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Khanna et al.

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[54] **NOVEL ETHER SUBSTITUTED FLUORESCEIN POLYAMINO ACID COMPOUNDS AS FLUORESCERS AND QUENCHERS**

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[52] **U.S. Cl.** ..... **260/112 B**; 23/230 B; 260/112 R; 260/112.5 R; 260/112.7; 260/121; 424/8; 424/12; 424/85; 424/88; 435/7; 435/188; 525/420; 260/335

[58] **Field of Search** ..... 260/112 R, 112 B, 121; 424/85, 88; 525/420; 435/188

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,174,384	12/1979	Ullman et al.	260/112 B
4,220,450	9/1980	Maggio	435/7 X
4,220,722	9/1980	Rowley et al.	435/7 X

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Attorney, Agent, or Firm—Bertram I. Rowland

[57] **ABSTRACT**

Diether symmetrically substituted fluoresceins are provided having at least one anionic group and a linking functionality. Depending upon the site of substitution, the compounds can be used as fluorescers absorbing at wavelengths in excess of 500 nm or as quenchers, absorbing at wavelengths in excess of 500 nm and exhibiting substantially no fluorescence. The compounds find wide application, particularly as labels in fluorescent immunoassays.

22 Claims, No Drawings

NOVEL ETHER SUBSTITUTED FLUORESCEIN POLYAMINO ACID COMPOUNDS AS FLUORESCERS AND QUENCHERS

BACKGROUND OF THE INVENTION

1. Field of the Invention

Fluorescing compounds find wide application, because of their ability to emit light upon excitation with energy within certain energy ranges. By virtue of this ability, fluorescers have found employment in advertising, novelty items, and as labels in chemical or biological processes, e.g. assays. That is, various compounds can be conjugated to a fluorescing compound, the conjugate subjected to some type of partitioning, and the fate of the conjugate determined by irradiating the sample with light and detecting the zone in which the conjugate exists.

This technique can be employed in immunoassays, involving specific binding pairs, such as antigens and antibodies. By conjugating a fluorescer to one of the members of the specific binding pair and employing various protocols, one can provide for partitioning of the fluorescer conjugate between a solid phase and a liquid phase in relation to the amount of antigen in an unknown sample. By measuring the fluorescence of either of the phases, one can then relate the level of fluorescence observed to a concentration of the antigen in the sample.

Alternatively, one can avoid partitioning of the fluorescent label by providing for a mechanism which varies the fluorescence of the label, depending upon the label environment in a liquid medium. For example, in addition to labeling one of the members of the specific binding pair with the fluorescer, one may label the other member with a quencher, that is, a molecule which is able to absorb the excitation energy of the fluorescer molecule, preventing the emission of a photon. The quenching then will occur only when the two members of the specific binding pair are associated, so that fluorescer and quencher have the required spatial proximity for quenching.

In preparing fluorescers, there are many desiderata. For a fluorescer, one desires a high extinction co-efficient, a high quantum efficiency, preferably approaching or equal to one, chemical stability, a large Stokes shift, and, where the fluorescence is to be affected by another agent, an efficient response to such reagent. Furthermore, where the fluorescer is to be used in the presence of serum or other composition, which is in itself fluorescent, it is desirable that the fluorescer absorb energy in a substantially different range from that absorbed by the other compounds in the medium. In the case of serum, it is desirable to have fluorescers which absorb light substantially in excess of 450 nm, preferably in excess of 500 nm.

For quencher molecules, it is desirable that the quencher efficiently quench the fluorescer molecule, that is, that there be substantial overlap between the wavelength range of emission of the fluorescer and the wavelength range of absorption by the quencher. In addition, the quencher should be chemically stable, preferably non-fluorescent, and provide a fluorescer-quencher pair with a high quenching efficiency.

In addition, any compounds of interest should be susceptible to reasonable modes of synthesis to provide the desired product in substantially pure form.

