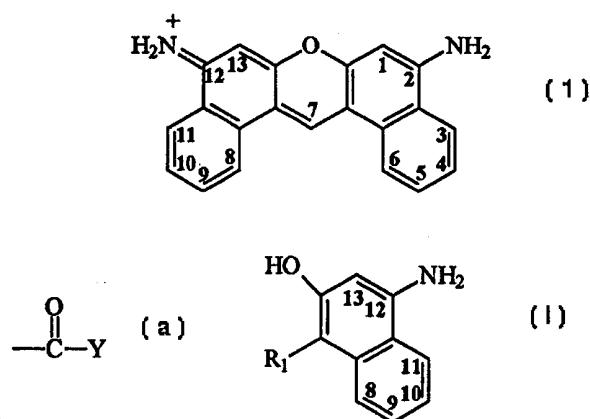




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(54) Title: DIBENZORHODAMINE DYES USEFUL AS FLOURESCENT LABELLING AGENTS



(57) Abstract

Dibenzorhodamine compounds having structure (1) are disclosed, including nitrogen- and aryl-substituted forms thereof. In addition, intermediates useful for synthesizing such compounds are disclosed, such intermediates having structure formula (I). In formula (I), R₁ is H or (a) wherein Y is H, lower alkyl, lower alkene, lower alkyne, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, or linking group, including substituted forms thereof. When R₁ is H, the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 7 members. The compounds of formula (I) further include aryl- and nitrogen-substituted forms thereof. The invention further includes energy transfer dyes comprising the dibenzorhodamine compounds, nucleosides labeled with the dibenzorhodamine compounds, and nucleic acid analysis methods employing the dibenzorhodamine compounds.

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DIBENZORHODAMINE DYES USEFUL AS FLUORESCENT LABELLING AGENTS

FIELD OF THE INVENTION

5 This invention relates generally to fluorescent dye compounds. More specifically, this invention relates to modified rhodamine dyes useful as fluorescent labeling reagents.

BACKGROUND

10 The non-radioactive detection of biological analytes utilizing fluorescent labels is an important technology in modern molecular biology. By eliminating the need for radioactive labels, safety is enhanced and the environmental impact and costs associated with reagent disposal is greatly reduced. Examples of methods utilizing such non-radioactive fluorescent detection include 4-color automated DNA sequencing, oligonucleotide hybridization methods, detection of polymerase-chain-reaction products, immunoassays, and the like.

15 In many applications it is advantageous to employ multiple spectrally distinguishable fluorescent labels in order to achieve independent detection of a plurality of spatially overlapping analytes, e.g., single-tube multiplex DNA probe assays and 4-color automated DNA sequencing methods. In the case of multiplex DNA probe assays, by employing spectrally distinguishable fluorescent labels, the number of reaction tubes may be reduced thereby simplifying experimental protocols and facilitating the production of application-specific reagent kits. In the case of 4-color automated DNA sequencing, multicolor fluorescent labeling allows for the analysis of multiple bases in a single lane thereby increasing throughput over single-color methods and reducing uncertainties associated with inter-lane electrophoretic mobility variations.

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30 Assembling a set of multiple spectrally distinguishable fluorescent labels is problematic. Multi-color fluorescent detection imposes at least six severe constraints on the selection of dye labels, particularly for applications requiring a single excitation light source, an electrophoretic separation, and/or treatment with enzymes, e.g., automated DNA sequencing. First, it is difficult to find a set of structurally similar dyes whose emission spectra are spectrally resolved, since the typical emission band half-width for

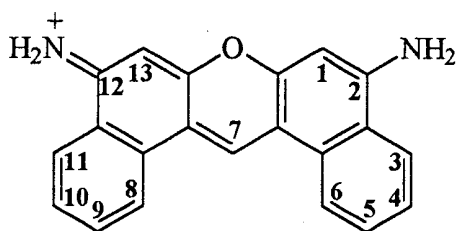
organic fluorescent dyes is about 40-80 nanometers (nm). Second, even if dyes with non-overlapping emission spectra are identified, the set may still not be suitable if the respective fluorescent quantum efficiencies are too low. Third, when several fluorescent dyes are used concurrently, simultaneous excitation becomes difficult because the absorption bands of the dyes are usually widely separated. Fourth, the charge, molecular size, and conformation of the dyes must not adversely affect the electrophoretic mobilities of the analyte. Fifth, the fluorescent dyes must be compatible with the chemistry used to create or manipulate the analyte, e.g., DNA synthesis solvents and reagents, buffers, polymerase enzymes, ligase enzymes, and the like. Sixth, the dye must have sufficient photostability to withstand laser excitation.

Currently available multiplex dye sets suitable in 4-color automated DNA sequencing applications require blue or blue-green laser light to adequately excite fluorescence emissions from all of the dyes making up the set, e.g., argon-ion lasers. Use of Blue or blue-green lasers in commercial automated DNA sequencing systems is disadvantageous because of the high cost and limited lifetime of such lasers.

SUMMARY

The present invention is directed towards our discovery of a class of dibenzorhodamine dye compounds suitable for the creation of sets of spectrally-resolvable fluorescent labels useful for multi-color fluorescent detection. The subject dye compounds are particularly well suited for use in automated 4-color DNA sequencing systems using an excitation light source having a wavelength greater than about 630 nm, e.g., a helium-neon gas laser or a solid state diode laser.

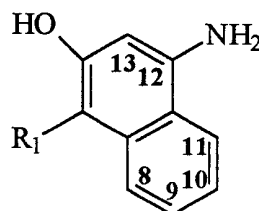
In a first aspect, the invention comprises dibenzorhodamine dye compounds having the structure



including nitrogen- and aryl-substituted forms thereof.

In a second aspect, the invention comprises intermediates useful for the synthesis of dibenzorhodamine compounds having the structure

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In Formula I, R_1 is H or $\text{—}\overset{\text{O}}{\parallel}\text{C—Y}$ wherein Y is H, lower alkyl, lower alkene, lower alkyne, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, or linking group, including substituted forms thereof. When R_1 is H, the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 7 members. The compounds of Formula I further include aryl- and nitrogen-substituted forms thereof.

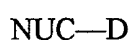
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In a third aspect, the invention includes energy transfer dye compounds comprising a donor dye, an acceptor dye, and a linker linking the donor and acceptor dyes. The donor dye is capable of absorbing light at a first wavelength and emitting excitation energy in response, and the acceptor dye is capable of absorbing the excitation energy emitted by the donor dye and fluorescing at a second wavelength in response. The linker serves to facilitate the efficient transfer of energy between the donor dye and the acceptor dye. According to the present invention, at least one of the donor and acceptor dyes is a dibenzorhodamine dye having the structure set forth above.

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wherein NUC is a nucleoside/tide or nucleoside/tide analog and D is a dibenzorhodamine dye compound having the structure set forth above. According to

the invention, NUC and D are connected by a linkage wherein the linkage is attached to D at one of the substituent positions. Furthermore, if NUC comprises a purine base, the linkage is attached to the 8-position of the purine, if NUC comprises a 7-deazapurine base, the linkage is attached to the 7-position of the 7-deazapurine, and if NUC comprises a
5 pyrimidine base, the linkage is attached to the 5-position of the pyrimidine.

In a fifth aspect, the invention includes polynucleotide analysis methods comprising the steps of forming a set of labeled polynucleotide fragments labeled with a dibenzorhodamine dye having the structure set forth above, subjecting the labeled
10 polynucleotide fragments to a size-dependent separation process, e.g., electrophoresis, and detecting the labeled polynucleotide fragments subsequent to the separation process.

Various aspects of the above-described invention achieve one or more of the following important advantages over known fluorescent dye compounds useful for
15 multiplex fluorescent detection: (1) the subject dye compounds may be efficiently excited by a low-cost red laser using wavelengths at or above 630 nm; (2) the emission spectra of the subject dye compounds can be modulated by minor variations in the type and location of nitrogen and/or aryl-substituents, allowing for the creation of dye sets having similar absorption characteristics yet spectrally resolvable fluorescence emission
20 spectra; (3) the subject dye compounds may be easily attached to nucleosides/tides or polynucleotides without compromising their favorable fluorescence properties; (4) the subject dye compounds have narrow emission bandwidths, i.e., the emission bandwidth has a full-width at half the maximum emission intensity of below about 70 nm; (5) the subject dye compounds are highly soluble in buffered aqueous solution while retaining a
25 high quantum yield; (6) the subject dye compounds are relatively photostable; and (7) the subject dye compounds have relatively large extinction coefficients, i.e., greater than about 50,000.

These and other features and advantages of the present invention will become
30 better understood with reference to the following description, figures, and appended claims.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1-3 show exemplary synthetic pathways for the synthesis of the 1-amino-3-hydroxynaphthalene intermediates of the invention.

5 FIG. 4 shows a generalized synthetic pathway for the synthesis of the dibenzorhodamine dye compounds of the invention.

FIGS. 5 and 6 show exemplary synthetic pathways for the synthesis of the dibenzorhodamine dye compounds of the invention.

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FIG. 7 shows the structures of several exemplary dibenzorhodamine dye compounds of the invention.

FIG. 8 shows a synthesis of compound 18.

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FIG. 9 shows a synthesis of compounds 57-61.

FIG. 10 shows a synthesis of compound 67.

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FIG. 11 shows a synthesis of compound 72.

FIG. 12 shows a synthesis of compound 76.

FIG. 13 shows a synthesis of compound 81.

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FIG. 14 shows a synthesis of compounds 82-84.

FIG. 15 shows a synthesis of compound 85.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings. While the

invention will be described in conjunction with the preferred embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the invention as defined by the appended
5 claims.

Generally, the present invention comprises a novel class of dibenzorhodamine dye compounds useful as fluorescent labels, methods and intermediates for synthesis of such dyes, reagents employing such dyes, and methods utilizing such dyes and reagents in the
10 area of analytical biotechnology. The compounds of the present invention find particular application in the area of fluorescent nucleic acid analysis, e.g., automated DNA sequencing and fragment analysis, detection of probe hybridization in hybridization arrays, detection of nucleic acid amplification products, and the like.

15 I. DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

"Spectral resolution" in reference to a set of dyes means that the fluorescent
20 emission bands of the dyes are sufficiently distinct, i.e., sufficiently non-overlapping, that reagents to which the respective dyes are attached, e.g. polynucleotides, can be distinguished on the basis of a fluorescent signal generated by the respective dyes using standard photodetection systems, e.g. employing a system of band pass filters and photomultiplier tubes, charged-coupled devices and spectrographs, or the like, as
25 exemplified by the systems described in U.S. Pat. Nos. 4,230,558, 4,811,218, or in Wheelless et al, pgs. 21-76, in *Flow Cytometry: Instrumentation and Data Analysis* (Academic Press, New York, 1985).

"Heterocycle" means cyclic compounds in which one or more ring atoms are not
30 carbon, i.e., are hetero atoms. Exemplary heterocycles include but are not limited to pyrrole, indole, furan, benzofuran, thiophene, benzothiophene and other like structures.

“Linking group” means a moiety capable of reacting with a “complementary functionality” attached to a reagent or member of an energy transfer dye pair, such reaction forming a “linkage” connecting the dye to the reagent or member of the energy transfer dye pair. Preferred linking groups include isothiocyanate, sulfonyl chloride, 4,6-dichlorotriazinyl, succinimidyl ester, or other activated carboxylates whenever the complementary functionality is amine. Preferably the linking group is maleimide, halo acetyl, or iodoacetamide whenever the complementary functionality is sulfhydryl. See R. Haugland, *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*, Molecular probes, Inc. (1992). In a particularly preferred embodiment, the linking group is a N-hydroxysuccinimidyl (NHS) ester and the complementary functionality is an amine, where to form the NHS ester, a dye of the invention including a carboxylate group is reacted with dicyclohexylcarbodiimide and N-hydroxysuccinimide.

“Substituted” as used herein refers to a molecule wherein one or more hydrogen atoms are replaced with one or more non-hydrogen atoms, functional groups or moieties. For example, an unsubstituted nitrogen is —NH_2 , while a substituted nitrogen is —NHCH_3 . Exemplary substituents include but are not limited to halo, e.g., fluorine and chlorine, lower alkyl, lower alkene, lower alkyne, sulfate, sulfonate, sulfone, amino, ammonium, amido, nitrile, lower alkoxy, phenoxy, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, and linking group.

“Polycyclic aromatic” means aromatic hydrocarbons having multiple ring structures including biaryls and condensed benzenoid hydrocarbons. The biaryls are benzenoid compounds where two or more rings are linked together by a single bond. The parent system of this class is biphenyl. The condensed benzenoid compounds are characterized by two or more benzene rings fused together at ortho positions in such a way that each pair of rings shares two carbons. The simplest members of this group are naphthalene, with two rings, and anthracene and phenanthrene, each with three rings.

“Lower alkyl” denotes straight-chain and branched hydrocarbon moieties containing from 1 to 8 carbon atoms, e.g., methyl, ethyl, propyl, isopropyl, tert-butyl, isobutyl, sec-butyl, neopentyl, tert-pentyl, and the like.

"Lower alkene" denotes a hydrocarbon containing from 1 to 8 carbon atoms wherein one or more of the carbon-carbon bonds are double bonds.

5 "Lower alkyne" denotes a hydrocarbon containing from 1 to 8 carbon atoms wherein one or more of the carbons are bonded with a triple bond.

"Nucleoside" refers to a compound consisting of a purine, deazapurine, or pyrimidine nucleoside base, e.g., adenine, guanine, cytosine, uracil, thymine, deazaadenine, deazaguanosine, and the like, linked to a pentose at the 1' position. When the nucleoside
10 base is purine or 7-deazapurine, the sugar moiety is attached at the 9-position of the purine or deazapurine, and when the nucleoside base is pyrimidine, the sugar moiety is attached at the 1-position of the pyrimidine, e.g., Kornberg and Baker, *DNA Replication*, 2nd Ed. (Freeman, San Francisco, 1992). The term "nucleotide" as used herein refers to a phosphate ester of a nucleoside, e.g., triphosphate esters, wherein the most common site of
15 esterification is the hydroxyl group attached to the C-5 position of the pentose. The term "nucleoside/tide" as used herein refers to a set of compounds including both nucleosides and nucleotides. "Analog" in reference to nucleosides/tides include synthetic analogs having modified base moieties, modified sugar moieties and/or modified phosphate moieties, e.g. described generally by Scheit, *Nucleotide Analogs* (John Wiley, New York, 1980).
20 Phosphate analogs comprise analogs of phosphate wherein the phosphorous atom is in the +5 oxidation state and one or more of the oxygen atoms is replaced with a non-oxygen moiety. Exemplary analogs include but are not limited to phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, boronophosphates, including associated counterions,
25 e.g., H^+ , NH_4^+ , Na^+ , if such counterions are present. Exemplary base analogs include but are not limited to 2,6-diaminopurine, hypoxanthine, pseudouridine, C-5-propyne, isocytosine, isoguanine, 2-thiopyrimidine, and other like analogs. Exemplary sugar analogs include but are not limited to 2'- or 3'-modifications where the 2'- or 3'-position is hydrogen, hydroxy, alkoxy, e.g., methoxy, ethoxy, allyloxy, isopropoxy, butoxy, isobutoxy
30 and phenoxy, amino or alkylamino, fluoro, chloro and bromo. The term "labeled nucleoside/tide" refers to nucleosides/tides which are covalently attached to the dye compounds of Formula I through a linkage.

“Water solublizing group” means a substituent which increases the solubility of the compounds of the invention in aqueous solution. Exemplary water-solubilizing groups include but are not limited to quaternary amine, sulfate, sulfonate, carboxylate, phosphate, polyether, polyhydroxyl, and boronate.

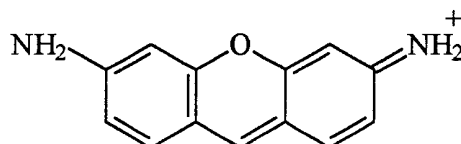
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“Polynucleotide” or “oligonucleotide” means polymers of natural nucleotide monomers or analogs thereof, including double and single stranded deoxyribonucleotides, ribonucleotides, α -anomeric forms thereof, and the like. Usually the nucleoside monomers are linked by phosphodiester linkages, where as used herein, the term “phosphodiester linkage” refers to phosphodiester bonds or bonds including phosphate analogs thereof, including associated counterions, e.g., H^+ , NH_4^+ , Na^+ , if such counterions are present. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, to several thousands of monomeric units. Whenever a polynucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'->3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes deoxythymidine, unless otherwise noted.

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“Rhodamine dye” refers to dyes including the general polycyclic structure



including any and all substituted versions thereof.

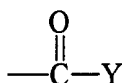
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II. 1-AMINO-3-HYDROXYNAPHTHALENE INTERMEDIATES

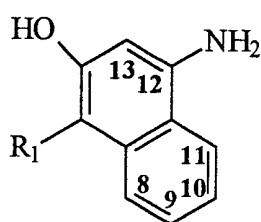
A. Structure

In a first aspect, the present invention comprises a novel class of 1-amino-3-hydroxynapthalene compounds useful as intermediates in the synthesis of dibenzorhodamine dyes. These compounds have the general structure shown in Formula I immediately below, including substituted forms thereof. In Formula I, R_1 is H or

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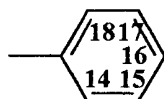


wherein Y is H, lower alkyl, lower alkene, lower alkyne, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, or linking group, including substituted forms thereof. When R₁ is H, the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 7 members. The compounds of Formula I further include aryl- and nitrogen-substituted forms thereof. (Note that all molecular structures provided herein are intended to encompass not only the exact electronic structures presented, but also include all resonant structures, protonation states and associated counterions thereof.)



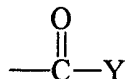
FORMULA I

In one preferred embodiment of the compounds of Formula I, Y is phenyl or substituted phenyl, the phenyl having the structure



Substituents at any one of positions C-14 to C-18 may be any suitable substituent. Preferably, the substituents are chlorine, fluorine, lower alkyl, carboxylic acid, sulfonic acid, -CH₂OH, alkoxy, phenoxy, linking group, water solubilizing group, and substituted forms thereof. In a particularly preferred embodiment, a substituent at position C-18 is selected from the group consisting of carboxylic acid and sulfonate, most preferably carboxylic acid. In another particularly preferred embodiment, a substituent at positions C-14 and C-17 is chlorine. In another preferred embodiment, a substituent at positions C-14 to C-17 is chlorine or fluorine. In yet another particularly preferred embodiment, a substituent at one of positions C-15 and C-16 is linking group and the other is hydrogen, a substituent at positions C-14 and C-17 is chlorine, and a substituent at position C-18 is carboxy.

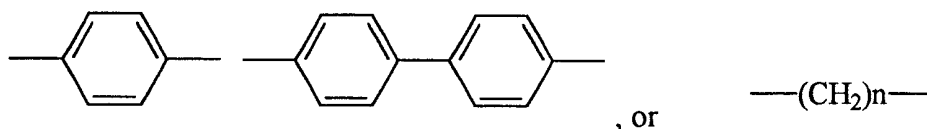
In another preferred embodiment of the compounds of Formula I, the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 7 members. More preferably, the first and/or second ring structure has five members, where preferably the five membered ring structure includes one gem disubstituted carbon. Preferably, the gem substituents are lower alkyl, e.g., methyl. In another preferred embodiment, the five membered ring is substituted with linking group. In this preferred embodiment wherein the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 7 members, R₁ may be H or



wherein Y is H, lower alkyl, lower alkene, lower alkyne, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, or linking group, including substituted forms thereof.

In yet another preferred embodiment of the compounds of Formula I, the compounds include a substituted nitrogen. Preferred nitrogen substituents include lower alkyl, lower alkene, lower alkyne, phenyl, aromatic, heterocycle, polycyclic aromatic, water-solubilizing group, linking group, and substituted forms thereof. More preferably, the nitrogen substituents are lower alkyl, phenyl, or substituted forms thereof, wherein preferred substituents are linking group, sulfonate or water-solubilizing group. Exemplary water-solubilizing groups are carboxylate, sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer. In a particularly preferred

embodiment, the nitrogen substituent is ---L---R , wherein L may be any linker and R is linking group or water-solubilizing group. In a particularly preferred embodiment, L is,



where n ranges from 1 to 8. In another particularly preferred embodiment, the water solubilizing group is carboxylate, sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

5 In yet another preferred embodiment, the compounds of Formula I include one or more substituents at one or more of positions C-8 to C-11, and C-13. Preferred substituents include fluorine, chlorine, lower alkyl, lower alkene, lower alkyne, sulfate, sulfonate, sulfone, sulfonamide, sulfoxide, amino, ammonium, amido, nitrile, lower alkoxy, phenoxy, aromatic, phenyl, polycyclic aromatic, water-solubilizing group, heterocycle, and
10 linking group, including substituted forms thereof. In a particularly preferred embodiment, the compound includes a fused aromatic ring bonded across the C-9 and C-10 carbons, or across the C-10 and C-11 carbons, including substituted forms thereof, where a particularly preferred substituent is sulfonate.

15 Several representative 1-amino-3-hydroxynaphthalene compounds of the invention are shown in FIGS. 1-3, i.e., compounds 4, 9, 15, 17, 22, 27 and 29, and FIGS. 10-13, i.e., compounds 62, 63, 68, 73 and 80.

B. Synthetic Methods

20 Several synthetic methods are available for the synthesis of the 1-amino-3-hydroxynaphthalene compounds described above, different methods being preferred depending on the nature of the ring structure and the nitrogen substituents of the particular compound to be synthesized.

25 A first preferred synthesis method suitable for the synthesis of 1-substituted-amino-3-hydroxynaphthalene compounds, e.g., 1-diethylamino-3-hydroxynaphthalene 4, is shown in FIG. 1. In this first method, a 3-methoxy-1-hydroxy naphthalene 1 is reacted with dry triethylamine and trifluoromethanesulfonic anhydride to form a crude 3-methoxynaphthalene-1-triflate 2. The triflate 2 is then reacted with an amine, e.g., a
30 secondary amine, e.g., diethylamine, using palladium catalyzed triflate/amine coupling to form the substituted amine compound 3. Compound 3 is then deprotected using a boron tribromide deprotection procedure to produce the 1-amino-3-hydroxynaphthalene

product, e.g., 1-diethylamino-3-hydroxynaphthalene **4**. An example of this synthesis is provided in Example 1 below.

