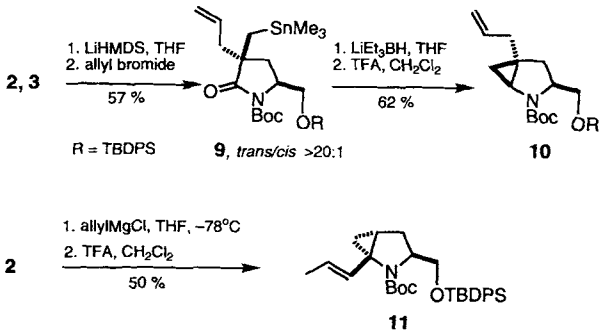
Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).



	N-Boc-L-proline [20]	**6**	**8**
$\tau(NC_\alpha)$	−17	−5.6	−14.4
$\tau(C_\alpha C_\beta)$	+31	+4.8	+15.3
$\tau(C_\beta C_\gamma)$	−35	−2.6	−11.4
$\tau(C_\gamma C_\delta)$	+24	−0.7	+2.9
$\tau(C_\delta N)$	−4	+4.1	+7.6
$\tau(BocNC_\alpha CO_2H)$	−72	−64.0	−67.1
rms deviation of fitted atoms	0.018 C_α, N, C_δ, C_γ	0.003 C_β, C_γ, C_δ, N	0.013 C_β, C_γ, C_δ, N
	δ(*cis*/*trans*)	*δ*(*cis*/*trans*)	*δ*(*cis*/*trans*)
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C_α	58.8	60.8/60.1	59.5/59.1
C_β	30.75/29.53	32.0	31.5/29.1

from **6** and **8**, in which the out-of-plane carbon atom was the one bearing the carboxyl group (0.082 Å and 0.235 Å respectively).

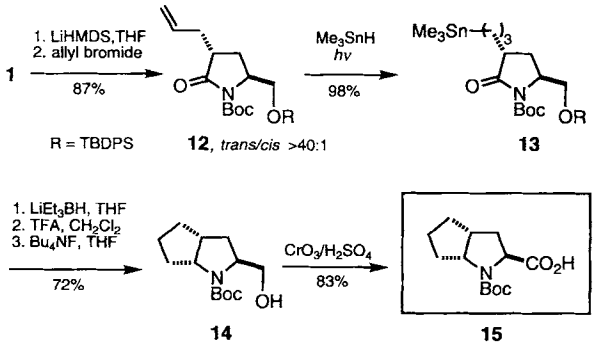
Intermediates **2** and **3** could also be subjected to further stereocontrolled branching leading to the α-C-allyl derivative **9**, which upon controlled reduction and acid-catalyzed destannylation led to the branched 4,5-methano-L-proline precursor **10** ($[\alpha]_D = +4.3°$, $c = 0.72$ in $CHCl_3$; Scheme 2). Treatment of **2**



Scheme 2.

with allylmagnesium chloride, followed by trifluoroacetic acid (TFA), led to the (*S*)-5-(2-propenyl)-4,5-methano-L-proline derivative **11** ($[\alpha]_D = -27.0$, $c = 0.57$ in $CHCl_3$) on migration of the double bond. Compounds **10** and **11** represent uniquely functionalized precursors to constrained ω-methanoprolines.