2. Description of the Prior Art

U.S. Pat. No. 3,998,943 discloses an immunoassay involving a ligand-fluorescer conjugate employing steric inhibition of simultaneous binding of antibody for ligand and antibody for fluorescer, where the antibody for fluorescer substantially quenches the fluorescence. U.S. Pat. No. 3,996,345 describes an immunoassay involving fluorescer-quencher pairs, where a fluorescer is bonded to one member of a specific binding pair and a quencher bonded to the same or different member of a specific binding pair. The assay is dependent upon the degree to which the quencher and fluorescer are brought within quenching proximity based on the amount of analyte in the medium.

There is an extensive list of compounds involving derivatives of fluorescers. Known compounds include 4',5'-dihydroxyfluorescein and 4',5'-dihydroxy-2',7'-dibromofluorescein (C.A. 61, 7407d). Isothiocyanate derivatives of fluorescein are commercially available, while isocyanate derivates are described in C.A. 59, 563b and sulfonic derivatives are described in C.A. 58, 9012a.

SUMMARY OF THE INVENTION

Di(chalcogen ether) symetrically substituted fluoresceins are provided having at least one anionic group and one functionality for linking to another molecule. The compounds are linked to other materials for reagents in immunoassays, particularly immunoassays involving serum samples. The fluorescein compounds may also be halogenated.

The fluorescers have large extinction coefficients, high quantum yields, have absorption maxima above 500 nm, have Stokes shifts, normally in excess of 10 nm and are stable by themselves and when bonded to other compounds. The quenchers have absorption maxima above 500 nm, have little or no observable fluorescence and efficiently quench a broad spectrum of fluorescent compounds.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The subject invention concerns chromogenic di(chalcogen ether) symmetrically substituted fluorescein compounds capable of accepting or donating electronic energy, which find particular use when conjugated to other compounds, particularly polypeptides, or soluble or insoluble supports for use as reagents in immunoassays. The fluorescein compounds are unsubstituted at 1',8' and are symmetrically disubstituted on the xanthene ring at either the 4',5'- or the 2',7'-positions. The compounds are normally 2,7-di(aliphatic ether substituted) or 4,5-di(aliphatic ether substituted)-9-phenyl-6-hydroxy-3H-xanthene-3-ones.

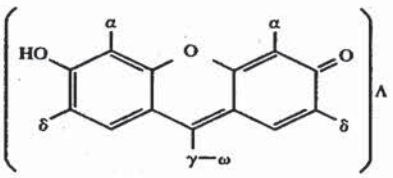
The molecules will have at least 15 carbon atoms, usually at least 16 carbon atoms, and not more than about 45 carbon atoms, usually not more than about 35 carbon atoms. There will be at least 5 chalcogen atoms (atomic number of 8 to 16, oxygen and sulfur), of which at least 3 will be oxygen. In addition to the chalcogen atoms, there may be from 0 to 8, usually from 0 to 6 heteroatoms, such as nitrogen, halogen of atomic number 9 to 53, particularly of 17 to 53, that is, fluorine, chlorine, bromine and iodine, or other heterofunctionalities which may be present to provide specific effects. There will usually be at least one anionic group, normally carboxylate or sulfonate, and one linking group, inter alia non-oxo-carbonyl, including isothiocyanate and isocyanate; sulfonamide, mercapto, and

amino, which may or may not be bonded directly to an annular carbon atom. For the most part, the linking group will be on the group, usually phenyl, substituted at the nine position of the xanthene, although linking groups may also be present as substituents on the ether group. These compounds are conjugated to haptens and antigens to provide conjugates which are capable of fluorescing or of quenching a fluorescer when the quencher is in close spatial proximity to the fluorescer.

The subject compositions have absorption maxima above 500 nm, usually above 510 nm, with relatively narrow bands, usually at least 50% of the area of the longest wave length absorption being over a wavelength range of about 50 nm. The fluorescing compounds are characterized by having good chemical stability, large Stokes shifts and extinction coefficients in excess of 65,000, usually in excess of 75,000. The Stokes shifts will be at least 10 nm, and preferably at least about 20 nm. The quenching compounds, will fluoresce with a quantum efficiency less than 10%, preferably less than 5%, in 0.05 molar phosphate when irradiated with light at the absorption maximum.