A second preferred synthesis method suitable for the synthesis of benzindoline
5 compounds, e.g., N-phenyl-3,3-dimethyl-4-hydroxy-benzindoline **9**, is also shown in
FIG. 1. In this method, the 3-methoxynaphthalene-1-triflate **2** is derivatized with a
primary amine, e.g., aniline, using a palladium catalyzed triflate coupling reaction to
give a secondary amine, e.g., 1-anilino-3-methoxynaphthalene **5**. The secondary amine **5**
is acetylated using an acid chloride, e.g., an haloacetylchloride, to give a disubstituted
10 amide, e.g., 1-amido-3-methoxynaphthalene **6**. The tertiary amide **6** is cyclized using a
Lewis-acid-catalyzed Friedel-Crafts cyclization procedure to give compound **7**, e.g.,
using AlCl_3 . Compound **7** is then reduced, e.g., using (lithium aluminum hydride)
LAH, to give compound **8**. Subsequent methoxy group deprotection by a boron
tribromide deprotection procedure gives the benzindoline, e.g., N-phenyl-3,3-
15 dimethyl-4-hydroxy-benzindoline **9**. An example of this synthesis is provided in
Example 2 below.

A third preferred synthesis method suitable for the synthesis of N-substituted-5-
hydroxy-(tetrahydro)benzoquinoline compounds, e.g., N-methyl-5-hydroxy-
20 (tetrahydro)benzoquinoline **15**, is shown in FIG. 2. In this method, compound **10** is
synthesized from methoxy-naphthaldehyde by condensation with malonic acid using a
piperidine catalyst in pyridine. Compound **10** is then reduced with hydrogen, followed
by LAH reduction, and reacted with trifluoromethanesulfonic anhydride to give the
triflate **11**. The triflate **11** is reacted with NaN_3 to give compound **12**. Compound **12** is
25 complexed with a Lewis acid, e.g., AlCl_3 , and refluxed yielding the cyclized
benzoquinoline derivative **13**. Next, a nitrogen substituent is added, e.g., the nitrogen is
alkylated using a conventional alkylation procedure, e.g., the benzoquinoline derivative
13 is reacted with with n-butyl lithium and an alkylating agent, e.g., MeI to give
compound **14** or propane sultone to give compound **16**. The methoxy group is then
30 removed by a boron tribromide procedure giving a N-alkylbenzoquinoline derivative,
e.g., compound **15** or **17**. An example of this synthesis is provided in Example 3 below.

A fourth preferred synthesis method suitable for the synthesis of N-substituted-2,2,4-trimethyl-5-hydroxy-benzoquinoline compounds, e.g. of N-methyl-2,2,4-trimethyl-5-hydroxy-(tetrahydro)benzoquinoline **22**, is shown in FIG. 3. In this method, following the procedure of A. Rosowsky and E.J. Modest (J.O.C. 30 1832
5 1965, and references therein), 1-amino-3-methoxynaphthalene **18** is reacted with acetone catalyzed by iodine and then quenched with saturated $\text{Na}_2\text{S}_2\text{O}_3$ to give the benzoquinoline compound **19**. Compound **19** is then alkylated with an alkylating agent, e.g., MeI, according to a general alkylation procedure to give compound **20**. The alkylated compound **20** is reduced with H_2 catalyzed by Pd/C to give a N-methyl-
10 methoxyquinoline intermediate **21**, and subsequent methoxy group deprotection by a general boron tribromide procedure yields the N-substituted-2,2,4-trimethyl-5-hydroxy-benzoquinoline compound, e.g., N-methyl-2,2,4-trimethyl-5-hydroxy-(tetrahydro)benzoquinoline **22**. An example of this synthesis is provided in Example 4 below.

15

A fifth preferred general synthesis method suitable for the synthesis of N-substituted-3,3-dimethyl-4-hydroxy-benzoindoline compounds, e.g. N-methyl-3,3-dimethyl-4-hydroxy-benzoindoline **27**, is also shown in FIG. 3. In this method, a 1-amino-3-methoxynaphthalene **18** is acetylated with an acid chloride, e.g., 2-bromo-2-methylpropionyl chloride, to give compound **23**. Compound **23** is cyclized by reaction
20 with AlCl_3 to give compound **24**. Compound **24** is then reduced with LAH to give the 3,3-dimethyl-4-methoxybenzoindoline **25**. Compound **25** is then alkylated with an alkylating agent, e.g., methyl iodide, to give a N-methyl-3,3-dimethyl-4-methoxybenzoindoline, e.g., compound **26**. Subsequent methoxy group deprotection
25 by with boron tribromide gives compound **27**. An example of this synthesis is provided in Example 5 below.

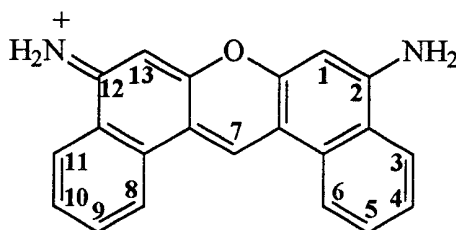
III. DIBENZORHODAMINE DYE COMPOUNDS

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A. Structure

In a second aspect, the present invention comprises a novel class of dibenzorhodamine dye compounds useful as molecular labels having the general structure

shown in Formula II immediately below, including aryl- and nitrogen-substituted forms thereof.

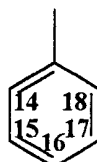


FORMULA II

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In one preferred embodiment of the compound of Formula II, the compound includes a first bridging group which when taken together with the C-12-bonded nitrogen and the C-12 and C-13 carbons forms a first ring structure having from 4 to 7 members, and/or a second bridging group which when taken together with the C-2-bonded-nitrogen and the C-1 and C-2 carbons forms a second ring structure having from 4 to 7 members. More preferably, one or both of the first and second ring structures has five members. In yet a more preferred embodiment, the five membered ring structure includes one gem disubstituted carbon, wherein the gem substituents are lower alkyl, e.g., methyl. In an alternative preferred embodiment, the five membered ring is substituted with linking group. In another preferred embodiment, the five membered ring includes one or more nitrogen substituents, as described below.

In another preferred embodiment, the compound of Formula II includes a C-7 substituent selected from the group consisting of acetylene, lower alkyl, lower alkene, cyano, phenyl, heterocyclic aromatic, heterocycle, and substituted forms thereof. In a more preferred embodiment, the C-7 substituent is a phenyl or substituted phenyl having the structure



wherein aryl substituents at positions C-14 to C-18 taken separately may be selected from the group consisting of hydrogen, chlorine, fluorine, lower alkyl, carboxylic acid, sulfonic

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acid, $-\text{CH}_2\text{OH}$, alkoxy, phenoxy, linking group, and substituted forms thereof. Preferably, the phenyl substituent at C-18 is selected from the group consisting of carboxylic acid and sulfonate, and is most preferably carboxylic acid. In another preferred embodiment, substituents at positions C-14 and C-17 are chlorine. In yet another preferred embodiment, substituents at positions C-14 to C-17 are all chlorine or all fluorine. In a particularly preferred embodiment, substituents at one of positions C-15 and C-16 is linking group and the other is hydrogen, substituents at positions C-14 and C-17 are chlorine, and a substituent at position C-18 is carboxy.

In an additional preferred embodiment, the compound of Formula II comprises a hydrogen radical at the C-7 position, i.e., the C-7 position is unsubstituted.

In yet another preferred embodiment of the invention, the compound of Formula II includes one or more nitrogen substituents. Preferably, such substituents are selected from the group consisting of lower alkyl, lower alkene, lower alkyne, phenyl, aromatic, heterocycle, polycyclic aromatic, water-solubilizing group, linking group, and substituted forms thereof. More preferably, the nitrogen substituents are selected from the group consisting of lower alkyl, phenyl, polycyclic aromatic, and substituted forms thereof, where exemplary substituents include linking group, and water-solubilizing group.

In another preferred embodiment of this second aspect of the invention, the compound of Formula II includes a third bridging group which when taken together with the C-12-bonded nitrogen and the C-11 and C-12 carbons forms a third ring structure having from 5 to 7 members, and/or a fourth bridging group which when taken together with the C-2-bonded nitrogen and the C-2 and C-3 carbons forms a fourth ring structure having from 5 to 7 members. Preferably, one or both of the third and fourth ring structures has six members. More preferably, the six membered ring structure includes one gem disubstituted carbon, wherein the gem substituents are lower alkyl, e.g., methyl.

In another preferred embodiment of the invention, the compound of Formula II includes aryl substituents at one or more of carbons C-1, C-3 through C-6, C-8 through

C-11, and C-13. Exemplary aryl substituents include but are not limited to fluorine, chlorine, lower alkyl, lower alkene, lower alkyne, sulfate, sulfonate, sulfone, sulfonamide, sulfoxide, amino, ammonium, amido, nitrile, lower alkoxy, phenoxy, aromatic, phenyl, polycyclic aromatic, water-solubilizing group, heterocycle, and linking group, including substituted forms thereof. In a particularly preferred embodiment, at least one substituent is sulfonate. In another particularly preferred embodiment, the compound of Formula II includes a fused aromatic ring bonded across the C-3 and C-4 carbons, the C-4 and C5 carbons, the C-9 and C-10 carbons, or the C-10 and C-11 carbons, including substituted forms thereof. Most preferably, the fused aromatic ring is bonded across the C-3 and C-4 carbons and the C-10 and C11 carbons, or across the C-9 and C-10 carbons and the C-4 and C-5 carbons, including substituted forms thereof.

Several exemplary dye compounds according to this second aspect of the invention are shown in FIGS. 7 and 10-13, i.e., compounds 41-47 and 67, 72, 76 and 81.

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B. Synthetic Methods

Generally, the dibenzorhodamine dyes of the present invention are synthesized as follows. See FIG. 4. An anhydride derivative 30, e.g., a phthalic anhydride, is mixed with 1-amino-3-methoxy intermediates 31 and 32, and Lewis acid, e.g., $ZnCl_2$, where the R- substituents in compound 30 may be the same or different, but are preferably the same. Exemplary R-substituents include but are not limited to acetylene, lower alkyl, lower alkene, phenyl, heterocycle, and substituted forms thereof. The mixture is heated briefly until melting is observed. A solvent, e.g., 1,2-dichlorobenzene, is added to the reaction mixture, and the heterogeneous mixture is heated to about 130°C to about 180°C. The crude reaction mixture is cooled and purified by normal phase flash chromatography to yield dye compound 33. When the anhydride is part of a substituted phthalic anhydride, e.g., compound 34, two isomers are formed. See FIG 5. The isomers 35 and 36 are separated by PTLC. The isomerically pure dyes are identified by single spots on normal and reverse phase TLC and by their UV/Visible absorption spectra and their long wavelength fluorescent excitation and emission spectra.

30

An alternative procedure for the synthesis of asymmetrical dye compounds is shown in FIG. 6. In this process, an anhydride derivative, e.g., phthalic anhydride **34**, is mixed with dry nitrobenzene and heated. The mixture is cooled to room temperature and anhydrous AlCl_3 is added with stirring. Subsequently a 1-amino-3-methoxy intermediate **31** is added with stirring and the reaction is heated. The reaction is cooled and suspended in EtOAc. The organic layer is washed with saturated NH_4Cl , brine, dried over Na_2SO_4 , filtered, and the solvent removed in vacuo. The resulting ketone intermediates **37/38** are purified and separated into distinct isomers **37** and **38** (except where substituents at C-14 and C-17 are the same and substituents at C-15 and C-16 are the same) by flash chromatography or recrystallization. The methoxy group of the isomerically pure ketone intermediate **37** or **38** is removed according to a general boron tribromide deprotection procedure. Thus, compound **38** gives the amino-hydroxynaphthalene ketone intermediate **39**. Amino-hydroxynaphthalene ketone intermediate **39** is then reacted with a 1-amino-3-methoxy intermediate **32**. The reaction is cooled, giving isomerically pure and asymmetrically substituted product **40** that may be further purified by PTLC.

In another method for synthesizing the dibenzorhodamine dyes of the present invention particularly suited to the synthesis of dyes not substituted at the C-7 position, i.e., pyronine dyes, the dyes are synthesized from hydroxybenzoindoline intermediates generated from O-protected N-substituted 3-hydroxybenzoindoline compounds following deprotection of the oxygen protecting group, e.g. methyl group deprotection by a demethylating reagent i.e., aluminum chloride, and isolated by normal phase chromatographic purification. According to the synthesis, a hydroxybenzoindoline intermediate, corresponding to one half of a dye molecule, is first reacted with a formylating reagent, e.g. methylformanilide/ POCl_3 , and the ensuing formylated hydroxybenzoindoline intermediate is reacted directly with a different (or same) hydroxybenzoindoline intermediate, corresponding to the other half of the dye molecule. The reaction is run under acidic dehydrating conditions, i.e. POCl_3 , and heat, i.e. 120-160 °C to give the crude carboxylic acid ester derivatized benzopyronine dye. In some cases, the methoxybenzoindoline intermediate is formylated prior to methyl group deprotection and then the methyl group of the formylated methoxyindoline derivative is

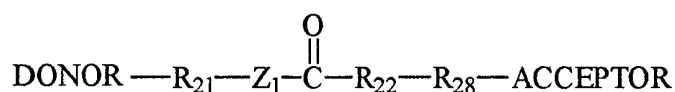
deprotected to give the formylated hydroxybenzoinoline intermediate prior to reaction with the second equivalent of hydroxyindoline to give the pyronine dye. The pure dye is isolated after aqueous work-up and normal phase chromatography. The intermediate dye carboxylic acid ester is then hydrolysed with acid, i.e. HBr, to give the free acid dye derivatives after aqueous work-up and normal phase chromatography. N-phenyl substituted dyes may be subsequently sulfonated with a sulfonating agent, e.g., ClSO₃H to give a final preferred dye derivative after aqueous work-up and normal phase chromatography.

10 V. ENERGY TRANSFER DYES INCORPORATING THE DIBENZORHODAMINE DYES

In another aspect, the present invention comprises energy transfer dye compounds incorporating the dibenzorhodamine dye compounds of Formula I. Generally, the energy transfer dyes of the present invention include a donor dye which absorbs light at a first wavelength and emits excitation energy in response, an acceptor dye which is capable of absorbing the excitation energy emitted by the donor dye and fluorescing at a second wavelength in response, and a linker which attaches the donor dye to the acceptor dye, the linker being effective to facilitate efficient energy transfer between the donor and acceptor dyes. A through discussion of the structure, synthesis and use of such energy transfer dyes is provided by Lee et al., U.S. Patent No. 5,800,996, and Mathies et al., U.S. Patent No. 5,654,419.

In a preferred embodiment, the linker includes a functional group which gives the linker some degree of structural rigidity, such as an alkene, diene, an alkyne, a five and six membered ring having at least one unsaturated bond or a fused ring structure.

One preferred linker according to the present invention for linking a donor dye to an acceptor dye in an energy transfer fluorescent dye has the general structure

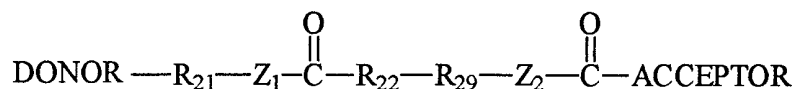


30 where R₂₁ is a lower alkyl attached to the donor dye, Z₁ is either NH, sulfur or oxygen,

R₂₂ is a substituent which includes an alkene, diene, alkyne, a five and six membered ring having at least one unsaturated bond or a fused ring structure which is attached to the carbonyl carbon, and R₂₈ includes a functional group which attaches the linker to the acceptor dye.

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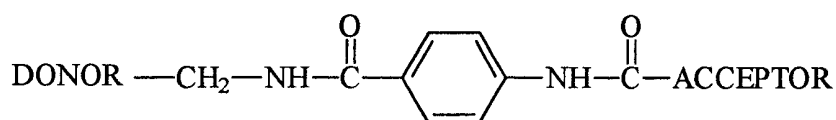
In one embodiment of this linker, illustrated below, the linker has the general structure



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where R₂₁ and R₂₂ are as detailed above, Z₁ and Z₂ are each independently either NH, sulfur or oxygen, R₂₉ is a lower alkyl, and the terminal carbonyl group is attached to a ring structure of the acceptor dye. In the variation where Z₂ is nitrogen, the C(O)R₂₂R₂₉Z₂ subunit forms an amino acid subunit. Particular examples of five or six membered rings which may be used as R₂₂ in the linker include, but are not limited to cyclopentene, cyclohexene, cyclopentadiene, cyclohexadiene, furan, thiofuran, pyrrole, pyrazole, isoimidazole, pyran, pyrone, benzene, pyridine, pyridazine, pyrimidine, triazine, pyrazine and oxazine. Examples of fused ring structures include, but are not limited to indene, benzofuran, thionaphthene, indole and naphthalene. A preferred embodiment of this linker is where R₂₁ and R₂₉ are methylene, Z₁ and Z₂ are NH, and R₂₂ is benzene, as shown below.

15



20

In another preferred embodiment of the energy-transfer-dye aspect of the present invention, the linker attaches to the dibenzorhodamine dye component of the energy transfer dye at the C-1 or 13 positions, or, alternatively, where the C-7 substituent is phenyl or substituted phenyl, at one of the C-15 or C-16 positions. In a

particularly preferred embodiment, both members of the energy transfer pair are dibenzorhodamine dyes, and the first member is linked through the C-1 position and the second member is linked through one of the C-15 or C-16 positions.

5 In yet another preferred embodiment of the energy-transfer-dye aspect of the present invention, a first member the dye pair is a dibenzorhodamine dye, and a second member of the dye pair is cyanine, phthalocyanine, squaraine, bodipy, fluorescein, or dibenzorhodamine dye having different substitutions than the first member.

10 VI. REAGENTS INCORPORATING THE DIBENZORHODAMINE DYES

In another aspect, the present invention comprises reagents labeled with the dibenzorhodamine dye compounds of Formula I. Reagents of the invention can be virtually anything to which the dyes of the invention can be attached. Preferably, the dye is covalently attached to the reagent. Reagents may include but are not limited to
15 proteins, polypeptides, polysaccharides, nucleotides, nucleosides, polynucleotides, lipids, solid supports, organic and inorganic polymers, and combinations and assemblages thereof, such as chromosomes, nuclei, living cells, such as bacteria or other microorganisms, mammalian cells, tissues, and the like.

20 A. Nucleoside/tide Reagents

A preferred class of labeled reagents comprise nucleoside/tides that incorporate the dibenzorhodamine dyes of the invention. Such nucleoside/tide reagents are particularly useful in the context of labeling polynucleotides formed by enzymatic synthesis, e.g., nucleotide triphosphates used in the context of PCR amplification, Sanger-type
25 polynucleotide sequencing, and nick-translation reactions.

Generally, the structure of the labeled nucleoside/tide reagent is

NUC—D

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FORMULA III

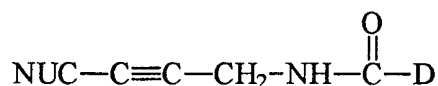
where NUC is a nucleoside/tide or nucleoside/tide analog and D is a dibenzorhodamine dye compound of Formula II.

5 The linkage linking the nucleoside/tide and the dye may be attached to the dye at any one of substituent positions C-1 to C-18 or at a C-2 bonded nitrogen or a C-12 bonded nitrogen. Preferably, the dye includes a phenyl or substituted phenyl substituent at the C-7 position and is attached to the nucleoside/tide at one of the C-15 or C-16 substituent positions, the other position being a hydrogen atom.

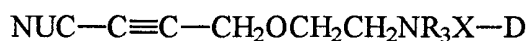
10 When NUC includes a purine base, the linkage between NUC and D is attached to the N⁸-position of the purine, when NUC includes a 7-deazapurine base, the linkage is attached to the N⁷-position of the 7-deazapurine, and when NUC includes a pyrimidine base, the linkage is attached to the N⁵-position of the pyrimidine.

15 Nucleoside labeling can be accomplished using any one of a large number of known nucleoside/tide labeling techniques employing known linkages, linking groups, and associated complementary functionalities. Generally, the linkage linking the dye and nucleoside should (i) not interfere with oligonucleotide-target hybridization, (ii) be compatible with relevant enzymes, e.g., polymerases, ligases, and the like, and (iii) not
20 adversely affect the fluorescence properties of the dye. Exemplary base labeling procedures suitable for use in connection with the present invention include the following: Gibson et al, *Nucleic Acids Research*, 15:6455-6467 (1987); Gebeyehu et al, *Nucleic Acids Research*, 15: 4513-4535 (1987); Haralambidis et al, *Nucleic Acids Research*, 15: 4856-4876 (1987); Nelson et al., *Nucleosides and Nucleotides*, 5(3): 233-241 (1986);
25 Bergstrom, et al., *JACS*, 111: 374-375 (1989); and U.S. Patent Nos. 4,855,225, 5,231,191, and 5,449,767.

Preferably, the linkages are acetylenic amido or alkenic amido linkages, the linkage
30 between the dye and the nucleoside/tide base being formed by reacting an activated N-hydroxysuccinimide (NHS) ester of the dye with an alkynylamino- or alkenylamino-derivatized base of a nucleoside/tide. More preferably, the resulting linkage is 3-(carboxy)amino-1-propyn-1-yl having the structure



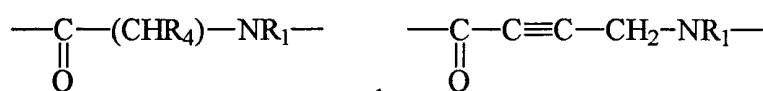
Alternative preferred linkages include substituted propargylethoxyamido linkages having the structure



5

wherein X is selected from the group consisting of $\text{---}\overset{\text{O}}{\parallel}{\text{C}}\text{---}(\text{CH}_2)_n\text{---NR}_1\text{---}$ where n

ranges from 1 to 5, $\text{---}\overset{\text{O}}{\parallel}{\text{C}}\text{---}\phi\text{---}(\text{CH}_2)_n\text{---NR}_1\text{---}$ where n ranges from 1 to 5,



, and

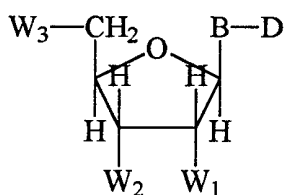
; R₁ is selected from the

group consisting of —H, lower alkyl and protecting group; and R₃ is selected from the group consisting of —H and lower alkyl. See Khan et al., U.S. Patent Application Serial No. 08/833,854 filed April 10, 1997.