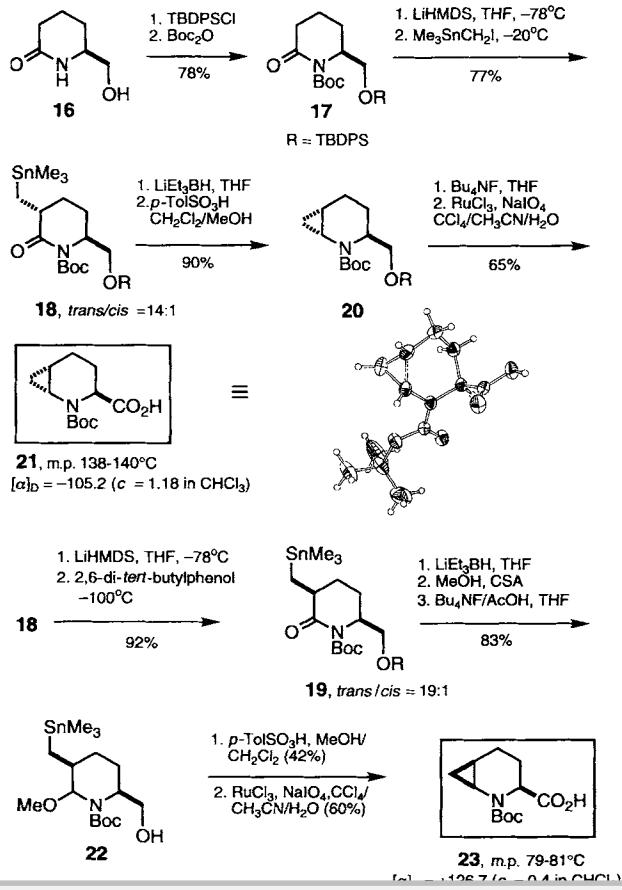
The versatility and generality of the intramolecular carbocyclization reaction with appended trimethylstannylalkyl groups via incipient iminium ions can be demonstrated in the synthesis of bicyclic proline congeners (Scheme 3). These compounds are related to the antihypertensive agent ramipril.[21] A highly stereoselective allylation of the enolate from **1** gave **12** ([α]$_D$ = −45.0, c = 1.0 in $CHCl_3$), which was subjected to a



Scheme 3.

$c = 0.92$ in $CHCl_3$). Formation of the hemiaminal, followed by acid-catalyzed cyclization and deprotection, led to the bicyclic prolinol derivative **14** ($[\alpha]_D = -97.3$, $c = 1.38$ in $CHCl_3$). Finally, oxidation under Jones conditions gave the immediate precursor to the *N*-Boc-(4*R*,5R)-ramipril diastereomer **15** (m.p. 61–63 °C; $[\alpha]_D = -126.7$, $c = 0.46$ in $CHCl_3$).

It is also of interest to view compounds **6**, **8**, **10**, and **11** as precursors to constrained analogues or precursors to L-pipecolic acid. The extension of the cyclopropanation reaction to the pipecolic acid series is shown in Scheme 4. Trimethylstannylmethylation of the lithium enolate derived from the readily available **17**[23] gave the *anti*-isomer **18**. Reduction to the hemiaminal and acid-catalyzed cyclization led to **20** ($[\alpha]_D = -56.0$, $c = 1.02$ in $CHCl_3$), which was deprotected and oxidized to the crystalline (5*R*,6*S*)-methano-*N*-Boc-L-pipecolic acid **21** (m.p. 138–140 °C; $[\alpha]_D = -105.2$, $c = 1.17$ in $CHCl_3$). Epimerization of **18** to the *syn*-isomer **19** by diastereoselective protonation, followed by functional group manipulation as described above, led to the crystalline diastereomeric (5*S*,6*S*)-methano-*N*-Boc-pipecolic acid **23** (m.p. 79–81 °C; $[\alpha]_D = -126.7$°, $c = 0.40$ in $CHCl_3$). The structure of crystalline **21** was unambiguously established by X-ray analysis. It is of interest to note that while the proline derivatives **6** and **8** adopt a *cis*-*N*-Boc proline orientation in the solid state (Table 1), the corresponding 4,5-methanopipecolic acid analogue **21** exhibits a *trans* orientation (Scheme 4). The presence of *cis* and *trans* isomers of **6** and **8** in $CDCl_3$ was evidenced by the corresponding ^{13}C NMR shifts, as in the case of *N*-Boc-L-proline (Table 1).