The compounds of the subject invention provide novel compounds having important spectroscopic and physical properties. The compounds have absorption maxima above 500 nm. By choosing the positions for the oxy substituents one can provide highly fluorescent compounds or compounds that are substantially non-fluorescent and can be used as quenchers. Compounds with ether substituents at the 2',7'-positions (fluorescein numbering) provide fluorescent compounds with high quantum efficiencies. Compounds with ether substituents at the 4',5'-positions provide compounds with substantially no fluorescence, while absorbing at long wavelengths so as to act as efficient quenchers.

For the most part the compounds of this invention will be water or base soluble compounds having the following formula:



wherein:

each of the α's and each of the δ's may be the same or different, either the α's or the δ's being bonded to an annular carbon atom through a chalcogen (atomic number 8 to 16, oxygen and sulfur); when not the chalcogen bonded pair the α's may be any substituent other than chalcogen and the δ's may be any convenient functionality including chalcogen;

one of the α's or δ's bonded through an ether or ω may be taken together with Λ to provide an active functionality for linking or, when not taken together with Λ, may be a linking functionality to Λ; when not taken together with Λ, ω may be any non-interfering functionality or hydrogen;

Λ is a ligand or receptor when not taken together to form an active functionality for linking;

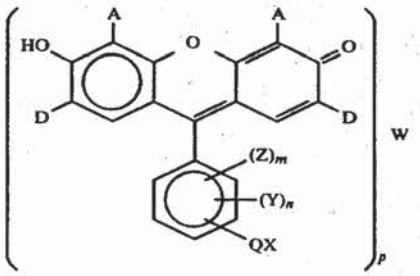
γ is a bond, or a spacer arm of from about 1 to 20 atoms, usually 1 to 16 carbon atoms; usually an aliphatic (includes cycloaliphatic) group of from 1 to 7 carbon atoms having more than 4 annular carbon atoms when cycloaliphatic or an aromatic group of from 6 to 16, usually 6 to 10 annular atoms;

there being one or more of the group in the parenthesis bonded to Λ when Λ is a ligand or receptor.

With the quencher molecules, the 4,5-diether-6-hydroxy-3H-xanthen-3-ones, the presence or absence of a substituent at the 2 and 7 positions does not affect the quenching, but can be used to modify the absorption characteristics of the molecule. Therefore, when the αs are ethers, the δs may be hydrogen or any convenient substituent such as alkyl of 1 to 6 carbon atoms, oxy (hydroxy and alkoxy of 1 to 6 carbon atoms), thio (mercapto, alkylthio of 1 to 6 and sulfonic acid, ester and amide), non-oxo-carbonyl of 1 to 6 carbon atoms (includes acid, esters and amides), cyano, nitro, halo, oxocarbonyl of 1 to 6 carbon atoms, or combinations thereof. The choice of substitution will be governed by the resulting absorption maximum, synthetic convenience and the effect on the physical and chemical properties of the molecule, such as water solubility, chemical reactivity, oxidation sensitivity and the like.

With the fluorescer molecule, the substituents at the 4,5-positions may be varied widely so long as the fluorescent efficiency is not significantly adversely affected. Therefore, while the substituents may be widely varied, the 4,5-position should not be substituted with chalcogen, which would have the effect of substantially reducing the fluorescence of the molecule. Therefore, the range of substituents for the 4,5-position of the fluorescer is more restricted than the range of substituents for the 2,7-position of the quencher.