The synthesis of alkynylamino-derivatized nucleosides is taught by Hobbs et al. in European Patent Application No. 87305844.0, and Hobbs et al., *J. Org. Chem.*, 54: 3420
 15 (1989). Briefly, the alkynylamino-derivatized nucleotides are formed by placing the appropriate halodideoxynucleoside (usually 5-iodopyrimidine and 7-iodo-7-deazapurine dideoxynucleosides as taught by Hobbs et al. (cited above)) and Cu(I) in a flask, flushing with argon to remove air, adding dry DMF, followed by addition of an alkynylamine, triethyl-amine and Pd(0). The reaction mixture is stirred for several hours,
 20 or until thin layer chromatography indicates consumption of the halodideoxynucleoside. When an unprotected alkynylamine is used, the alkynylamino-nucleoside can be isolated by concentrating the reaction mixture and chromatographing on silica gel using an eluting solvent which contains ammonium hydroxide to neutralize the hydrohalide generated in the coupling reaction. When a protected alkynylamine is used,
 25 methanol/methylene chloride can be added to the reaction mixture, followed by the

bicarbonate form of a strongly basic anion exchange resin. The slurry can then be stirred for about 45 minutes, filtered, and the resin rinsed with additional methanol/methylene chloride. The combined filtrates can be concentrated and purified by flash-chromatography on silica gel using a methanol-methylene chloride gradient. The triphosphates are
5 obtained by standard techniques.

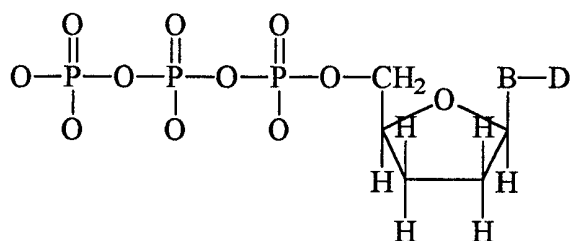
Particularly preferred nucleosides/tides of the present invention are shown below in Formula IV wherein



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FORMULA IV

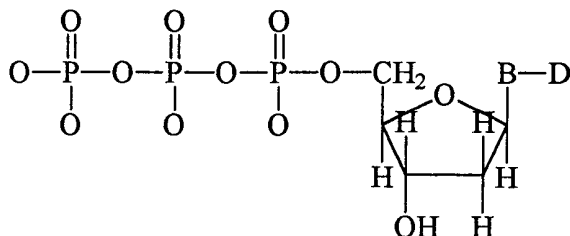
B is a nucleoside/tide base, e.g., uracil, cytosine, deazaadenine, or deazaguanosine; W_1 and W_2 taken separately are OH or a group capable of blocking polymerase-mediated template-directed polymerization, e.g., H, fluorine and the like; W_3 is OH, or mono-, di- or triphosphate or phosphate analog; and D is a dye compound of Formula I. In one
15 particularly preferred embodiment, the nucleotides of the present invention are dideoxynucleotide triphosphates having the structure shown in Formula IV.1 below, including associated counterions if present.



FORMULA IV.1

20 Labeled dideoxy nucleotides such as that shown in Formula IV.1 find particular application as chain terminating agents in Sanger-type DNA sequencing methods utilizing fluorescent detection.

In a second particularly preferred embodiment, the nucleotides of the present invention are deoxynucleotide triphosphates having the structure shown in Formula IV.2 below.



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FORMULA IV.2

Labeled deoxynucleotides such as that shown in Formula IV.2 find particular application as reagents for labeling polymerase extension products, e.g., in the polymerase chain reaction or nick-translation.

10 B. Polynucleotide Reagents

Yet another preferred class of reagents of the present invention comprise polynucleotides labeled with the dibenzorhodamine dyes of the invention. Such labeled polynucleotides are useful in a number of important contexts including as DNA sequencing primers, PCR primers, oligonucleotide hybridization probes, oligonucleotide ligation probes, and the like.

15

In one preferred embodiment, the labeled polynucleotides of the present invention include multiple dyes located such that fluorescence energy transfer takes place between a donor dye and an acceptor dye. Such multi-dye energy-transfer polynucleotides find application as spectrally-tunable sequencing primers, e.g., Ju et al., *Proc. Natl. Acad. Sci. USA* 92: 4347-4351 (1995), and as hybridization probes, e.g., Lee et al. *Nucleic Acids Research*, 21: 3761-3766 (1993).

20

Labeled polynucleotides may be synthesized either enzymatically, e.g., using a DNA polymerase or ligase, e.g., Stryer, *Biochemistry*, Chapter 24, W.H. Freeman and Company (1981), or by chemical synthesis, e.g., by the phosphoramidite method, the phosphite-triester method, and the like, e.g., Gait, *Oligonucleotide Synthesis*, IRL Press (1990). Labels may be introduced during enzymatic synthesis utilizing labeled nucleotide

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triphosphate monomers as described above, or introduced during chemical synthesis using labeled non-nucleotide or nucleotide phosphoramidites as described above, or may be introduced subsequent to synthesis.

5 Generally, if the labeled polynucleotide is made using enzymatic synthesis, the following procedure may be used. A template DNA is denatured and an oligonucleotide primer is annealed to the template DNA. A mixture of deoxynucleotide triphosphates is added to the mixture including dGTP, dATP, dCTP, and dTTP where at least a fraction of the deoxynucleotides is labeled with a dye compound of the invention as described above.

10 Next, a polymerase enzyme is added under conditions where the polymerase enzyme is active. A labeled polynucleotide is formed by the incorporation of the labeled deoxynucleotides during polymerase-mediated strand synthesis. In an alternative enzymatic synthesis method, two primers are used instead of one, one primer complementary to the + strand and the other complementary to the - strand of the target, the polymerase is a

15 thermostable polymerase, and the reaction temperature is cycled between a denaturation temperature and an extension temperature, thereby exponentially synthesizing a labeled complement to the target sequence by PCR, e.g., *PCR Protocols*, Innis et al. eds., Academic Press (1990).

20 Labeled polynucleotides may be chemically synthesized using the phosphoramidite method. Detailed descriptions of the chemistry used to form polynucleotides by the phosphoramidite method are provided elsewhere, e.g., Caruthers et al., U.S. Pat. Nos. 4,458,066 and 4,415,732; Caruthers et al., *Genetic Engineering*, 4: 1-17 (1982); *Users Manual Model 392 and 394 Polynucleotide Synthesizers*, pages 6-1 through 6-

25 22, Applied Biosystems, Part No. 901237 (1991).

 The phosphoramidite method of polynucleotide synthesis is the preferred method because of its efficient and rapid coupling and the stability of the starting materials. The synthesis is performed with the growing polynucleotide chain attached

30 to a solid support, so that excess reagents, which are in the liquid phase, can be easily removed by filtration, thereby eliminating the need for purification steps between synthesis cycles.

The following briefly describes the steps of a typical polynucleotide synthesis cycle using the phosphoramidite method. First, a solid support including a protected nucleotide monomer is treated with acid, e.g., trichloroacetic acid, to remove a 5'-hydroxyl protecting group, freeing the hydroxyl for a subsequent coupling reaction. An activated intermediate is then formed by simultaneously adding a protected phosphoramidite nucleoside monomer and a weak acid, e.g., tetrazole, to the reaction. The weak acid protonates the nitrogen of the phosphoramidite forming a reactive intermediate. Nucleoside addition is complete within 30 s. Next, a capping step is performed which terminates any polynucleotide chains that did not undergo nucleoside addition. Capping is preferably done with acetic anhydride and 1-methylimidazole. The internucleotide linkage is then converted from the phosphite to the more stable phosphotriester by oxidation using iodine as the preferred oxidizing agent and water as the oxygen donor. After oxidation, the hydroxyl protecting group is removed with a protic acid, e.g., trichloroacetic acid or dichloroacetic acid, and the cycle is repeated until chain elongation is complete. After synthesis, the polynucleotide chain is cleaved from the support using a base, e.g., ammonium hydroxide or t-butyl amine. The cleavage reaction also removes any phosphate protecting groups, e.g., cyanoethyl. Finally, the protecting groups on the exocyclic amines of the bases and the hydroxyl protecting groups on the dyes are removed by treating the polynucleotide solution in base at an elevated temperature, e.g., 55 °C.

Any of the phosphoramidite nucleoside monomers may be dye-labeled phosphoramidites as described above. If the 5'-terminal position of the nucleotide is labeled, a labeled non-nucleotidic phosphoramidite of the invention may be used during the final condensation step. If an internal position of the oligonucleotide is labeled, a labeled nucleotidic phosphoramidite of the invention may be used during any of the condensation steps.

Subsequent to synthesis, the polynucleotide may be labeled at a number of positions including the 5'-terminus, e.g., *Oligonucleotides and Analogs*, Eckstein ed., Chapter 8, IRL Press (1991) and Orgel et al., *Nucleic Acids Research* 11(18): 6513 (1983); U.S.

Patent No. 5,118,800; the phosphodiester backbone, e.g., *ibid.*, Chapter 9; or at the 3'-terminus, e.g., Nelson, *Nucleic Acids Research* 20(23): 6253-6259, and U.S. Patent Nos. 5,401,837 and 5,141,813. For a through review of oligonucleotide labeling procedures see R. Haugland in *Excited States of Biopolymers*, Steiner ed., Plenum Press, NY (1983).

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As shown in FIGS. 14 and 15, in one preferred post-synthesis chemical labeling method an oligonucleotide is labeled as follows. A dye including a carboxy linking group is converted to the N-hydroxysuccinimide ester by reacting with approximately 1 equivalent of 1,3-dicyclohexylcarbodiimide and approximately 3 equivalents of N-hydroxysuccinimide in dry ethyl acetate for 3 hours at room temperature. The reaction mixture is washed with 5
10 % HCl, dried over magnesium sulfate, filtered, and concentrated to a solid which is resuspended in DMSO. The DMSO dye stock is then added in excess (10-20 x) to an aminohexyl derivatized oligonucleotide in 0.25 M bicarbonate/carbonate buffer at pH 9.4 and allowed to react for 6 hours, e.g., U.S. Patent No. 4,757,141. The dye labeled
15 oligonucleotide is separated from unreacted dye by passage through a size-exclusion chromatography column eluting with buffer, e.g., 0.1 molar triethylamine acetate (TEAA). The fraction containing the crude labeled oligonucleotide is further purified by reverse phase HPLC employing gradient elution.

20 VII. METHODS UTILIZING THE DIBENZORHODAMINE DYES

The dyes and reagents of the present invention are well suited to any method utilizing fluorescent detection, particularly methods requiring the simultaneous detection of multiple spatially-overlapping analytes. Dyes and reagents of the invention are particularly well suited for identifying classes of polynucleotides that have been
25 subjected to a biochemical separation procedure, such as electrophoresis, or that have been distributed among locations in a spatially-addressable hybridization array.

In a preferred category of methods referred to herein as "fragment analysis" or "genetic analysis" methods, labeled polynucleotide fragments are generated through
30 template-directed enzymatic synthesis using labeled primers or nucleotides, e.g., by ligation or polymerase-directed primer extension; the fragments are subjected to a size-dependent separation process, e.g., electrophoresis or chromatography; and, the separated fragments

are detected subsequent to the separation, e.g., by laser-induced fluorescence. In a particularly preferred embodiment, multiple classes of polynucleotides are separated simultaneously and the different classes are distinguished by spectrally resolvable labels.

5 One such fragment analysis method known as amplified fragment length polymorphism detection (AmpFLP) is based on amplified fragment length polymorphisms, i.e., restriction fragment length polymorphisms that are amplified by PCR. These amplified fragments of varying size serve as linked markers for following mutant genes through families. The closer the amplified fragment is to the mutant gene on the chromosome, the
10 higher the linkage correlation. Because genes for many genetic disorders have not been identified, these linkage markers serve to help evaluate disease risk or paternity. In the AmpFLPs technique, the polynucleotides may be labeled by using a labeled polynucleotide PCR primer, or by utilizing labeled nucleotide triphosphates in the PCR.

15 In another such fragment analysis method known as nick translation, a reaction is used to replace unlabeled nucleoside triphosphates in a double-stranded DNA molecule with labeled ones. Free 3'-hydroxyl groups are created within the unlabeled DNA by "nicks" caused by deoxyribonuclease I (DNAase I) treatment. DNA polymerase I then catalyzes the addition of a labeled nucleotide to the 3'-hydroxyl terminus of the nick. At the same
20 time, the 5' to 3'-exonuclease activity of this enzyme eliminates the nucleotide unit from the 5'-phosphoryl terminus of the nick. A new nucleotide with a free 3'-OH group is incorporated at the position of the original excised nucleotide, and the nick is shifted along by one nucleotide unit in the 3' direction. This 3' shift will result in the sequential addition of new labeled nucleotides to the DNA with the removal of existing unlabeled nucleotides.
25 The nick-translated polynucleotide is then analyzed using a separation process, e.g., electrophoresis.

Another exemplary fragment analysis method is based on variable number of tandem repeats, or VNTRs. VNTRs are regions of double-stranded DNA that contain
30 adjacent multiple copies of a particular sequence, with the number of repeating units being variable. Examples of VNTR loci are pYNZ22, pMCT118, and Apo B. A subset of VNTR methods are those methods based on the detection of microsatellite repeats, or short tandem

repeats (STRs), i.e., tandem repeats of DNA characterized by a short (2-4 bases) repeated sequence. One of the most abundant interspersed repetitive DNA families in humans is the (dC-dA)_n--(dG-dT)_n dinucleotide repeat family (also called the (CA)_n dinucleotide repeat family). There are thought to be as many as 50,000 to 100,000 (CA)_n repeat regions in the human genome, typically with 15-30 repeats per block. Many of these repeat regions are polymorphic in length and can therefore serve as useful genetic markers. Preferably, in VNTR or STR methods, label is introduced into the polynucleotide fragments by using a dye-labeled PCR primer.

In a particularly preferred fragment analysis method, classes identified in accordance with the invention are defined in terms of terminal nucleotides so that a correspondence is established between the four possible terminal bases and the members of a set of spectrally resolvable dyes. Such sets are readily assembled from the dyes of the invention by measuring emission and absorption bandwidths with commercially available spectrophotometers. More preferably, the classes arise in the context of the chemical or chain termination methods of DNA sequencing, and most preferably the classes arise in the context of the chain termination methods, i.e., dideoxy DNA sequencing, or Sanger-type sequencing.

Sanger-type sequencing involves the synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded or double-stranded DNA template whose sequence is to be determined. Synthesis is initiated at a defined site based on where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by incorporation of a nucleotide analog that will not support continued DNA elongation. Exemplary chain-terminating nucleotide analogs include the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) which lack the 3'-OH group necessary for 3' to 5' DNA chain elongation. When proper proportions of dNTPs (2'-deoxynucleoside 5'-triphosphates) and one of the four ddNTPs are used, enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP is incorporated. If labeled primers or labeled ddNTPs are used for each reaction, the sequence information can be detected by fluorescence after separation by high-resolution electrophoresis. In the chain termination method, dyes of the invention can be attached to either sequencing primers or

dideoxynucleotides. Dyes can be linked to a complementary functionality on the 5'-end of the primer, e.g. following the teaching in Fung et al, U.S. Pat. No. 4,757,141; on the base of a primer; or on the base of a dideoxynucleotide, e.g. via the alkynylamino linking groups disclosed by Hobbs et al, supra.

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In each of the above fragment analysis methods labeled polynucleotides are preferably separated by electrophoretic procedures, e.g. Gould and Matthews, cited above; Rickwood and Hames, Eds., *Gel Electrophoresis of Nucleic Acids: A Practical Approach*, IRL Press Limited, London, 1981; Osterman, *Methods of Protein and Nucleic Acid Research*, Vol. 1 Springer-Verlag, Berlin, 1984; or U.S. Patents 5,374,527, 5,624,800 and/or 5,552,028. Preferably the type of electrophoretic matrix is crosslinked or uncrosslinked polyacrylamide having a concentration (weight to volume) of between about 2-20 weight percent. More preferably, the polyacrylamide concentration is between about 4-8 percent. Preferably in the context of DNA sequencing in particular, the electrophoresis matrix includes a denaturing agent, e.g., urea, formamide, and the like. Detailed procedures for constructing such matrices are given by Maniatis et al., "Fractionation of Low Molecular Weight DNA and RNA in Polyacrylamide Gels Containing 98% Formamide or 7 M Urea," in *Methods in Enzymology*, 65: 299-305 (1980); Maniatis et al., "Chain Length Determination of Small Double- and Single-Stranded DNA Molecules by Polyacrylamide Gel Electrophoresis," *Biochemistry*, 14: 3787-3794 (1975); Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, pgs. 179-185 (1982); and *ABI PRISM™ 377 DNA Sequencer User's Manual, Rev. A*, January 1995, Chapter 2 (p/n 903433, The Perkin-Elmer Corporation, Foster City, CA). The optimal electrophoresis conditions, e.g., polymer concentration, pH, temperature, concentration of denaturing agent, employed in a particular separation depends on many factors, including the size range of the nucleic acids to be separated, their base compositions, whether they are single stranded or double stranded, and the nature of the classes for which information is sought by electrophoresis. Accordingly application of the invention may require standard preliminary testing to optimize conditions for particular separations.

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Subsequent to electrophoretic separation, the dye-polynucleotide conjugates are detected by measuring the fluorescence emission from the dye labeled polynucleotides. To perform such detection, the labeled polynucleotides are illuminated by standard means, e.g. high intensity mercury vapor lamps, lasers, or the like. Preferably the illumination means is a laser having an illumination beam at a wavelength above about 600 nm. More preferably, the dye-polynucleotides are illuminated by laser light generated by a He-Ne gas laser or a solid-state diode laser. The fluorescence is then detected by a light-sensitive detector, e.g., a photomultiplier tube, a charged coupled device, or the like. Exemplary electrophoresis detection systems are described elsewhere, e.g., U.S. Patent Nos. 5,543,026; 5,274,240; 4,879,012; 5,091,652 and 4,811,218.

VIII. EXAMPLES

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the invention and not to in any way limit its scope.

MATERIALS AND METHODS

All chemicals were purchased from Aldrich Chemical Company unless otherwise noted. Martius yellow was purchased from Fluka. Acetone was dried over CaSO_4 and distilled. Dichloromethane (CH_2Cl_2) and nitrobenzene were dried over CaH_2 and distilled. Tetrahydrofuran (THF) was dried over LAH and distilled as needed. Triethylamine (Et_3N) was dried over sodium and distilled. DMSO (99.9%) and N,N-diisopropylethylamine (99.5%) were dried and stored over activated molecular sieves. Silica gel (220-400 mesh) from VWR was used for normal phase flash chromatography. Reverse phase chromatography was performed on octadecyl functionalized silica gel from Aldrich. Preparative thin layer chromatography (PTLC) was performed on 1 and 2 mm pre-made silica gel plates from EM science (VWR). TLC was performed on aluminum back silica gel 60 plates from EM science (VWR). Developed spots were visualized with both long and short wavelength UV irradiation.

Absorption spectroscopy was performed on a Hewlett Packard model 8451A UV/Vis diode array spectrophotometer. Fluorescence measurements were made on a

Perkin-Elmer LS-50B luminescence spectrophotometer. NMR spectra were determined on a Varian 300 MHz NMR referenced relative to a solvent peak at 7.26 ppm in CD₃Cl or 3.31 ppm in CD₃OD. HPLC purification of oligomer labeled dye fragments was performed on a Perkin-Elmer Series 200 pump, employing a reverse phase C-18 column, with both UV and fluorescence emission detection. Fluorescence detection was performed by a Perkin-Elmer LC 240 fluorescence detector equipped with a red sensitive PMT, and UV detection was performed with a Model LC 295 UV/Vis detector. Pump and detectors were all interfaced with a Perkin-Elmer Model 1022 computer run in two-channel mode. Buffers were made up fresh from the following concentrated stock: 10 x TBE (0.89 M tris-(hydroxymethyl)aminomethane, 0.89 M borate, 0.02 M ethylenediaminetetraacetic acid disodium salt), and 0.1 M TEAA (triethylamine acetate).

All reactions were run in an oven-dried round bottom flask, under argon atmosphere, and capped with a rubber septum. Anhydrous solvents were manipulated under an argon atmosphere with oven-dried syringes. As used herein, the term "aqueous workup" refers to a purification method comprising the following steps: (i) adding a reaction mixture to a saturated aqueous NH₄Cl solution, a 5% HCl solution, or a saturated Na₂S₂O₃ solution, (ii) extracting the solution three times with an organic solvent, e.g., EtOAc, or CH₂Cl₂, (iii) washing the combined organic layer once with saturated NaCl, (iv) drying the solution with Na₂SO₄, (v) filtering the drying agent, and (vi) removing the solvent in vacuo. 3-Methoxy-1-hydroxynaphthalene **1** was synthesized from 1,3-dihydroxynaphthalene by the method of K.H.Bell and L.F.McCaffery (Aust. J. Chem. 46: 731 (1993)). 1-Amino-3-methoxynaphthalene **18** was synthesized according to the procedure of G.T.Morgan and E.D.Evans (J. Chem. Soc. 115: 1126 (1919)), or, as described below in Example 10.

EXAMPLE 1

Synthesis of 1-Diethylamino-3-Hydroxynaphthalene **4** (FIG. 1)

3-methoxy-1-hydroxy naphthalene **1** (1 gm) was suspended in dry CH₂Cl₂ (30 mL). Dry triethylamine (1.2 equivalents) was added and the reaction was cooled to -5 °C. Trifluoromethanesulfonic anhydride (1.1 equivalents) suspended in CH₂Cl₂ (15 mL)

was added dropwise with vigorous stirring over a period of 2 hours. The reaction was allowed to come to room temperature and subjected to aqueous work up using 5% HCl and CH₂Cl₂. The resulting crude 3-methoxynaphthalene-1-triflate **2** was purified by normal phase flash chromatography employing an EtOAc/Hexane (1:10) mobile phase.

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The purified 3-methoxynaphthalene-1-triflate **2** was converted to the 1-diethylamino-3-methoxynaphthalene **3** using the palladium-catalyzed triflate/amine coupling procedure of Wolfe as follows (J.P.Wolfe and S.L.Buchwald, *JOC*, 61: 1133 (1996)). The 3-methoxy-naphthalene-1-triflate **2** (1 gram) was suspended in 100 mL of dry toluene with 0.015 equivalents of (S)-(-)-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP), 0.005 equivalents of tris(dibenzylideneacetone)dipalladium (Pd₂(dba)₃), and 3 equivalents of dry diethyl amine. The reaction was purged with argon, and 3.3 equivalents of solid sodium t-butoxide was added with stirring. The reaction was then heated, and stirred for 16 hours at 80 °C in an oil bath. The reaction was allowed to come to room temperature and subjected to aqueous work up using 5% HCl and CH₂Cl₂ resulting in a crude 1-diethylamino-3-methoxynaphthalene **3**, which was purified by normal phase flash chromatography employing EtOAc: hexane (1: 49) as the mobile phase (¹HNMR: CD₃Cl d 8.20 (broad d, 1 H, J = 9 Hz), 7.72(broad d, 1 H, J = 7.8 Hz), 7.43 (dt, 1 H, J = 7.2, 1.2 Hz), 7.34 (dt, 1 H, J = 7.7, 1.2 Hz), 6.88 (d, 1 H, J = 2.4 Hz),), 6.82 (d, 1 H, J = 2.4 Hz), 3.93 (s, 3 H), 3.21 (q, 4 H, J = 7.2 Hz), 1.08 (t, 6 H, J = 7.2 Hz)).