Pipecolic acid is an important constituent of the immunosuppressive agents FK-506[24] and rapamycin,[25] in which its α-ketoamide portion is intimately involved in an "active complex" with the target enzyme.[26] It is also involved in the metabolism of L-lysine, an essential amino acid for mammalian growth and development.[27] Functionalized pipecolic acids are also considered strained analogues of lysine with applications in drug design and peptidomimetic research,[28] as well as in the inhibition of L-pipecolate oxidase.[27]

It is our belief that the replacement of L-proline and L-pipecolic acid by conformationally altered ring variants represented by the methano congeners described in this work could have important consequences in biological recognition, in *cis*–*trans* conformational changes, in the susceptibility of the secondary amide bonds to enzymatic cleavage, and in related processes or phenomena. Studies that address these issues will be reported in due course.

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1,8-Bis(dimethylamino)-4,5-dihydroxynaphthalene, a Neutral, Intramolecularly Protonated "Proton Sponge" with Zwitterionic Structure**

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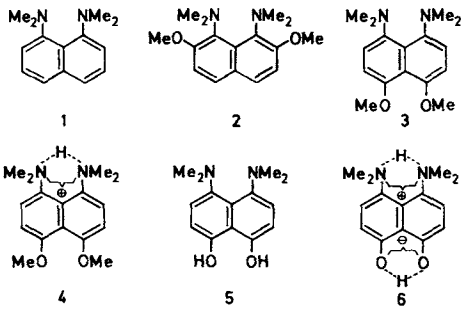
The interaction of basic groups in close proximity to each other may lead, as in the case of 1,8-bis(dimethylamino)-naphthalene (**1**), to unusually high basicities ("proton sponges").[1] The influences of gradually changed distances and orientations of the basic centers as well as of inductive, mesomeric, and steric effects on the basicity of such compounds have been thoroughly studied.[2] In comparison to the basicity of **1** [$pK_a \approx 12.1$ (H_2O); 7.5 (DMSO)],[3] that of 2,7-dimethoxy-1,8-bis(dimethylamino)naphthalene (**2**) is found to be increased by four powers of ten [$pK_a \approx 16.1$ (H_2O); 11.5 (DMSO)].[3] To separate the mesomeric effect of the two methoxy groups from their steric effect on the dimethylamino groups, we were interested in **3**, an isomer of **2** in which the two methoxy groups are not in the 2,7-positions but in the opposite *peri*-positions. In fact, 1,8-bis(dimethylamino)-4,5-dimethoxynaphthalene (**3**) is considerably less basic [$pK_a \approx 13.9$ (H_2O); 9.3 (DMSO)] than the isomer **2**, indicating that the main reason for the high basicity of **2** is the steric effect of the methoxy groups in *ortho*-positions to the dimethylamino groups. Irrespective of this primarily intended basicity comparison of **2** and **3**, the synthesis of **3** should allow the easy preparation of the corresponding 4,5-dihydroxy compound **5**, which by intramolecular proton displacement may lead to a new type of neutral, yet zwitterionic "proton sponge" (formula **6**).



For the synthesis of **3**, 1,8-dihydroxynaphthalene[4] was methylated to give 1,8-dimethoxynaphthalene, which was nitrated (conc. nitric acid, glacial acetic acid/dichloromethane, 9:5) to yield 1,8-dimethoxy-4,5-dinitronaphthalene (39%; m.p. 278 °C); the isomeric 2,5-dinitro product (m.p. 151–153 °C) was separated by chromatography on silica gel with dichloromethane as eluent. Catalytic hydrogenation (10% Pd/C, tetrahydrofuran (THF), 20 °C) resulted in the formation of 1,8-diamino-4,5-dimethoxynaphthalene (97%; m.p. 83–95 °C, decomp), which was N-methylated according to the method of Quast et al.[5] to give **3** (71%; m.p. 75 °C, from *n*-hexane/ethyl

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