For the most part, the compounds of this invention having γ as aromatic will have the following formula:



wherein:

the two As are the same or different, normally being the same, when other than the functionality for linking;

the two Ds are the same or different, normally being the same, when other than the functionality for linking;

either the As or the Ds are chalcogen ethers (chalcogen of atomic number 8 to 16), usually oxyethers, of the formula —JMX, where J is oxygen or sulfur;

when other than —JMX, the As are hydrogen or halo of atomic number 9 to 53 i.e. fluorine, chlorine, bromine, or iodine, particularly chloro and iodo, while the Ds may be hydrogen or any substituent, particularly having carbon and hydrogen and as heteroatoms, oxygen, sulfur, nitrogen and halogen, normally chemically inert under conditions of usage;

M is a divalent hydrocarbon group, normally saturated aliphatic, of from 1 to 8, usually 1 to 6 and

preferably 1 to 3 carbon atoms, usually straight chain;

one of the X's is an active functionality for linking to a ligand, receptor, or support or a functionality linked to said ligand or support;

wherein when X is taken together with W to provide an active functionality for linking, XW can be a non-oxo-carbonyl functionality including the sulfur and nitrogen analogs thereof, e.g. carboxylic acid, carboxylic acid ester, e.g. lower alkyl (1–3 carbon atoms) or active ester capable of forming amide bonds in an aqueous medium, e.g. N-oxy succinimide and p-nitrophenyl, isocyanate, isothiocyanate, imidate lower alkyl ester; activated olefin, e.g. maleimido; mercaptan (—SH); formyl (—CHO); sulfonyl chloride; amino; active halo e.g. haloacetyl or halotriazine, with the proviso that XW is non-oxo-carbonyl or sulfonyl when bonded to M;

when X is not taken together with W, one of the Xs is a linking functionality bonded to W and depending upon the particular active functionality will be non-oxo-carbonyl (including the nitrogen and sulfur analogs thereof) having one valence to carbon; carbamyl, thiocarbamyl; substituted ethylene from activated olefin; thio; methylene (from formyl by reductive amination); amido nitrogen or sec-amino; sulfonyl; or oxo-carbonyl methyl from active halo; when X is a linking functionality bonded to M, X is non-oxo-carbonyl; when not a linking group, X is hydrogen or non-oxo-carbonyl, e.g. carboxylic acid, ester or amide, sulfonamide, sulfonic acid or, particularly when bonded to an annular carbon atom, halo;

p is one when W is taken together with X and is otherwise on the average 1 to the molecular weight of W divided by 500, usually 1000, more usually 1500 and most usually 2000, generally p ranges from about 1 to 200, usually 1 to 100;

when W is not taken together with X, W is a ligand, including receptors, of at least about 125 molecular weight, being haptenic or antigenic, generally being from about 125 to 2000 molecular weight when haptenic and from about 5000 to 1×10^7 when antigenic, although combinations of antigens and other materials may have a much higher composite molecular weight; the ligand will be joined to X, normally through amino, hydroxy, mercapto or active ethylene, to form amido, amidine, thioamide, ether, or thioether, although other linkages may be employed, or W is a soluble or insoluble support which may be a polysaccharide, naturally occurring or synthetic, modified or unmodified, a naturally occurring or synthetic polymer, glass, inorganic solids, liposomes, or the like;

Q is a bond or spacer arm (linking chain), usually aliphatic, aromatic, heterocyclic, or combination thereof, generally aliphatically saturated, where the arm will usually have from 1 to 16, more usually 1 to 12, preferably 1 to 8 atoms in the chain, which are carbon, nitrogen, oxygen and sulfur, wherein the nitrogen is amido or bonded solely to carbon and hydrogen, e.g. tert-amino, oxygen is oxy, and sulfur is thioether, with the chalcogen bonded solely to carbon and heteroatoms being separated by at least two carbon atoms when bonded to saturated carbon atoms; the total number of carbon atoms being generally 1 to 20 usually 1 to 12 and the total number of heteroatoms being 0 to 10, usually 0 to 8; oxygen may be present as non-oxo-carbonyl or oxy, there being from 0 to 9, usually 0 to 4 heterofunctionalities; when X is not a linking functionality or group, Q will normally be a bond;

Y is halogen of atomic number 9 to 53, particularly chloro;

n is an integer of from 0 to 4 wherein m plus n is not greater than 4;

Z is an acidic anionic group, such as carboxylic acid or sulfonic acid; and

m is an integer of from 0 to 3, usually 1 to 3.