Next, the methyl group of the 1-diethyl--amino-3-methoxy-naphthalene **3** was removed by boron tribromide deprotection as follows. The 1-amino-3-methoxy-naphthalene (100 mg) was suspended in dry CH₂Cl₂ (5 mL) and the mixture was cooled to -70 °C in a dry ice / acetone bath. Boron tribromide (10 equivalents) was added dropwise and the reaction was stirred for 30 minutes, then placed in a refrigerator (0 °C) overnight. The reaction was quenched at -70 °C by careful addition of MeOH (10 mL). Solid NaHCO₃ (30 equivalents) was added and the reaction was warmed to room temperature, then briefly heated to reflux. The mixture was cooled and filtered, the filtrate was acidified with AcOH, and the solvent was removed in vacuo to give the

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crude 1-diethylamino-3-hydroxynaphthalene 4, which was purified by normal phase flash chromatography employing EtOAc: hexane (1:4) as the mobile phase.

EXAMPLE 2

5 Synthesis of N-Phenyl-3,3-Dimethyl-Hydroxy-Benzoindoline 9 (FIG. 1)

The 3-methoxynaphthalene-1-triflate 2 was derivatized with aniline according to the palladium catalyzed triflate/amine coupling reaction described above in Example 1 to give the 1-anilino-3-methoxynaphthalene 5.

10

The 1-anilino-3-methoxynaphthalene 5 was acetylated by an amino group acetylation procedure as follows. The 1-amino-3-methoxynaphthalene 5 (500 mg) and 1.2 equivalents of dry Et₃N were suspended in 10 mL of dry CH₂Cl₂ and cooled to -5 °C using an ice / NaCl bath. 1.1 equivalent of 2-bromo-2-methylpropionylchloride was added dropwise and the reaction was stirred for 1 hour at -5 °C and stirred at room temperature for an additional 1 hour. The reaction was allowed to come to room temperature and subjected to aqueous work up using 5% HCl and EtOAc resulting in the crude intermediate 1-(bromoalkyl)amido-3-methoxy-naphthalene 6, which was purified by normal phase flash chromatography employing EtOAc: hexane (1: 9) as the mobile phase.

20

The 1-(bromoalkyl)amido-3-methoxy-naphthalene 6 was cyclized using an AlCl₃ catalyzed Friedel-Crafts cyclization procedure as follows. 1 to 3 equivalents of AlCl₃ in nitrobenzene was added to the 1-(bromoalkyl)amido-3-hydroxy-naphthalene 6. The reaction was heated to 130 °C and reacted for 1 hour. Aqueous work-up using NH₄Cl and EtOAc gave the crude N-phenyl-benzoindolinone intermediate 7, which was purified by normal phase flash chromatography employing EtOAc: hexane (1: 4) as the mobile phase. The amide carbonyl group of the N-phenyl-benzoindoline intermediate 7 was then reduced with LAH to give compound 8 (¹HNMR: CD₃Cl d 7.71 (d, 1 H, J = 7.8 Hz), 7.32 (m, 2 H), 7.24 (m, 2 H), 7.07 (bt, 1 H, J = 6.6 Hz), 6.96 (m, 3 H), 6.84 (s, 1 H), 3.97 (s, 3 H), 3.92 (s, 2 H), 1.44 (s, 6 H).

30

Methoxy group deprotection of compound 8 was effected using the boron tribromide deprotection procedure described in Example 1, resulting in the N-phenyl-3,3-dimethyl-hydroxy-benzoindoline 9.

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EXAMPLE 3

Synthesis of N-Methyl-5-Hydroxy-(Tetrahydro)benzoquinoline 15 (FIG. 2)

Compound 10 was synthesized by condensation of methoxy-naphthaldehyde and malonic acid employing piperidine catalysis in pyridine. Compound 10 was reduced with hydrogen over 10 % Pd/carbon, followed by LAH reduction, and reacted as outlined for the synthesis of compound 2 above with trifluoromethanesulfonic anhydride to give the triflate 11. Triflate 11 was then reacted with NaN_3 (3 equiv.) in DMF at 100 °C for 6 hours. Then, the reaction was allowed to come to room temperature and subjected to aqueous work up using pure water and EtOAc resulting in pure compound 12. Compound 12 was suspended in dry CH_2Cl_2 , complexed with 3 to 5 equivalents of solid AlCl_3 , and refluxed for 2 hours yielding compound 13.

Compound 13 was alkylated with MeI according to a general amino group alkylation procedure as follows. The 3-methoxybenzoquinoline derivative (100 mg) 13 was suspended in 5 mL of dry THF and cooled to -5 °C (ice / NaCl). 1.1 equivalents of n-butyl lithium (1 M) was added dropwise, and the reaction was stirred for 1 hour. 3 equivalents of the MeI alkylating agent was added slowly and the reaction was allowed to stir at room temperature for 2 hours. Aqueous work-up using NH_4Cl and EtOAc gave a crude alkylated 3-methoxybenzoquinoline intermediate 14. Intermediate 14 was then purified by normal phase flash chromatography employing EtOAc: hexane (1:19) as the mobile phase (^1H NMR: CD_3Cl d 8.1 (broad d, 1 H, J = 8.1 Hz), 7.68(dd, 1 H, J = 8.1, 1.8 Hz), 7.34 (m, 2 H), 6.8 (s, 1 H), 3.92 (s, 3 H), 3.21 (m, 2 H), 2.94 (s, 3 H), 2.77 (t, 2 H, J = 6.6 Hz), 1.92 (m, 2 H)). Subsequent methoxy group deprotection by the general boron tribromide procedure described above in Example 1 resulted in the N-methyl-hydroxybenzoquinoline derivative 15.

EXAMPLE 4

Synthesis of 3-(5-Hydroxybenzoquinolin-1-yl) propanesulfonic acid 17 (FIG. 2)

Compound **13** was synthesized according to the procedure outlined above in Example 3 for the synthesis of the N-methyl-hydroxybenzoquinoline derivative **15**. Compound **13** was then alkylated according to the general amino group alkylation procedure described above in Example 3, this time using 1,3-propane sultone as the alkylating agent rather than MeI, to give a 5-methoxybenzoquinoline-N-propanesulfonic acid intermediate **16** (¹HNMR: CD₃OD d 7.94 (d, 1 H, J = 8.7 Hz), 7.65 (d, 1 H, J = 8.4 Hz), 7.32 (t, 1 H), 7.27 (t, 1 H), 6.85 (s, 1 H), 4.89 (s, 3 H), 3.20 (m, 2 H), 3.08 (bt, 2 H, J = 6 Hz), 2.91 (m, 2 H), 2.72 (t, 2 H, J = 6.6 Hz), 2.33 (m, 2 H), 1.89 (m, 2 H). Subsequent methoxy group deprotection of compound **16** by the general boron tribromide procedure described above in Example 1 resulted in the 3-(5-hydroxybenzoquinolin-1-yl) propanesulfonic acid **17**.

EXAMPLE 5

15 Synthesis of N-Methyl-2,2,4-Trimethyl-5-Hydroxy-(Tetrahydro)benzoquinoline **22** (FIG.3)

Following the procedure of A.Rosowsky and E.J.Modest (J.O.C., 30: 1832 (1965)), 1-amino-3-methoxynaphthalene **18** (1 gm) was dissolved in dry acetone (50 mL), and 0.01 equivalent of iodine was added to the solution. The reaction was heated and stirred for 16 hours, cooled, and then quenched with saturated Na₂S₂O₃. The reaction mixture was then subjected to aqueous work up using saturated Na₂S₂O₃ and EtOAc resulting in the crude methoxybenzoquinoline **19**. The methoxybenzoquinoline **19** was purified by flash chromatography using an EtOAc/hexane 1:9 mobile phase. Compound **19** was then alkylated with MeI according to the general amino group alkylation procedure described above in Example 3 to give compound **20**. Compound **20** was reduced with H₂ in a Parr hydrogenator at 70 psi and 10% Pd/C catalysis to give a N-methyl-2,2,4-trimethyl-5-methoxybenzoquinoline intermediate **21** (¹HNMR: CD₃Cl d 8.20 (bd, 1 H, J = 7.5 Hz), 7.65 (bd, 1 H, J = 7.5 Hz), 7.33 (m, 2 H), 6.89 (s, 1 H), 3.94 (s, 3 H), 3.14 (b sextet, 1 H, J = 6.6 Hz), 2.80 (3, 3 H), 1.89 (d, 2 H, J = 8.7), 1.42 (d, 3 H, J = 6.9 Hz), 1.34 (s, 3 H), 1.05 (s, 3 H). Subsequent methoxy group deprotection of compound **21** by the general boron tribromide procedure described above in Example 1 gave the N-methyl-5-hydroxy-(tetrahydro)benzoquinoline **22**.

EXAMPLE 6Synthesis of N-Methyl-3,3-Dimethyl-4-Hydroxy-Benzoindoline **27** (FIG. 3)

5 1-Amino-3-methoxynaphthalene **18** was acetylated with 2-bromo-2-methylpropionyl chloride according to the general amino group acylation procedure described above in Example 2 to give compound **23**. Compound **23** was cyclized by the Friedel-Crafts cyclization procedure described above in Example 2 to give compound **24**. Next, compound **24** was reduced with 3 equivalents LAH in THF to give
10 the 4-methoxybenzoindoline **25**. Compound **25** was alkylated using the general amino group alkylation procedure described above in Example 3 using methyl iodide as the alkylating agent to give a N-methyl-3,3-dimethyl-4-methoxybenzoindoline intermediate **26** (¹HNMR: CD₃Cl d 8.07 (bd, 1 H, J = 8.4 Hz), 7.69 (bd, 1 H, J = 8.1 Hz), 7.33 (bt, 1 H, J = 7.8 Hz), 7.22 (bt, 1 H, J = 8.1 Hz), 6.70 (s, 1 H), 3.92 (s, 3 H), 3.32 (s, 2 H),
15 3.32 (s, 3 H), 1.44 (s, 6 H). Subsequent methoxy group deprotection of compound **26** by the general boron tribromide procedure described in Example 1 resulted in the N-methyl-3,3-dimethyl-4-hydroxy-benzoindoline **27**.

EXAMPLE 7Synthesis of N-Ethyl-3,3-Dimethyl-4-Hydroxy-Benzoindoline **29** (FIG. 3)

The 4-methoxybenzoindoline **25** was synthesized as described above in Example 6. Compound **25** was alkylated by the general amino group alkylation procedure described in Example 3 employing ethyl iodide as the alkylating agent to
25 give the N-ethyl-3,3-dimethyl-4-methoxybenzoindoline intermediate **28** (¹HNMR: CD₃Cl d 7.90 (d, 1 H, J = 8.7 Hz), 7.68 (d, 1 H, J = 8.1 Hz), 7.32 (bt, 1 H, J = 7.5 Hz), 7.22 (bt, 1 H, J = 6.9 Hz), 6.69 (s, 1 H), 3.83 (s, 3 H), 3.52 (q, 2 H J = 7.5 Hz), 3.38 (s, 2 H), 1.46 (s, 6 H), 1.27 (t, 3 H, J = 7.5 Hz). Subsequent methoxy group deprotection of compound **28** by the general boron tribromide procedure described in
30 Example 1 yielded the N-ethyl-3,3-dimethyl-4-hydroxy-benzoindoline **29**.

EXAMPLE 8

Synthesis of Selected Dibenzorhodamine Dye Compounds

General Procedure A (FIG. 5). A solid phthalic anhydride derivative **34** was mixed with 1.4 equivalents of an aminohydroxy intermediate **31** and 2.8 equivalents of ZnCl_2 . The oven dried reaction vessel was capped with a rubber septa and purged with Argon. The solid mixture was heated briefly at $130\text{ }^\circ\text{C}$ until melting was observed, e.g.,
5 after approximately 15 minutes. 1,2-Dichlorobenzene (approximately 10 equivalents) was added by syringe to the reaction mixture, and the heterogeneous mixture was heated to $130\text{ }^\circ\text{C}$ to $170\text{ }^\circ\text{C}$ for 4 hours. The crude reaction mixture was cooled, suspended in a minimal amount of $\text{MeOH}:\text{CH}_2\text{Cl}_2$ (1:19), loaded directly onto a normal phase flash chromatography column, and the crude dye was eluted with an
10 $\text{MeOH}:\text{CH}_2\text{Cl}_2$ (1:19) mobile phase. When necessary, the dye was purified and separated into distinct isomers **35** and **36** by PTLC developed with $\text{MeOH}:\text{CH}_2\text{Cl}_2$ (1:9). The isomerically pure dye, which migrated as a single spot on silica TLC eluting with 1:9 $\text{MeOH}:\text{CH}_2\text{Cl}_2$, was identified by its UV/Visible absorption spectra and its long wavelength fluorescent excitation and emission spectra.

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General Procedure B (FIG. 6). In the general procedure outlined in FIG. 6, a solid phthalic anhydride derivative **34** (100 mg) was placed in a round bottom flask capped with a rubber septa and purged with dry argon. Dry nitrobenzene (2 mL) was added and heated to dissolve the anhydride. The mixture was cooled to room
20 temperature and 3 to 6 equivalents of anhydrous AlCl_3 was added with stirring to dissolve the solid. Subsequently, 1 equivalent of a 1-amino-3-methoxynaphthalene intermediate **31** was added with stirring and the reaction was heated to $130\text{ }^\circ\text{C}$ for 1 hour. The reaction was then cooled and suspended in EtOAc . The organic layer was washed with saturated NH_4Cl and brine, dried over Na_2SO_4 , filtered, and the solvent
25 was removed in vacuo. When necessary, the resulting ketone intermediate **37/38** was purified and separated into distinct isomers **37** and **38** using normal phase flash chromatography using ($\text{MeOH}:\text{CH}_2\text{Cl}_2$, 1:19) as the mobile phase, or by recrystallization. The methoxy group of the isomerically pure intermediate **37** or **38** was removed according to the general boron tribromide deprotection procedure
30 described in Example 1 to give amino-hydroxynaphthalene ketone **39**. The amino-hydroxynaphthalene ketone **39** (100 mg) was then reacted at $130\text{ }^\circ\text{C}$ with 1 equivalent of a 1-amino-3-naphthalene intermediate **32** in dry 1,2-dichlorobenzene (2 mL) for 2

hours. The reaction was cooled, giving isomerically pure and asymmetrically substituted product **40** that was purified as in General Procedure A above.

Synthesis of Dibenzorhodamine Dye **41** (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, and 1-diethylamino-3-hydroxynaphthalene **4** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye **42** (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, and N-methyl-5-hydroxy-benzoquinoline **15** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye **43** (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, and 5-hydroxy-benzoquinoline **17** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye **44** (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, the N-methyl-2,2,4-trimethyl-5-hydroxy-benzoquinoline **22** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye **45** (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, and N-methyl-3,3-dimethyl-4-hydroxy-benzoinoline **27** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye 46 (FIG. 7). General procedure A was followed employing tetrafluorophthalic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 to C-17 are F, and N-ethyl-3,3-dimethyl-4-hydroxy-benzindoline **29** as the aminohydroxy intermediate **31**.

Synthesis of Dibenzorhodamine Dye 47 (FIG. 7). General procedure A was followed employing dichlorotrimellitic anhydride as the phthalic anhydride derivative, i.e., compound **34** where the substituents at C-14 and C-17 are Cl and the substituent at C-15 is CO₂H, and N-phenyl-3,3-dimethyl-4-hydroxy-benzindoline **9** as the aminohydroxy intermediate **31**.

EXAMPLE 9

Spectral Properties of Selected Dibenzorhodamine Dye Compounds

The following table presents important spectral properties of several representative dibenzorhodamine dye compounds of the invention. All spectra were recorded at room temperature, in 1 x TBE buffer and 8 M urea, for the free dye having 0.05 absorption at the dye's $\lambda_{\text{max, abs}}$. Dye concentration was approximately 10^{-6} M.

Dye	Absorption Maximum (nm)	Emission Maximum (nm)	Full Width at Half Max (nm)
41	585	614	59
42	609	634	42
43	597	637	47
44	598	640	50
45	639	650	31
46	639	652	33
47	632	676	66

EXAMPLE 10

Synthesis of 3,3-dimethyl-4-hydroxy-benzindoline **25** (FIGS. 8 and 9)

Compound **2** was converted to 1-amino-3-methoxynaphthalene **18** using the palladium-catalyzed triflate/imine coupling and hydrolysis procedure of Buchwald (Buchwald, S.L. et al Tetrahedron Letters, 1997, 38/36, 6367-6370) as follows (FIG. 8).

5

The 3-methoxy-naphthalene-1-triflate **2** (25 g) was mixed with 0.013 equivalents of tris(dibenzylideneacetone)-dipalladium ($\text{Pd}_2(\text{dba})_3$), 0.04 equivalents of racemic 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (\pm BINAP), 1.3 equivalents of potassium carbonate, 1.3 equivalents of cesium carbonate, and 1.5 equivalents of benzophenone imine. Alternatively, potassium or sodium t-butoxide (2.6 equivalents) could be used as a substitute base for the reaction. The reaction mixture was suspended in 75 mL of dry toluene and 75 mL of dry tetrahydrofuran and stirred for 24 hours at 120 °C in an oil bath. A mixture of 75 mL of hexane/ethyl acetate (9:1) was added to the reaction mixture, and the mixture was eluted through silica gel with hexane/ethyl acetate (3:2).
10 The elutant was collected and then concentrated under reduced pressure. To the concentrated oil, hexane/ethyl acetate (9:1) was added to crystallize out 1-benzophenone imino-3-methoxynaphthalene **49** as yellow crystals (20.0 g, 72.6 %). The mother liquor was concentrated and hexane/ethyl acetate (9:1) was added to crystallize out a second crop of **49** as yellow crystals (3.0 g, 10.9 %). (Total yield: 83.5 %).

20

Compound **49** was hydrolyzed under acid conditions to give **18** as follows. Compound **49** (27 gm) was suspended in 150 mL of 1,4-dioxane and 100 mL of 5% sulfuric acid (H_2SO_4). The reaction was then heated and stirred for 1 hour at 50 °C in an oil bath. The reaction mixture was cooled to room temperature and washed with
25 150 mL of hexane/ethyl acetate (3:2). The solution was then basified with ice cold aqueous NaOH to pH 10-11, and extracted three times with ethyl acetate. The combined organic extract was dried with sodium sulfate (Na_2SO_4) and evaporated under reduced pressure to give **18** as pale brown oil which crystallized upon standing (13.9 g, 100 %).

30

Compound **25**, 3,3-dimethyl-hydroxy-benzoinoline, was synthesized from **18** according to the procedure described above in Example 6.

EXAMPLE 11

Synthesis of N-Substituted Methoxybenzoindoline Intermediates 57-61 (FIG. 9)

Compound 25 was employed as a common intermediate to synthesize the N-substituted derivatives 57-61 by either alkylative substitution or palladium catalysed coupling to the secondary amine group (FIG. 9).

Synthesis of N-(6-hexanoic acid)-3,3-dimethyl-hydroxy-benzoindoline 57.

Compound 25 (2.3 g) was alkylated by treating with 3 equivalents of commercially available methyl-6-iodohexanoic acid 52 and 3 equivalents of diisopropylethylamine. The mixture was suspended in 23 mL of dry toluene and heated at 130 °C in an oil bath with stirring for 18 hours. The solvent was evaporated under reduced pressure to give crude N-(6-hexanoic acid)-3,3-dimethyl-hydroxy-benzoindoline 57. Crude 57 was dissolved in dichloromethane and purified by normal phase flash chromatography eluting with hexane/ethyl acetate (99:1) (yield 2.55 g, 74%).

Synthesis of N-(biphenyl)-3,3-dimethyl-hydroxy-benzoindoline 58.

Compound 25 (2.3 g) was N-arylated by palladium catalysed coupling according to the established procedure (Buchwald, et al J. Org., 1997, 62, 6066-6068) as follows: Compound 25 (2 g) was mixed with 1.4 equivalents of 4-bromo-biphenyl 7, sodium t-butoxide 1.3 equivalents), 0.013 equivalents of tris(dibenzylideneacetone)dipalladium (Pd₂(dba)₃), 0.04 equivalents of racemic 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (± BINAP). The reaction mixture was suspended in 100 mL of dry toluene, stirred well, and heated at 130 °C in an oil bath overnight (16 hrs.). The reaction mixture was quenched with aqueous ammonium chloride (NH₄Cl) and extracted (3x) with ethyl acetate. The organic extract was dried with anhydrous Na₂SO₄. Hexane (equal volume) was added to the crude reaction mixture and it was eluted through silica-gel with hexane/ethyl acetate (1:1). The solvent was evaporated under reduced pressure and the residue was purified by normal phase flash chromatography eluting with hexane/ethyl acetate (98:2) to give N-(biphenyl)-3,3-dimethyl-hydroxy-benzoindoline 58 (yield 85%) as white powder.

Synthesis of N-(carboxybiphenyl)-3,3-dimethyl-hydroxy-benzoindoline 59.

Compound **54** was synthesized from commercially available bromo-biphenyl-methyl ketone by a haloform reaction to give the bromo-biphenyl acid, followed by esterification employing established procedures. Compound **25** was N-arylated with **54** according to the established procedure (Buchwald, et al Tetrahedron Letters, 1997, **38/36**, 6359-6362) as follows: Compound **25** (0.4 g) was mixed with 1.4 equivalents of compound **54**, 0.013 equivalents of tris(dibenzylideneacetone)dipalladium (Pd₂(dba)₃), 0.04 equivalents of racemic 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (± BINAP), and 3 equivalents of cesium carbonate. The reaction mixture was suspended in 100 mL of dry toluene and 100 mL of dry tetrahydrofuran, then heated at 130 °C in an oil bath and stirred for 18 hours. The reaction was cooled and hexane (30 mL) was added to the crude mixture. The crude product was eluted through silica-gel with hexane/ethyl acetate (3:2). The solvent was removed under reduced pressure and the N-(carboxybiphenyl)-3,3-dimethyl-hydroxy-benzoindoline **59** was purified by normal phase flash chromatography eluting with hexane/ethyl acetate: (95:5) (bright yellow solid yield 0.25 g, 32%).

Synthesis of N-phenyl-3,3-dimethyl-hydroxy-benzoindoline 60. Compound **25** was N-arylated with iodobenzene or bromobenzene as described above for synthesis of compound **58** from compound **25**. Pure N-phenyl-3,3-dimethyl-hydroxy-benzoindoline **8** was isolated after normal phase flash chromatography employing hexane/ethyl acetate: (98:2).

Synthesis of N-carboxyphenyl-3,3-dimethyl-hydroxy-benzoindoline 61. Compound **25** was N-arylated with methyl iodobenzoate or methyl bromobenzoate as described above for synthesis of compound **59**. Pure N-carboxyphenyl-3,3-dimethyl-hydroxy-benzoindoline **61** was isolated after normal phase flash chromatography employing hexane/ethyl acetate: (95:5).

30

EXAMPLE 12

Synthesis of Dibenzorhodamine Dyes **67**, **72**, **76** and **81** (FIGS. 10-13)

Synthesis of Joda 4-Dye 67 (FIG 10). Hydroxyindoline **62** was obtained by demethylation of compound **58** using aluminium chloride (AlCl_3) according to the following procedure: Compound **58** (2 g), and aluminium chloride (6 g) were thoroughly mixed under a strong stream of argon and the reaction mixture purged for 10 minutes with argon. The solid mixture was heated under Argon in an oil bath from 90 °C to 120 °C over 20 min. The reaction mixture was cooled and the solids suspended in dichloromethane and transferred into a solution of ice and dilute H_2SO_4 . The aqueous layer was extracted five times, the combined organic extract was washed with saturated sodium chloride, and dried over sodium sulfate. After filtration, the solvent was evaporated under reduced pressure and pure **62** (0.13 g) was isolated after normal phase flash chromatography eluting with dichloromethane/methanol (99:1). Hydroxyindoline **63** was generated from compound **59** employing the same procedure as that for conversion of compound **58** to compound **62**.

Next, one equivalent of a 1:1 complex of N-methylformanilide and phosphorous oxychloride in dry dichloromethane was added to one equivalent of **62** in a small pear flask (100 mg in a 5 ml flask). The reaction mixture was heated at 80 °C in an oil bath under a stream of Argon until the dichloromethane was evaporated leaving a dark red oil. Nitrobenzene (300 ul) was added followed by addition of 2 equivalents of POCl_3 . To the above solution was added one equivalent of **63** suspended in nitrobenzene (500 ul). The reaction mixture was heated from 80 °C to 155 °C over 25 min. and then kept at 155 °C for 15 minutes. The reaction was cooled and transferred with dichloromethane to 5 % HCl. The aqueous layer was saturated with brine and extracted 3x with dichloromethane. The combined organic extracts were separated and evaporated under reduced pressure to give the crude dye derivative **64**. Crude **64** was suspended in acetic acid (3 ml) and 5 % HCl (3ml) and heated at 60 °C for 1 hour. The reaction mixture was then poured into a solution of ice, saturated aqueous sodium chloride, and extracted 3 times with dichloromethane. After evaporation of the combined organic layers, the pure mono-carboxylated asymmetric dye derivative **65** was then purified from the other two symmetric dye products, the bis-biphenyl and bis-carboxybiphenyl dyes, by normal phase flash chromatography eluting with dichloromethane/methanol (9:1).

Finally, the purified dye derivative **65** was sulfonated to give Joda-4 dye **67** as follows. Dye derivative **65** (30 mg) and 3 equivalents of anhydrous sodium sulfate were suspended in dry dichloromethane (50 mL) at 0 °C under Argon. To the above solution, 5 equivalents of chlorosulfonic acid (ClSO₃H) were added and the reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was quenched with saturated aqueous sodium chloride/5 % HCl and the aqueous layer extracted 3 times with dichloromethane. The organic extract was evaporated under reduced pressure to give crude Joda-4 dye **66**. Crude dye **66** was suspended in a solution of dioxane and 5 % HCl (2:1) and stirred at room temperature for 20 hours. The solution was then concentrated under reduced pressure, suspended in saturated aqueous sodium chloride and extracted 3 times with dichloromethane. The solvent was evaporated to give crude Joda 4 dye **67**. Pure Joda 4 dye **67** was isolated by normal phase preparative thin layer chromatography (PTLC) developed with methanol/dichloromethane (1:4) or flash chromatography eluting with methanol/dichloromethane (1:9).

Synthesis of Joda-3 Dye 72 (FIG 11). Compound **61** was demethylated to give compound **68**, and compound **8** was demethylated to give compound **9**, both employing the aluminum chloride deprotection procedure described above for conversion of compound **58** to compound **62**. Compound **68** was reacted with the methyl formanilide/POCl₃ complex, and subsequently with compound **9** to generate dye derivative **70** as described for the synthesis of dye derivative **64** from compounds **62** and **63**. Crude dye derivative **70** was hydrolysed by acid to give dye derivative **71** according to the procedure for generation of dye derivative **65** from **64** above. The pure asymmetric dye derivative **71** was purified by PTLC from the two symmetric bis-phenyl and bis-carboxyphenyl dye products. The purified dye **71** was then sulfonated and hydrolysed to give crude Joda-3 dye **72** as described above for generation of Joda-4 dye **67** from dye derivative **65**. Pure Joda-3 dye **72** was isolated by normal phase preparative thin layer chromatography (PTLC) developed with methanol/dichloromethane (1:4) or flash chromatography eluting with methanol/dichloromethane (1:9).

Synthesis of Joda-2 Dye 76. (FIG. 12) Compound 57 was demethylated to give compound 73 by boron tribromide deprotection, and compound 8 was demethylated to give 9 employing the aluminum chloride deprotection procedure described above for conversion of compound 58 to compound 62. Compound 57 reacted with the methyl formanilide/ POCl_3 complex under the standard conditions, and subsequently with compound 9 under the standard conditions to generate dye derivative 74 as described above in Example 7. Crude dye derivative 74 was hydrolysed by standard acid hydrolysis to give dye derivative 75. The pure asymmetric dye derivative 75 was purified by PTLC from the two symmetric bisphenyl and bis-hexanoic dye products. The purified dye 75 was then sulfonated by the standard procedure described for formation of 67 from 65 to give crude Joda-3 dye 76. Pure Joda-2 dye 76 was isolated by normal phase preparative thin layer chromatography (PTLC) developed with methanol/ dichloromethane (1:4) or flash chromatography eluting with methanol/ dichloromethane (1:9).

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Synthesis of Joda-1 Dye 81. (FIG. 13) Compound 57 (0.313 g) was saponified by treatment with 7 equivalents of 1M KOH in methanol at room temperature over 3 hours. The solution was concentrated to dryness and the oil extracted well with ethyl acetate and 5% hydrochloric acid solution. The organic layer was dried with sodium sulfate and concentrated to a colorless oil. Pure 77 was isolated by normal phase chromatography eluting with 5% methanol/ dichloromethane/ 0.1% acetic acid (yield 0.243g; 84%).

Next, compound 77 (0.360 gm) was dissolved in dry dichloromethane (2 ml) and a solution of dichloromethylmethyl ether (2.6 equivalents) and tin tetrachloride (4 equivalents). The solution was refluxed for 1.5 hrs. The mixture was extracted with ethyl acetate and 3M hydrochloric acid solution. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 , concentrated to an oil, and used without purification. There was an approximately equimolar mixture of compound 77 and 78 at this point, based on thin layer chromatography. This oil was dissolved in dry dichloromethane (8 ml), and cooled to -78°C . Demethylation of the mixture was carried out by established boron tribromide procedures to yield a mixture of

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Compounds **79** and **80**. The resultant crude oil was dissolved in dry dichloromethane (5 ml) and phosphorus oxychloride (2 equivalents) was added; nitrobenzene (5 ml) was then added. The mixture was heated from 80 to 150°C over 45 minutes and promptly removed from heat. The workup and isolation was performed as in Example 12 for the
5 synthesis of compound **67** to yield crude Joda 1 dye **81**. Pure dye **81** was isolated by PTLC as described in Example 12 for the synthesis of compound **67**, yielding 32 mg.

EXAMPLE 13

10 Synthesis of Dye Carboxy N-Hydroxysuccinimide Derivatives of Compounds **67**, **72**, **76**, and **81** and Coupling the Derivatives to Nucleotides and Polynucleotides (FIGS. 14 and 15)

The N-hydroxysuccinimide (NHS) derivatization of the carboxylic acid dyes **67**, **72**, **76**, and **81** was accomplished by one of two methods. For synthesis of the Joda
15 4 dye NHS **82**, Joda 3 dye NHS **83**, and Joda 2 dye NHS **84** the preferred method (Method A) employed O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate reagent, while for Joda 1 dye **85** the preferred method (Method B) employed dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide.

20 Method A: The dye was suspended in dry DMF (5 mg dye: 300 ul DMF) with 6 equivalents of diisopropyl-ethyl amine. To the dye solution was added 15 equivalents of O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate and the reaction was stirred for 15 minutes at room temperature. The reaction was transferred with dichloromethane into 5% HCl (20 ml). The aqueous layer was made salty by
25 addition of saturated NaCl and extracted 3 times with dichloromethane. The combined organic layers were concentrated to dryness and the crude dye-NHS purified by normal phase flash chromatography eluting with MeOH/CH₂Cl₂ (1: 9).

Method B: The dye and 20 equivalents of N-hydroxysuccinimide were
30 suspended in dry dichloromethane (5 mg: 500 ul CH₂Cl₂). Nine equivalents of dicyclohexylcarbodiimide were added and the reaction was stirred for 1 hour at room temperature. The reaction was quenched and the pure dye-NHS isolated as for Method A.

Attachment of the NHS derivatives to nucleotides and polynucleotides was performed as follows. Amino group substituted oligomer or terminator was suspended in formamide (5×10^{-4} M) and 15 equivalents of diisopropylethylamine were added. An excess (5-10 equivalents) of dye-NHS suspended in DMSO (5mg/ 60 ul) were added
5 with stirring at room temperature. The reaction was stirred at room temperature for 4 hours. To the reaction mixture was added 3 M NaOAc to give 0.5 M NaOAc. Four times the reaction volume of EtOH were added and the mixture was cooled. The precipitated dye labeled oliomer was isolated by centrifugation and purified by reverse phase HPLC eluting with 30% AcCN/0.1 x TEAA buffer.

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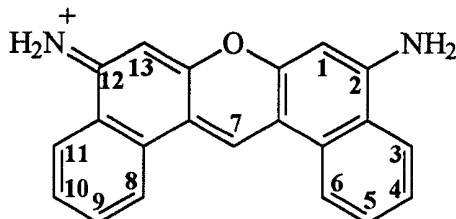
All publications, patents, and patent applications referred to herein are hereby incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

15

Although only a few embodiments have been described in detail above, those having ordinary skill in the chemical arts will clearly understand that many modifications are possible in these embodiments without departing from the teachings thereof. All such modifications are intended to be encompassed within the scope of
20 the following claims.

WE CLAIM:

1. A dibenzorhodamine compound having the structure



5 including nitrogen- and aryl-substituted forms thereof.

2. The compound of **claim 1** comprising a first bridging group which when taken together with the C-12-bonded nitrogen and the C-12 and C-13 carbons forms a first ring structure having from 4 to 7 members; and/or

10 a second bridging group which when taken together with the C-2-bonded-nitrogen and the C-1 and C-2 carbons forms a second ring structure having from 4 to 7 members.

15 3. The compound of **claim 2** wherein one or both of the first and second ring structures has five members.

4. The compound of **claim 3** wherein the five membered ring structure includes one gem disubstituted carbon.

20 5. The compound of **claim 4** comprising one or more nitrogen substituents selected from the group consisting of lower alkyl, lower alkene, lower alkyne, phenyl, aromatic, heterocycle, polycyclic aromatic, water-solubilizing group, linking group, including substituted forms thereof.

25 6. The compound of **claim 5** wherein the nitrogen substituents are selected from the group consisting of lower alkyl, phenyl, polycyclic aromatic and substituted forms thereof.

7. The compound of **claim 6** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl, substituted polycyclic aromatic and substituted phenyl, wherein the substituent is linking group.

5 8. The compound of **claim 6** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl, substituted polycyclic aromatic and substituted phenyl, wherein the substituent is sulfonate.

9. The compound of **claim 5** wherein the nitrogen substituents are water-
10 solubilizing group.

10. The compound of **claim 9** wherein the water-solubilizing group is selected from the group consisting of carboxylate, sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

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11. The compound of **claim 4** wherein the gem substituents are lower alkyl.

12. The compound of **claim 11** wherein the gem substituents are methyl.

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13. The compound of **claim 3** wherein the five membered ring is not aromatic.

14. The compound of **claim 3** wherein the five membered ring is substituted with linking group or water-solubilizing group.

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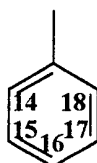
15. The compound of **claim 2** wherein the first and second ring structures are the same.

16. The compound of **claim 2** wherein the first and second ring structures are different.

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17. The compound of **claim 1** comprising a C-7 substituent selected from the group consisting of acetylene, lower alkyl, lower alkene, cyano, phenyl, heterocycle, and substituted forms thereof.

5 18. The compound of **claim 17** wherein the C-7 substituent is phenyl having the structure



including aryl-substituted forms thereof.

10 19. The compound of **claim 18** wherein substituents at any one of positions C-14 to C-18 are selected from the group consisting of chlorine, fluorine, lower alkyl, carboxylic acid, sulfonic acid, $-\text{CH}_2\text{OH}$, alkoxy, phenoxy, linking group, water solubilizing group, and substituted forms thereof.

15 20. The compound of **claim 19** wherein a substituent at position C-18 is selected from the group consisting of carboxylic acid and sulfonate.

21. The compound of **claim 19** wherein a substituent at position C-18 is carboxylic acid.

20

22. The compound of **claim 19** wherein a substituent at positions C-14 and C-17 is chlorine.

23. The compound of **claim 19** wherein a substituent at positions C-14 to C-17 is chlorine.

25

24. The compound of **claim 19** wherein a substituent at positions C-14 to C-17 is fluorine.

25. The compound of **claim 19** wherein a substituent at one of positions C-15 and C-16 is linking group and the other is hydrogen, a substituent at positions C-14 and C-17 is chlorine, and a substituent at position C-18 is carboxy.

5 26. The compound of **claim 1** comprising one or more nitrogen substituents selected from the group consisting of lower alkyl, lower alkene, lower alkyne, phenyl, aromatic, heterocycle, polycyclic aromatic, water-solubilizing group, linking group, including substituted forms thereof.

10 27. The compound of **claim 26** wherein the nitrogen substituents are selected from the group consisting of lower alkyl, phenyl, polycyclic aromatic and substituted forms thereof.

15 28. The compound of **claim 27** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl, substituted polycyclic aromatic and substituted phenyl, wherein the substituent is linking group.

20 29. The compound of **claim 27** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl, substituted polycyclic aromatic and substituted phenyl, wherein the substituent is sulfonate.

30 30. The compound of **claim 26** wherein the nitrogen substituents are water-solubilizing group.

25 31. The compound of **claim 30** wherein the water-solubilizing group is selected from the group consisting of sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

30 32. The compound of **claim 1** comprising a third bridging group which when taken together with the C-12-bonded nitrogen and the C-11 and C-12 carbons forms a third ring structure having from 5 to 7 members; and/or

a fourth bridging group which when taken together with the C-2-bonded nitrogen and the C-2 and C-3 carbons forms a fourth ring structure having from 5 to 7 members.

5 33. The compound of **claim 32** wherein one or both of the third and fourth ring structures has six members.

34. The compound of **claim 33** wherein the six membered ring structure includes one gem disubstituted carbon.

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35. The compound of **claim 34** wherein the gem substituents are lower alkyl.

36. The compound of **claim 35** wherein the gem substituents are methyl.

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37. The compound of **claim 33** wherein the six membered ring is not aromatic.

38. The compound of **claim 1** comprising one or more substituents at one or more of positions C-1, C-3 to C-6, C-8 to C-11, and C-13, selected from the group consisting of fluorine, chlorine, lower alkyl, lower alkene, lower alkyne, sulfate, sulfonate, sulfone, sulfonamide, sulfoxide, amino, ammonium, amido, nitrile, lower alkoxy, phenoxy, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, and linking group, including substituted forms thereof.

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39. The compound of **claim 38** comprising a fused aromatic ring bonded across the C-3 and C-4 carbons, the C-4 and C5 carbons, the C-9 and C-10 carbons, and/or the C-10 and C-11 carbons, including substituted forms thereof.

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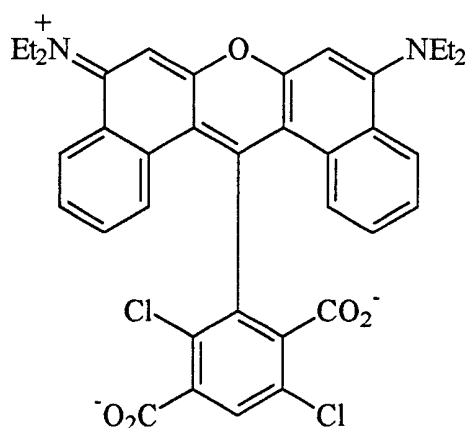
40. The compound of **claim 39** comprising a fused aromatic ring bonded across the C-3 and C-4 carbons and the C-10 and C11 carbons, including substituted forms thereof.

41. The compound of **claim 39** comprising a fused aromatic ring bonded across the C-9 and C-10 carbons and the C-4 and C-5 carbons, including substituted forms thereof.

42. The compound of **claim 38** wherein the one or more substituents are sulfonate.

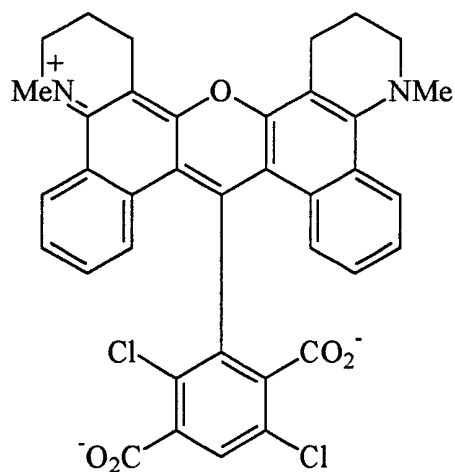
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43. The compound of **claim 1** having the structure

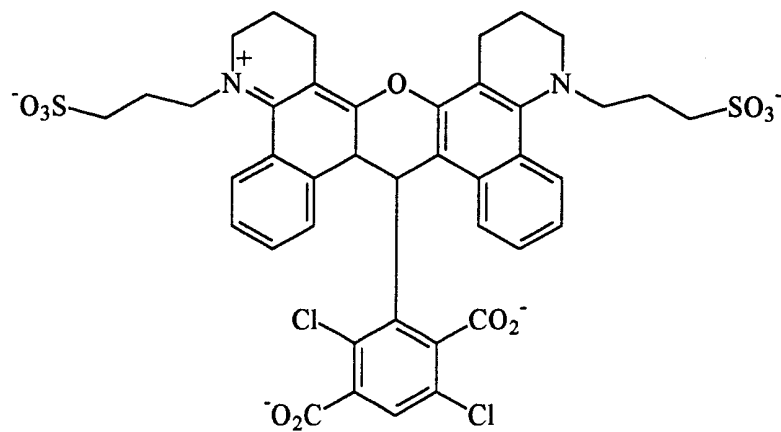


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44. The compound of **claim 1** having the structure

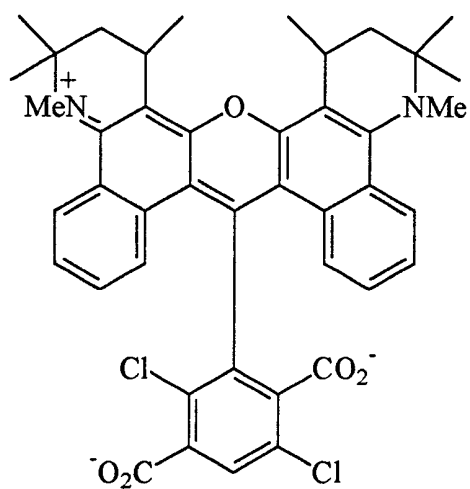


45. The compound of **claim 1** having the structure

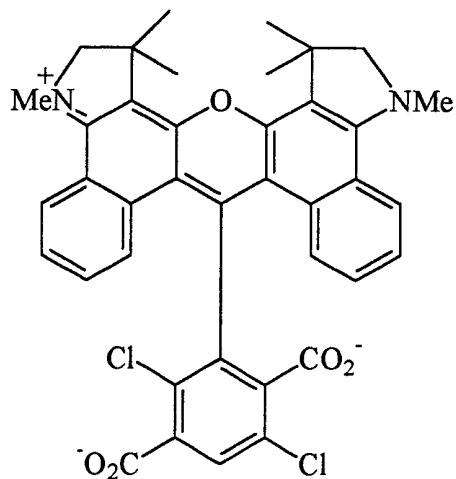


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46. The compound of **claim 1** having the structure

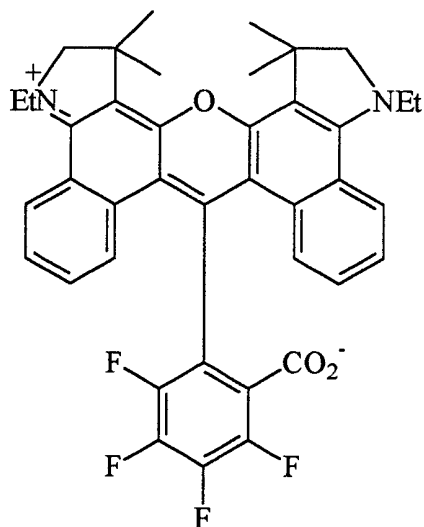


47. The compound of **claim 1** having the structure

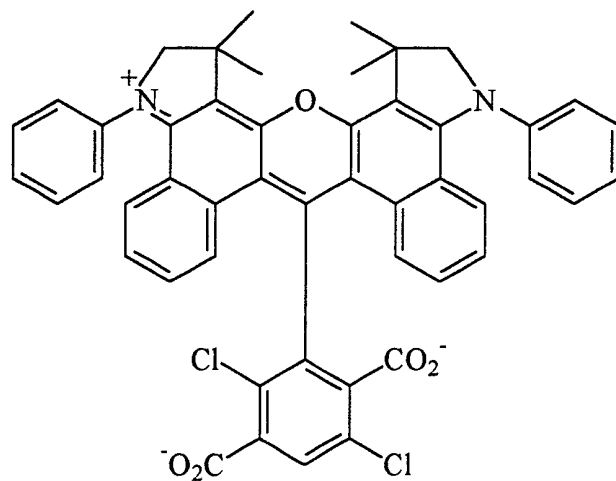


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48. The compound of **claim 1** having the structure

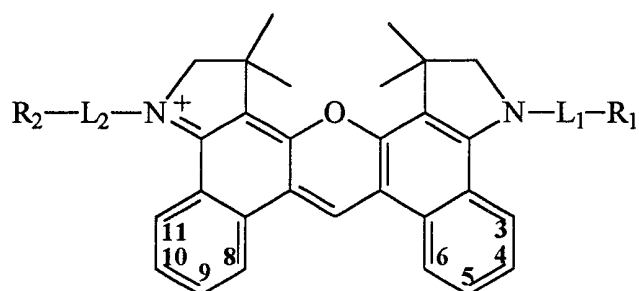


49. The compound of **claim 1** having the structure



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50. The compound of **claim 1** having the structure

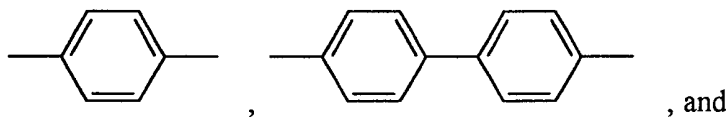


10

wherein

L_1 and L_2 are selected from the group consisting of

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—(CH₂)_n— where n ranges from 1 to 8; and

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R_1 and R_2 are selected from the group consisting of linking group and water-solubilizing group.