Quite obviously, the compounds of the subject invention can be modified so as not to be within the above formula, without significantly affecting the properties of the compounds. For example, one or more of the acidic anionic groups could be esterified or amidified, or alkyl groups can be substituted on the phenyl, as well as other groups, such as cyano, nitro, or the like. However, these changes will in most cases require additional synthetic steps which are not warranted by the degree of enhancement, if any, in the spectroscopic or chemical properties of the resulting product.

The subject compounds have many desirable properties. The products have significant water solubility which allow them to be conjugated to a wide variety of polypeptides, without significantly adversly affecting the water solubility of the polypeptide, nor having the polypeptide adversly affect the spectroscopic properties of the subject compounds.

As for the spectroscopic properties of the compounds, the compounds absorb at relatively long wavelengths, generally in excess of 500 nm, more usually in excess of 510 nm. Thus, naturally occurring fluorescence which may be encountered when working with physiological fluids is substantially avoided by employing exciting light at a wavelength range which does not significantly excite the naturally occurring fluorescers. In addition, the compounds have relatively sharp absorption peaks, and the fluorescers relatively sharp emission peaks. Because of this, efficient overlap can be obtained between fluorescers and quenchers which allow for efficient quenching up to distances of about 70 Å. The fluorescing compounds also have large Stokes shifts, so that the absorption band and emission band peaks are separated by at least 10 nm, frequently by at least 15 nm. The large Stokes shifts minimize background interference with the observed fluorescence.

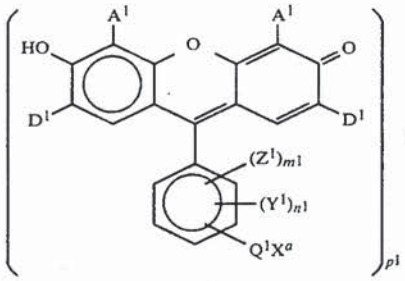
The quenchers have little or no fluorescence, so they do not contribute to background interference with the observed signal. By providing for fluorescer-quencher couples, where the absorption band of the quencher substantially overlaps the emission bands of the fluorescer, efficient systems are provided for performing immunoassays, which rely on quenching of fluorescence, when a quencher is brought into close proximity to the fluorescer due to binding of immunologically related materials.

In describing the subject invention, the simple monomeric spectroscopically active compounds used for conjugation will be considered first, followed by consideration of the various conjugates. The compounds are chemically stable, even at basic pHs, so that they maintain their spectroscopic properties during use.

The compounds employed for conjugation to other compounds will be characterized by having an active functionality which forms a stable covalent bond with another compound, usually an amide bond or thioether

bond. For the most part, the linking functionality will involve a non-oxo-carbonyl, including the nitrogen and sulfur analogues thereof, and may be bonded directly to an annular carbon atom of the phenyl group of the fluorescein, bonded through a linking group, or bonded directly or through a linking group to the oxy- or thioether functionality. Various functionalities may be employed which are compatible with the other functionalities in the molecule. The functionalities include carboxylic acid, which may be activated with carbodiimide or activating alcohols to provide active ester groups, isocyanates, isothiocyanates, imidates or the like which groups react with amino functionalities to form amides, thioamides or amidines. Alternatively, one can have amino groups as the functionality, which can be combined with carboxylic acids or derivatives to provide amide links. Finally, one can employ mercapto functionalities, which can be combined with ethylenic groups, particularly activated ethylenic groups, such as maleic acid derivatives, or vice versa, to provide thioethers.