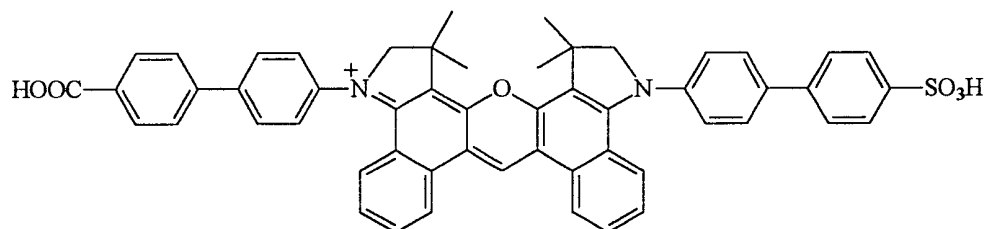
51. The compound of **claim 50** wherein the water solubilizing group is selected from the group consisting of carboxylate, sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

5 52. The compound of **claim 50** comprising a fused aromatic ring bonded across the C-3 and C-4 carbons, the C-4 and C5 carbons, the C-9 and C-10 carbons, or the C-10 and C-11 carbons, including substituted forms thereof.

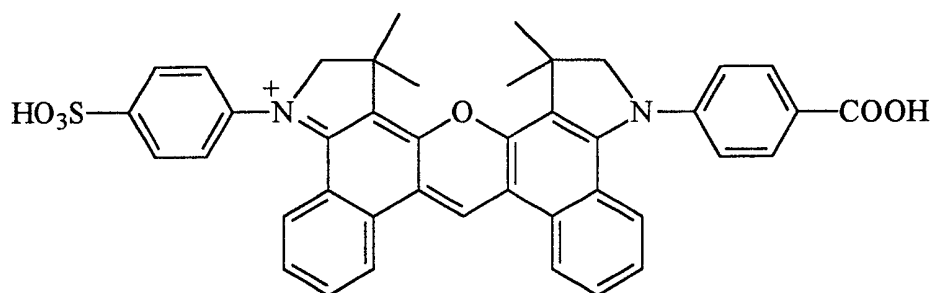
10 53. The compound of **claim 52** comprising a fused aromatic ring bonded across the C-3 and C-4 carbons and the C-10 and C11 carbons, including substituted forms thereof.

54. The compound of **claim 52** comprising a fused aromatic ring bonded across the C-9 and C-10 carbons and the C-4 and C-5 carbons, including substituted forms thereof

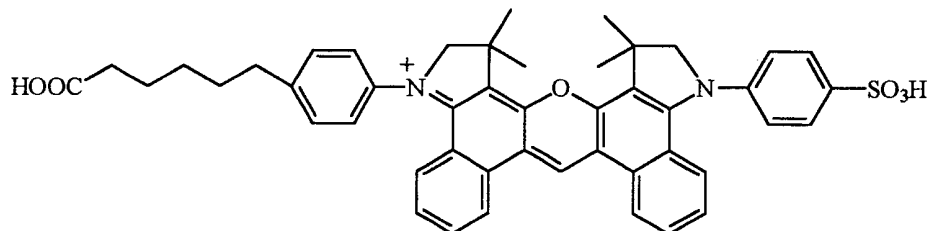
15 55. The compound of **claim 1** having the structure



20 56. The compound of **claim 1** having the structure

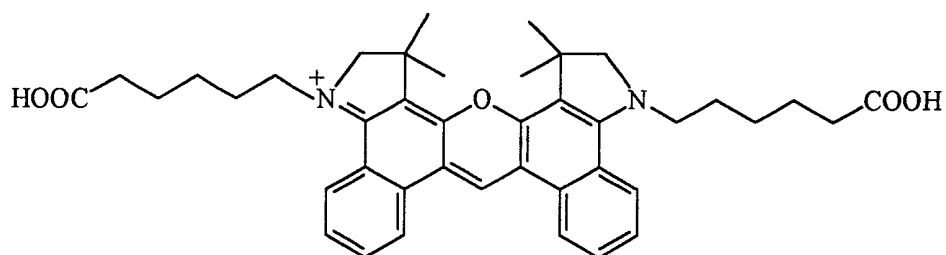


57. The compound of **claim 1** having the structure



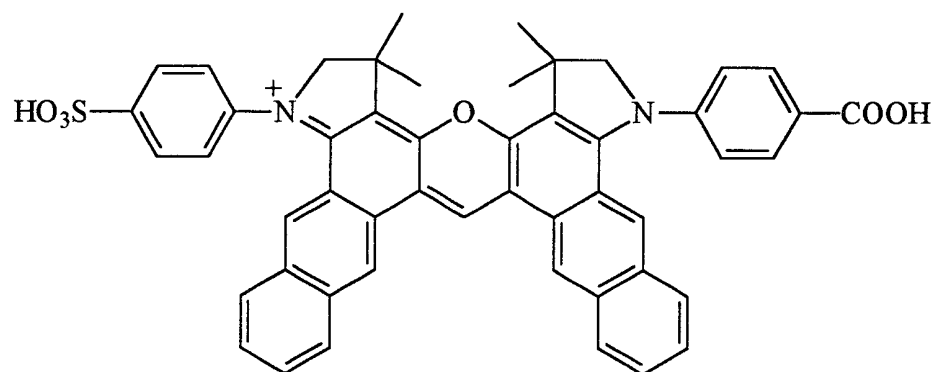
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58. The compound of **claim 1** having the structure



10

59. The compound of **claim 1** having the structure

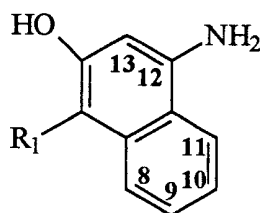


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60. An intermediate useful for the synthesis of dibenzorhodamine compounds having the structure

5



wherein

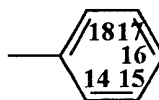
10 R_1 is H or $\text{—}\overset{\text{O}}{\parallel}{\text{C}}\text{—Y}$, wherein Y is selected from the group consisting of H, lower alkyl, lower alkene, lower alkyne, aromatic, phenyl, polycyclic aromatic, heterocycle, water-solubilizing group, and linking group;

wherein when R_1 is H, the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure having from 5 to 15 7 members; and

including aryl- and nitrogen-substituted forms thereof.

20

61. The compound of **claim 60** wherein Y is



62. The compound of **claim 61** wherein substituents at any one of positions C-14 to C-18 are selected from the group consisting of chlorine, fluorine, lower alkyl, carboxylic acid, sulfonic acid, $\text{—CH}_2\text{OH}$, alkoxy, phenoxy, linking group, water solubilizing group, and 25 substituted forms thereof.

63. The compound of **claim 62** wherein a substituent at position C-18 is selected from the group consisting of carboxylic acid and sulfonate.

64. The compound of **claim 63** wherein a substituent at position C-18 is carboxylic acid.

5 65. The compound of **claim 62** wherein a substituent at positions C-14 and C-17 is chlorine.

66. The compound of **claim 62** wherein a substituent positions C-14 to C-17 is chlorine.

10 67. The compound of **claim 62** wherein a substituent at positions C-14 to C-17 is fluorine.

68. The compound of **claim 62** wherein a substituent at one of positions C-15 and C-16 is linking group and the other is hydrogen, a substituent at positions C-14 and C-17 is chlorine, and a substituent at position C-18 is carboxy.

15 69. The compound of **claim 60** wherein the C-12-bonded nitrogen and the C-12 and C-13 carbons form a first ring structure having from 4 to 7 members, and/or the C-12-bonded nitrogen and the C-11 and C-12 carbons form a second ring structure
20 having from 5 to 7 members.

70. The compound of **claim 60** wherein the first and/or second ring structure has five members.

25 71. The compound of **claim 70** wherein the five membered ring structure includes one gem disubstituted carbon.

72. The compound of **claim 71** wherein the gem substituents are lower alkyl.

30 73. The compound of **claim 72** wherein the gem substituents are methyl.

74. The compound of **claim 70** wherein the five membered ring is not aromatic.

5 75. The compound of **claim 70** wherein the five membered ring is substituted with linking group.

10 76. The compound of **claim 60** comprising one or more nitrogen substituents selected from the group consisting of lower alkyl, lower alkene, lower alkyne, phenyl, aromatic, heterocycle, polycyclic aromatic, water-solubilizing group, linking group, and substituted forms thereof.

77. The compound of **claim 76** wherein the nitrogen substituents are selected from the group consisting of lower alkyl, phenyl, and substituted forms thereof.

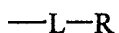
15 78. The compound of **claim 77** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl and substituted phenyl, wherein the substituent is linking group.

20 79. The compound of **claim 77** wherein the nitrogen substituents are selected from the group consisting of substituted lower alkyl and substituted phenyl, wherein the substituent is sulfonate.

25 80. The compound of **claim 76** wherein the nitrogen substituents are water-solubilizing group.

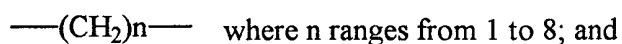
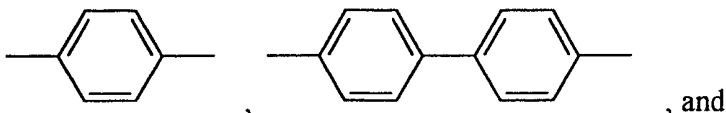
81. The compound of **claim 80** wherein the water-solubilizing group is selected from the group consisting of carboxylate, sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

82. The compound of **claim 76** wherein the nitrogen substituent is



wherein L is selected from the group consisting of

5



10

R is selected from the group consisting of linking group and water-solubilizing group.

83. The compound of **claim 82** wherein the water solubilizing group is selected from the group consisting of sulfonate, phosphate, quaternary amine, sulfate, polyhydroxyl, and water-soluble polymer.

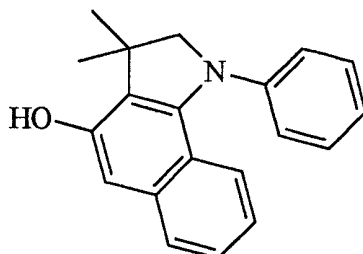
84. The compound of **claim 60** comprising one or more substituents at one or more of positions C-8 to C-11, and C-13, selected from the group consisting of fluorine, chlorine, lower alkyl, lower alkene, lower alkyne, sulfate, sulfonate, sulfone, sulfonamide, sulfoxide, amino, ammonium, amido, nitrile, lower alkoxy, phenoxy, aromatic, phenyl, polycyclic aromatic, water-solubilizing group, heterocycle, and linking group, including substituted forms thereof.

85. The compound of **claim 84** comprising a fused aromatic ring bonded across the C-9 and C-10 carbons, or the C-10 and C-11 carbons, including substituted forms thereof.

86. The compound of **claim 85** comprising a fused aromatic ring bonded across the C-10 and C11 carbons, including substituted forms thereof.

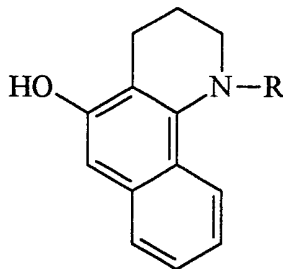
87. The compound of **claim 84** wherein the one or more substituents are sulfonate.

88. The compound of **claim 60** having the structure



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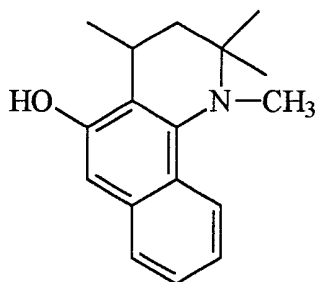
89. The compound of **claim 60** having the structure



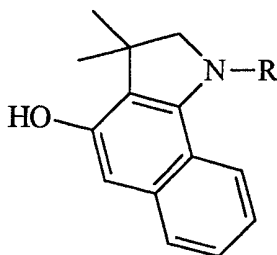
wherein R is selected from the group consisting of methyl and $-(\text{CH}_2)_3\text{SO}_3^-$.

10

90. The compound of **claim 60** having the structure



91. The compound of **claim 60** having the structure



wherein R is lower alkyl.

5

92. The compound of **claim 91** wherein R is selected from the group consisting of methyl or ethyl.

93. An energy transfer dye comprising:

10

a donor dye capable of absorbing light at a first wavelength and emitting excitation energy in response;

an acceptor dye capable of absorbing the excitation energy emitted by the donor dye and fluorescing at a second wavelength in response; and

15

a linker for linking the donor dye and the acceptor dye, the linker serving to facilitate the efficient transfer of energy between the donor dye and the acceptor dye;

wherein at least one of the donor dye and acceptor dye is a dibenzorhodamine compound of **claim 1**.

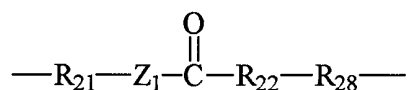
94. The energy transfer dye of **claim 93** wherein the linker includes a functional group selected from the group consisting of alkene, diene, alkyne, a five and six membered ring having at least one unsaturated bond, and a fused ring structure.

20

95. The energy transfer dye of **claim 93** wherein the first wavelength is greater than about 600 nm.

25

96. The energy transfer dye of **claim 93** wherein the linker has the structure



wherein

Z₁ is selected from the group consisting of —NH, sulfur and oxygen;

5 R₂₁ is a lower alkyl attached to the donor dye;

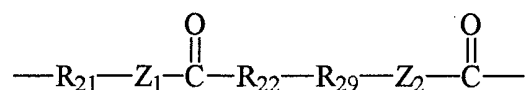
R₂₂ is a substituent selected from the group consisting of an alkene, diene, alkyne, a five and six membered ring having at least one unsaturated bond and a fused ring structure which is attached to the carbonyl carbon; and

R₂₈ includes a functional group which attaches the linker to the acceptor dye.

10

97. The energy transfer dye of **claim 96** wherein R₂₂ is a five or six membered ring selected from the group consisting of cyclopentene, cyclohexene, cyclopentadiene, cyclohexadiene, furan, thiofuran, pyrrole, isopyrrole, isoazole, pyrazole, isoimidazole, pyran, pyrone, benzene, pyridine, pyridazine, pyrimidine, pyrazine oxazine, indene, benzofuran,
15 thionaphthene, indole and naphthalene.

98. The energy transfer dye of **claim 96** wherein the linker has the structure



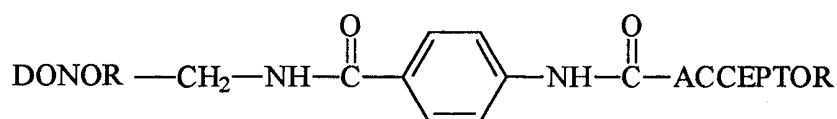
20

wherein

Z₂ is selected from the group consisting of —NH, sulfur and oxygen; and

R₂₉ is a lower alkyl.

99. The energy transfer dye of **claim 93** wherein the linker has the structure



5 100. A labeled nucleoside/side having the formula:



wherein

NUC is a nucleoside/tide or nucleoside/tide analog;

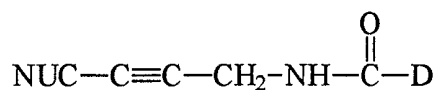
D is a dibenzorhodamine dye compound of **claim 1**, NUC and D being
10 connected by a linkage;

wherein if NUC comprises a purine base, the linkage is attached to the 8-position of the purine, if NUC comprises a 7-deazapurine base, the linkage is attached to the 7-position of the 7-deazapurine, and if NUC comprises a pyrimidine base, the linkage is attached to the 5-position of the pyrimidine.

15

101. The labeled nucleoside/tide of **claim 100** wherein NUC comprises a base selected from the group consisting of uracil, cytosine, deazaadenine, and deazaguanosine.

102. The labeled nucleoside/tide of **claim 100** wherein the linkage is



20

103. A method of polynucleotide sequencing comprising the steps of:

forming a mixture of a first, a second, a third, and a fourth class of polynucleotides such that:

25 each polynucleotide in the first class includes a 3'-terminal dideoxyadenosine and is labeled with a first dye;

each polynucleotide in the second class includes a 3'-terminal dideoxycytidine and is labeled with a second dye;

each polynucleotide in the third class includes a 3'-terminal dideoxyguanosine and is labeled with a third dye; and

each polynucleotide in the fourth class includes a 3'-terminal dideoxythymidine and is labeled with a fourth dye;

5 wherein at least one of the first, second, third, or fourth dyes is a dibenzorhodamine compound of **claim 1**;

the other of the dyes being spectrally resolvable from the dibenzorhodamine dye(s) and from each other;

10 electrophoretically separating the polynucleotides thereby forming bands of similarly sized polynucleotides;

illuminating the bands with an illumination beam capable of causing the dyes to fluoresce; and

identifying the classes of the polynucleotides in the bands by the fluorescence spectrum of the dyes.

15

104. A method of fragment analysis comprising:

forming labeled polynucleotide fragments, the fragments being labeled with a dibenzorhodamine compound of **claim 1**;

20 subjecting the labeled polynucleotide fragments to a size-dependent separation process; and

detecting the labeled polynucleotide fragments subsequent to the separation process.

25 105. The method of **claim 104** wherein the size-dependent separation process is electrophoresis and the labeled polynucleotide fragments are detected by fluorescence.

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Fig. 1

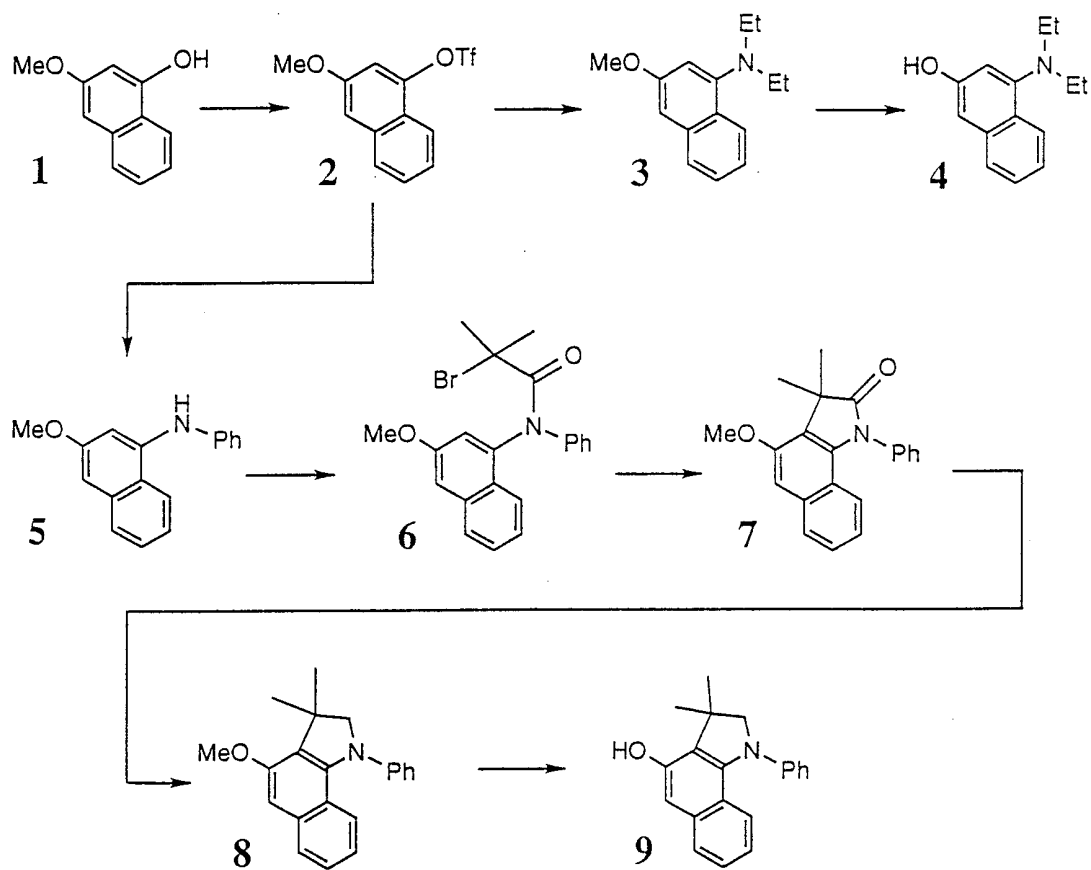


Fig. 2

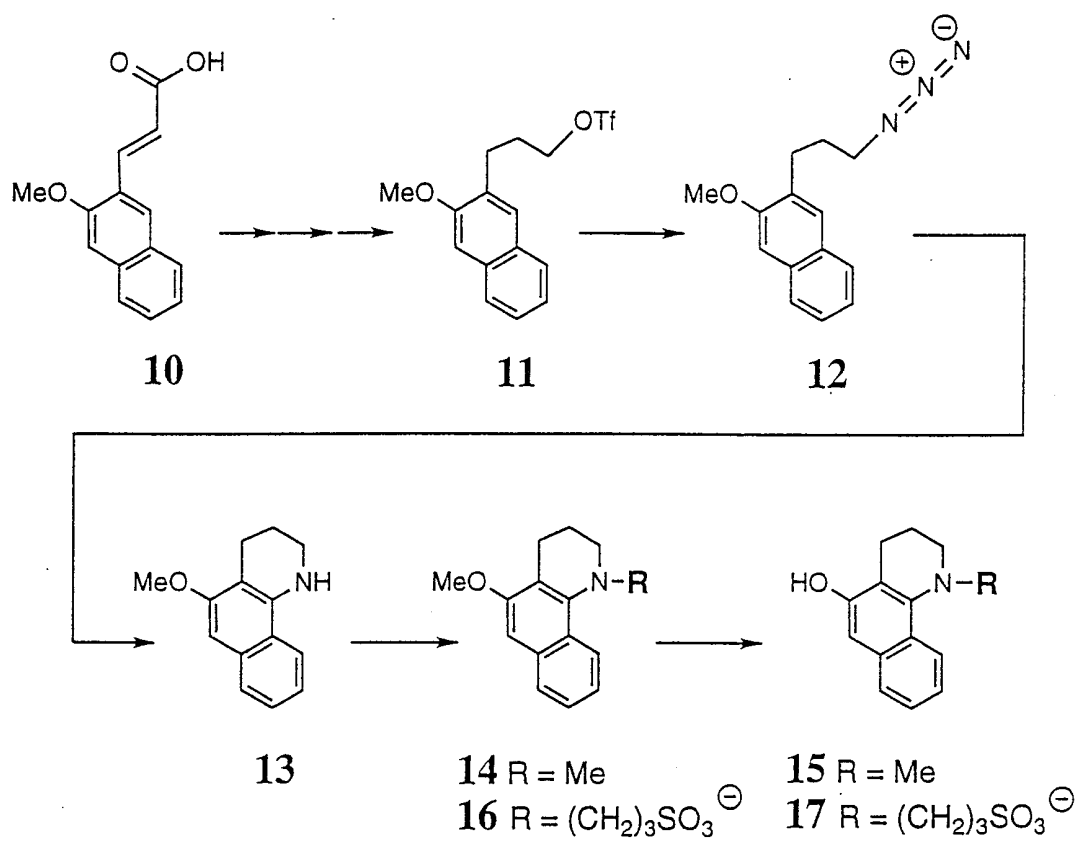
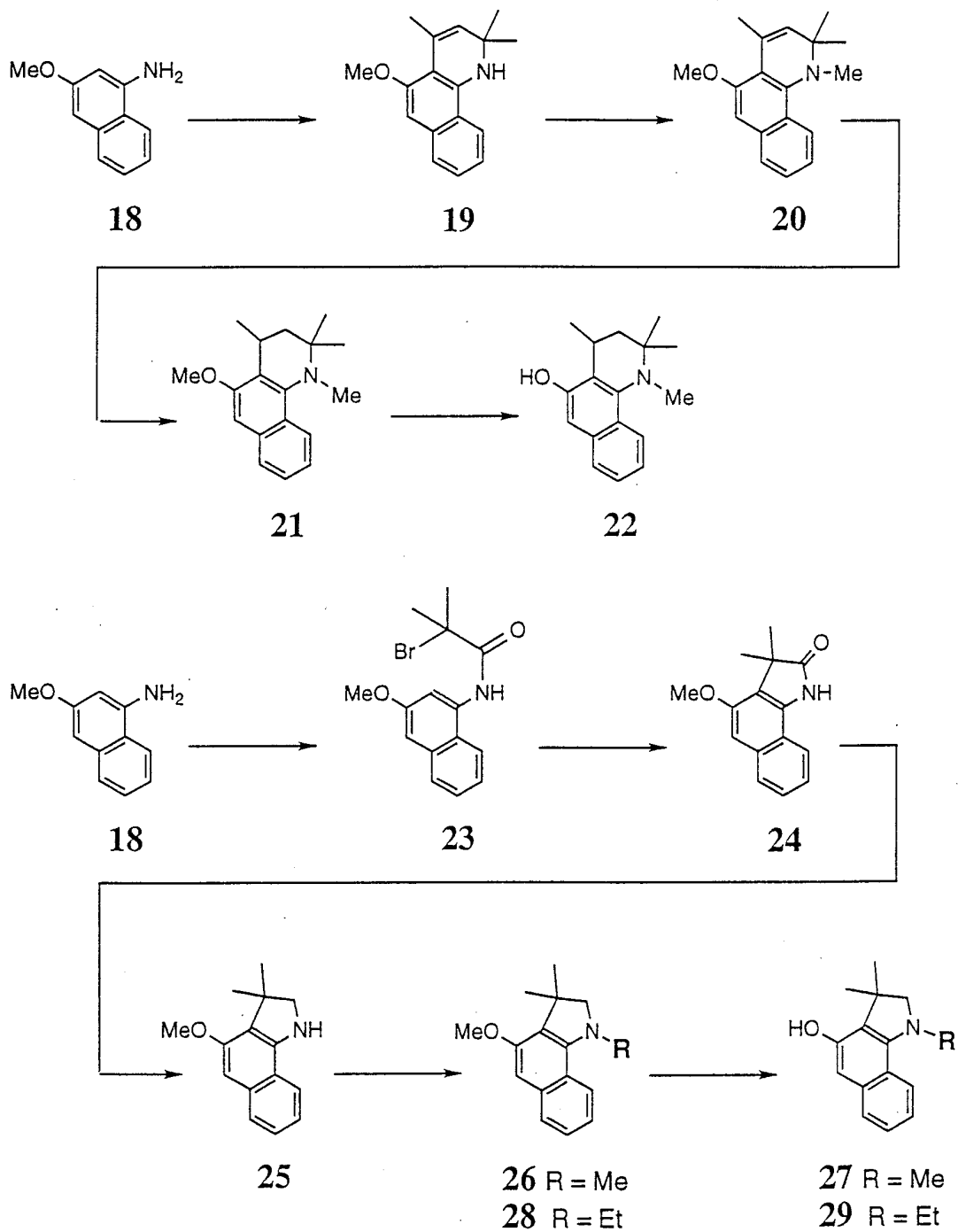


Fig. 3



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Fig. 4

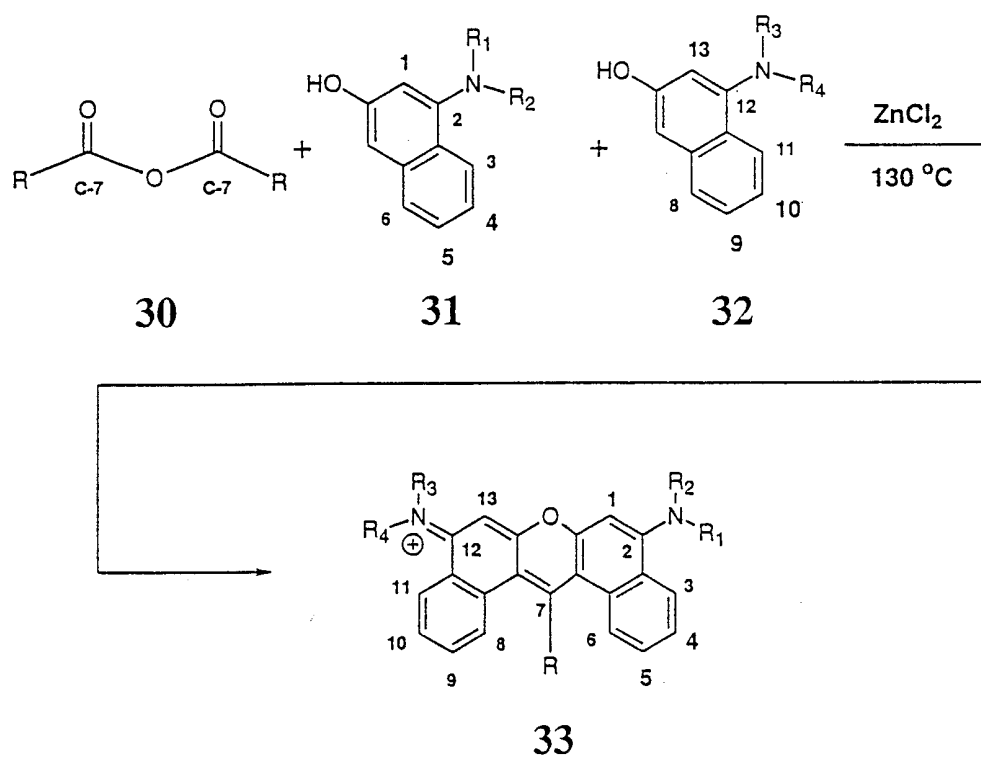
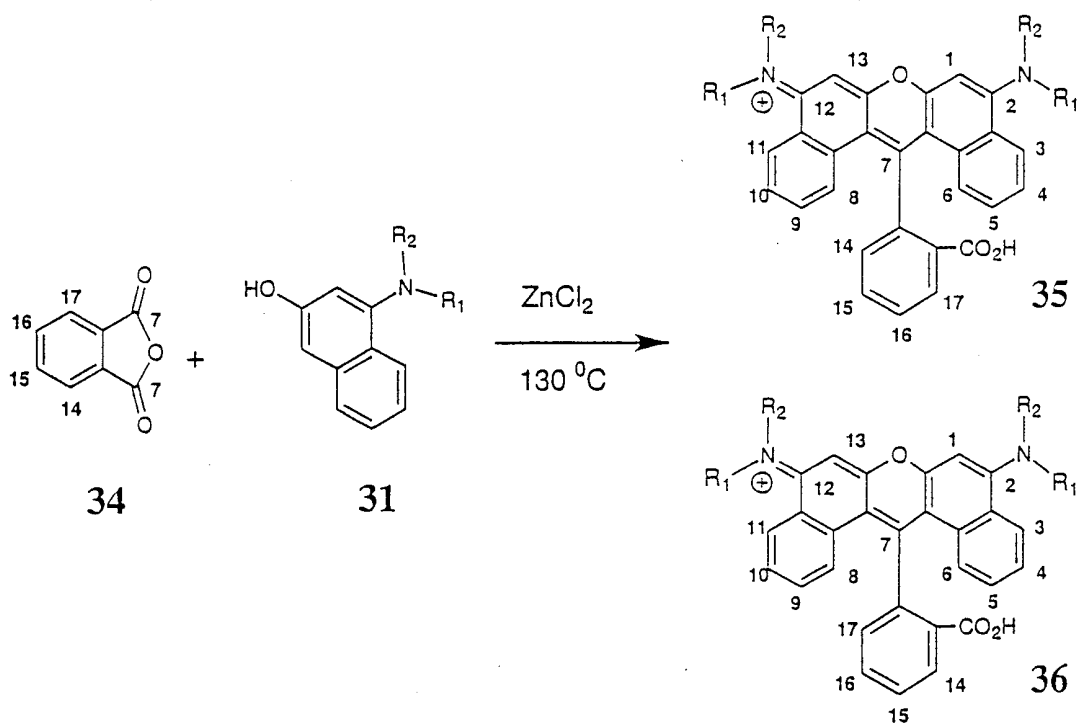
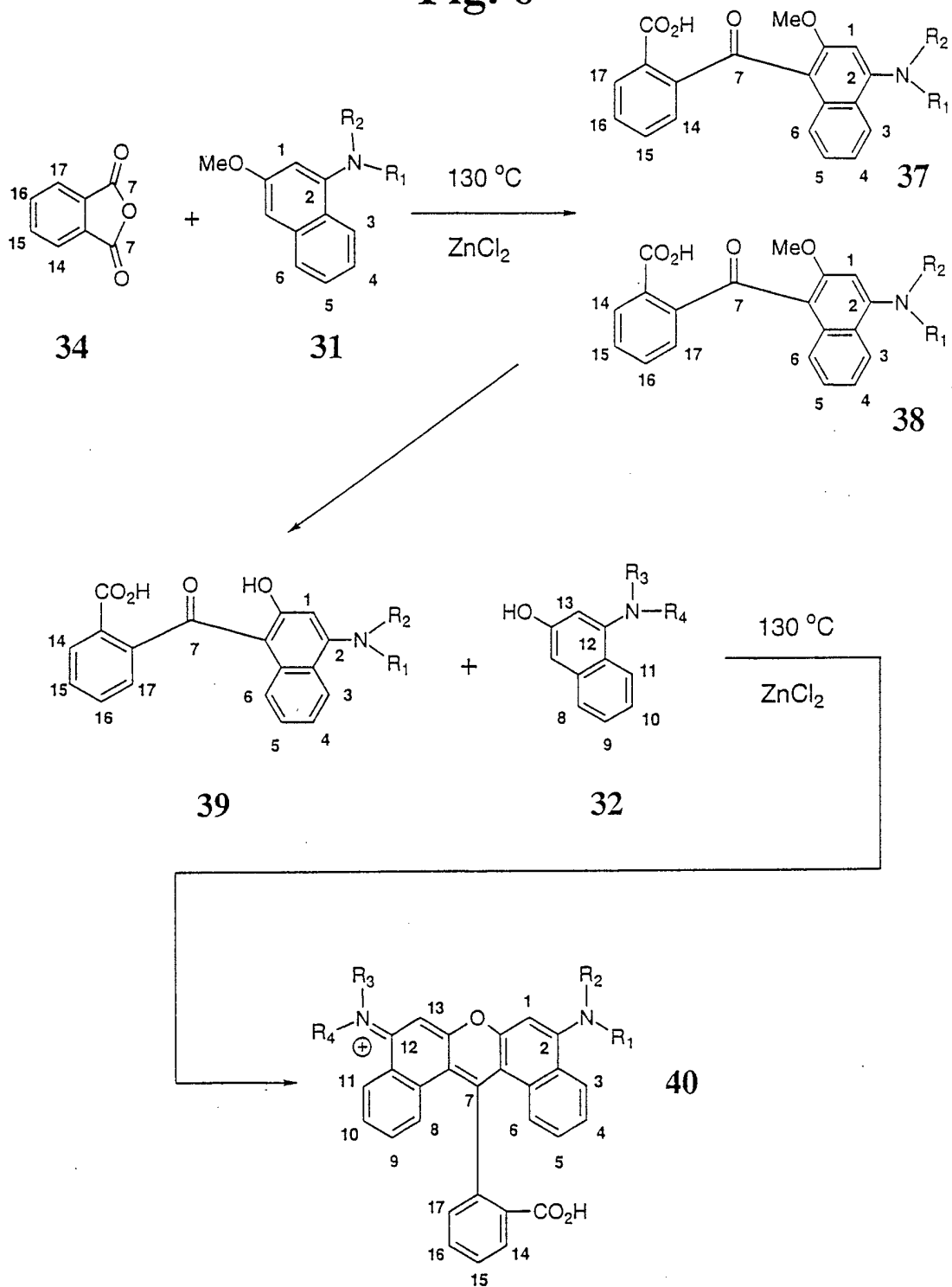


Fig. 5



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Fig. 6



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Fig. 7

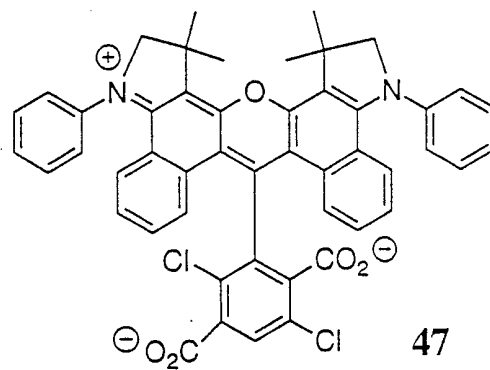
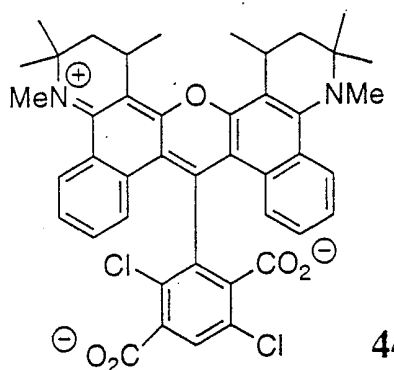
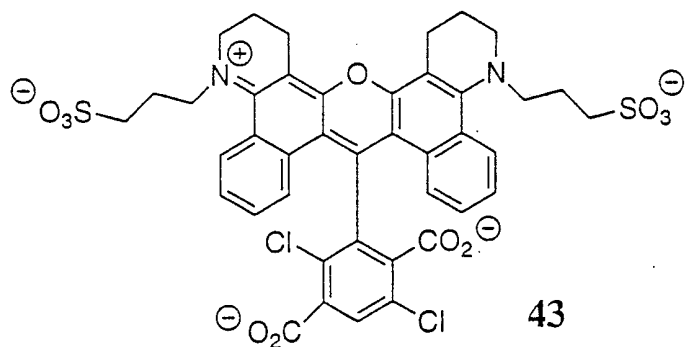
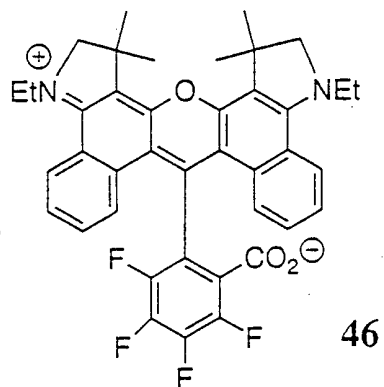
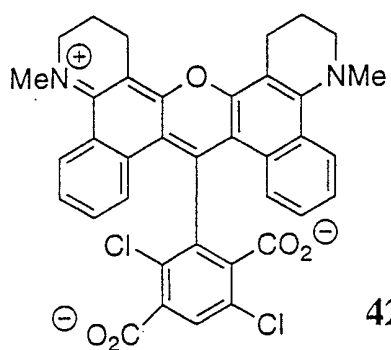
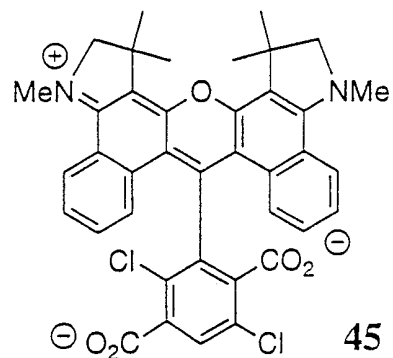
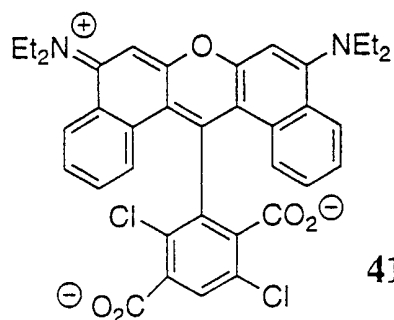
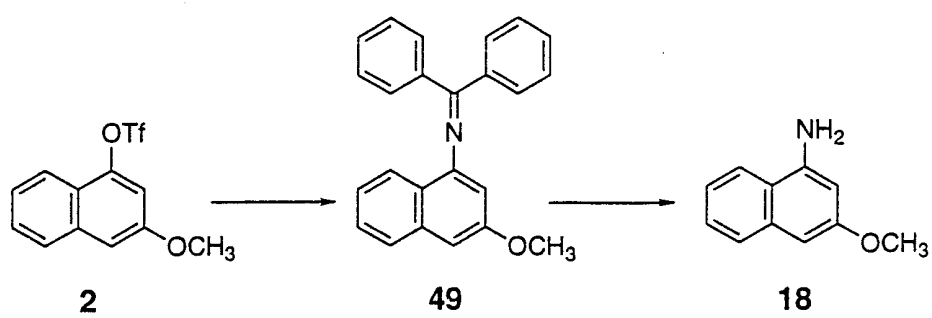