For the most part, a preferred group of compounds will have the following formula:



wherein:

the two A^1s are the same or different, usually being the same except when one is the functionality for linking;

the two D^1s are the same or different, usually being the same except when one is the functionality for linking;

either the A^1s or the D^1s are oxyethers of the formula $-OM^1X^b$, wherein M^1 is a saturated aliphatic hydrocarbylene group of from 1 to 6, usually 1 to 3 carbon atoms, preferably straight chain and of from 1 to 2 carbon atoms, i.e. methylene or ethylene (hydrocarbylene intends a divalent organic radical composed solely of carbon and hydrogen); when not oxyethers, the A^1s are preferably hydrogen or halo particularly of atomic number 9 to 53, more particularly chloro or iodo, and the D^1s are preferably hydrogen, halo, or alkyl of up to six carbon atom;

one of the X^a or X^bs, usually X^a, is taken together with W^1 to form an active functionality, which may have the same definition as —XW, but will usually be a non-oxo-carbonyl containing functionality (including sulfur-thiono-analogs thereof), such as mixed anhydride, e.g. with butyl chloroformate, carboxylic acid, activated ester, isocyanate or isothiocyanate, with the proviso that X^b when taken together with W^1 is a carboxylic acid or derivative thereof;

when not taken together with W^1, one of the X^a or X^bs is a linking group to W^1 which is carbonyl, forming an amide or ester with W^1, carbamyl forming a urea with W^1, or thiocarbamyl forming a thiourea with W^1;

when not an active or linking functionality X^a is hydrogen, or non-oxo-carbonyl e.g. carboxy, and X^b is hydrogen or carboxyl, usually hydrogen;

Q^1 may be the same as Q, a bond or spacer arm, but will usually be a bond or spacer arm of from 1 to 12, usually 2 to 12 atoms in the chain which are carbon, nitrogen and oxygen, generally having from 1 to 10, usually 1 to 8 carbon atoms and 0 to 8 heteroatoms which are nitrogen, oxygen and sulfur, wherein oxygen is present bonded solely to carbon e.g. non-oxo-carbonyl or oxy ether, sulfur is analogous to oxygen and nitrogen is amido or bonded solely to carbon e.g. tertiary amino; Q^1 is usually a bond when X^a is other than a reactive functionality or linking functionality;

when W^1 is not taken together with X^a or X^b, W^1 is a ligand, receptor or support, usually having amino or hydroxyl, particularly amino functionalities for linking;

p^1 may be the same as p, being 1 when one of X^a or X^bs are taken together with W^1, and is otherwise 1 to the molecular weight of W^1 divided by 500, usually divided by 1500, generally in the range of 1 to 200, usually in the range of 1 to 100 and more usually in the range of 1 to 50;

Z^1 is an acidic anionic group, such as a carboxylic acid or sulfonic acid;

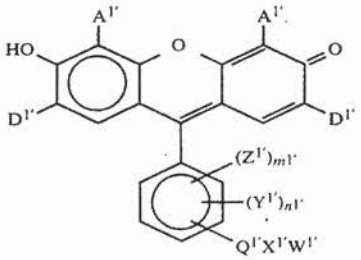
m^1 is an integer of from 1 to 3;

Y^1 is halogen of atomic number 9 to 53, particularly chloro;

n^1 is an integer of from 0 to 3, wherein m^1 plus n^1 is not greater than 4;

The compounds will normally have from 0 to 6, usually 0 to 5 halogen of atomic number 9 to 53, preferably chlorine or iodine, and usually from 0 to 4 chlorines, frequently 2 to 4 chlorines. The compounds will normally have at least two carboxylic acid groups and up to 5 carboxylic acids groups, preferably having from 2 to 3 carboxylic acid groups. The non-oxo-carbonyl linking functionality may or may not be bonded to a carbon atom, but is preferably bonded to a carbon atom.

The preferred compounds having the active functionality will for the most part have the following formula:



wherein:

either the $A^{1'}$s or $D^{1'}$s are alkoxy of from 1 to 3, usually 1 to 2 carbon atoms, when not alkoxy they are as previously described for As and Ds;

$Y^{1'}$, $Z^{1'}$, $m^{1'}$ and $n^{1'}$ have the same scope as the unprimed symbols;

$W^{1'}$ is an active functionality having a non-oxo-carbonyl group or sulfur analog (thiono) and includes

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