FIG. 8



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FIG. 9

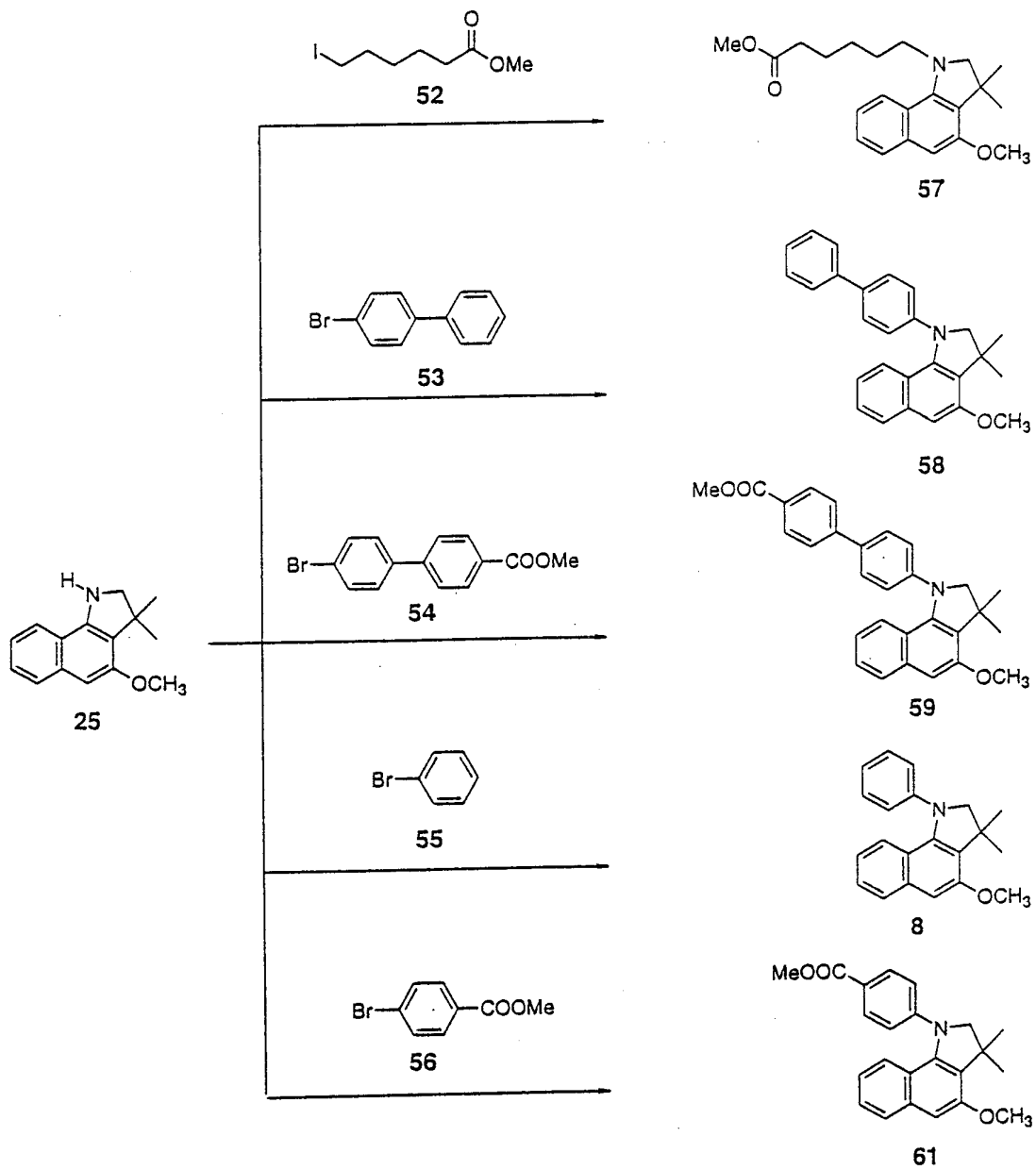


FIG. 10

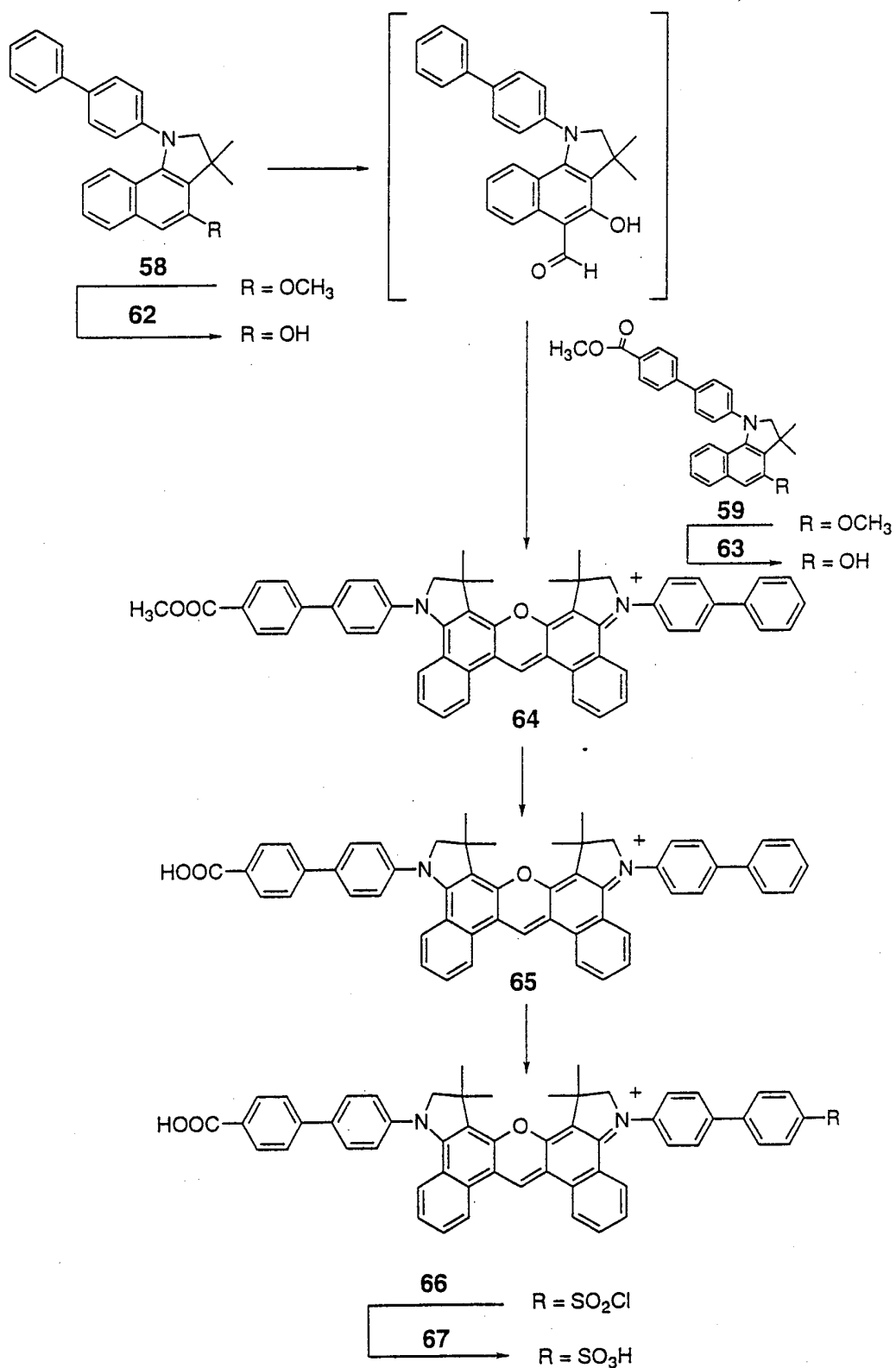


FIG. 11

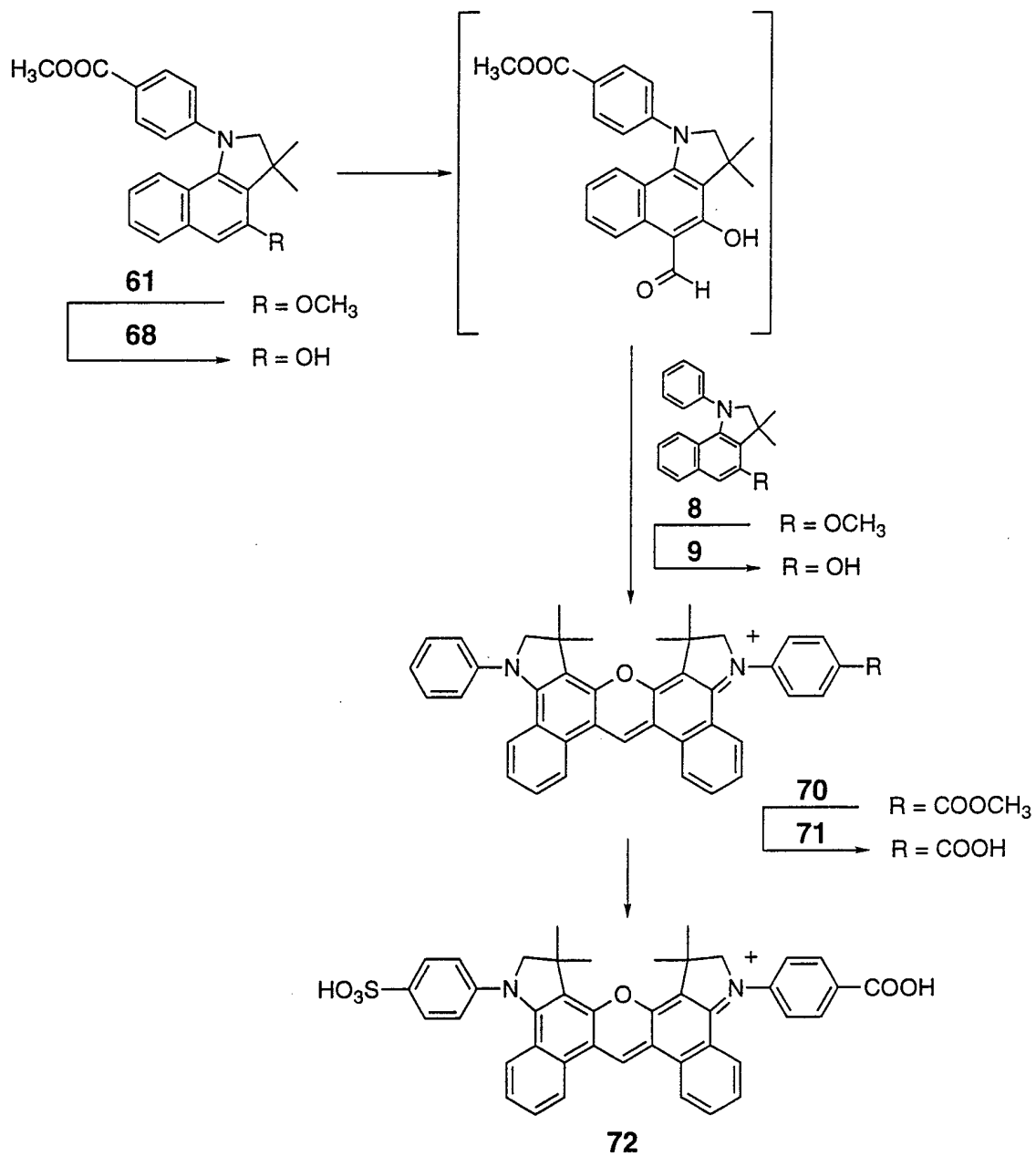


FIG. 12

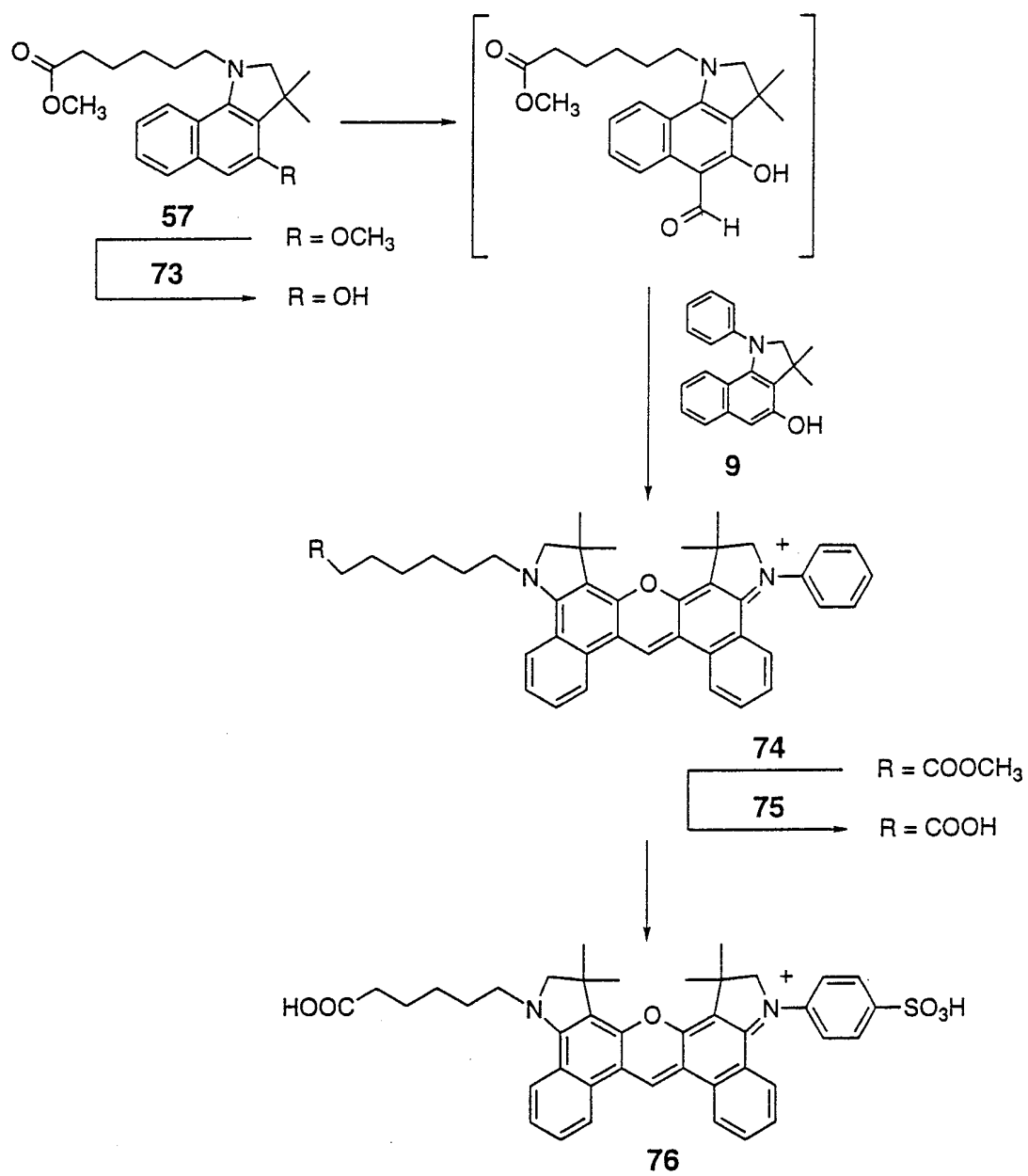


FIG. 13

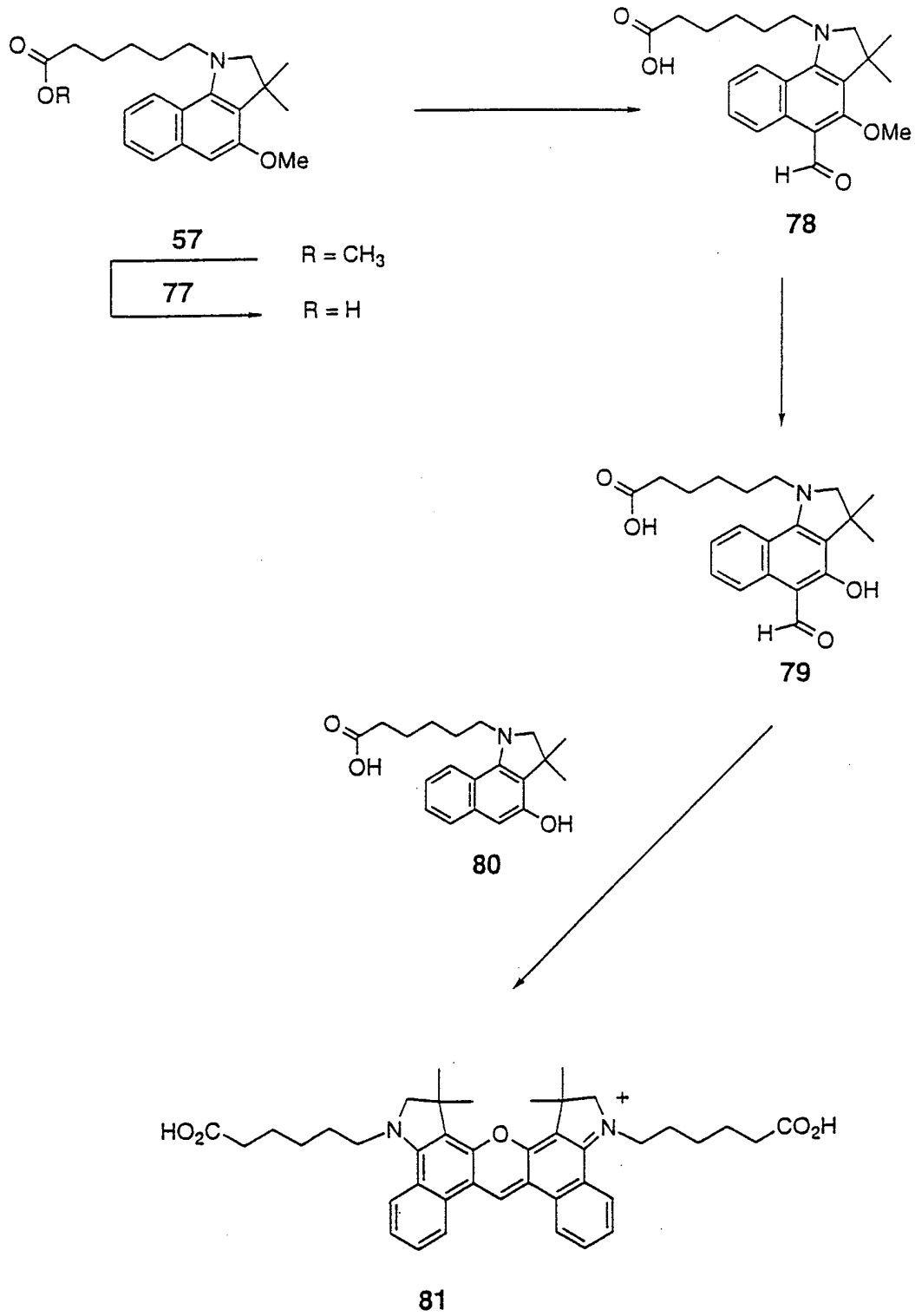


FIG. 14

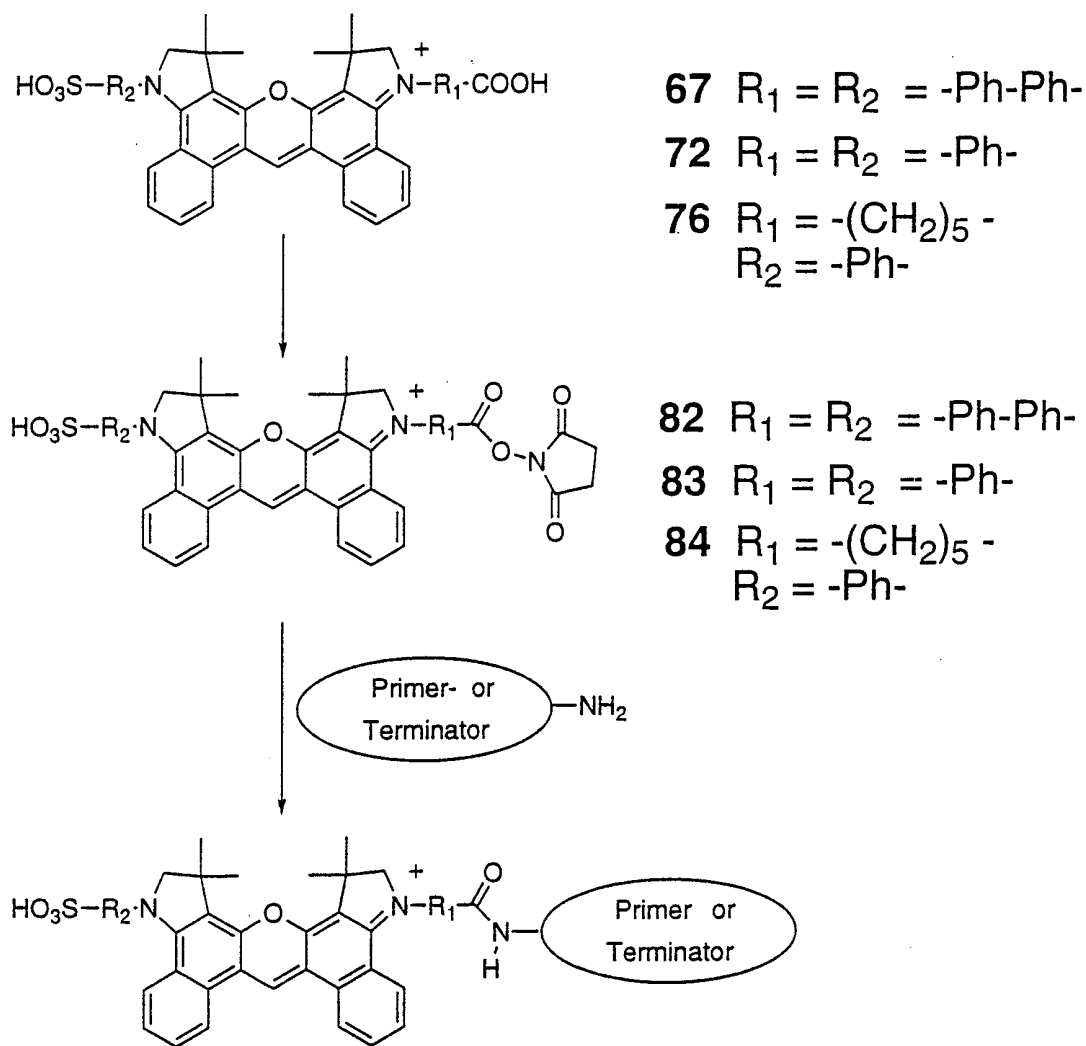
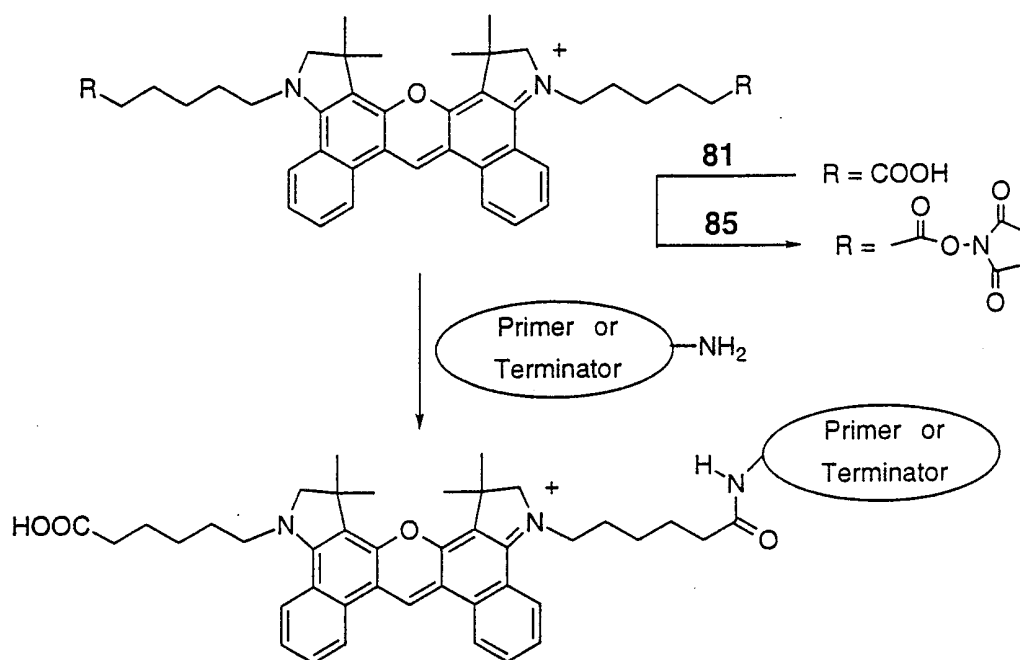


FIG. 15



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 98/24626

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C09B11/02 C09B11/24 C07H19/04 C07H21/00 C07D209/60
 C07D221/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C09B C07H C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP 0 543 333 A (BOEHRINGER MANNHEIM GMBH) 26 May 1993 see page 5, line 23 - page 11, line 29; claims; examples ---	1-105
A	WO 94 05688 A (APPLIED BIOSYSTEMS) 17 March 1994 see claims; examples ---	1-105
	-/--	

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

° Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search 19 March 1999	Date of mailing of the international search report 10/05/1999
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Ginoux, C
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INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/US 98/24626

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M.ONDA ET AL.: "Heterocycles. IV. Photolyses of..." CHEMICAL AND PHARMACEUTICAL BULLETIN., vol. 25, no. 11, 1977, pages 2935-2941, XP002097261 TOKYO JP see compound 12 ---	60,69,84
X	CHEMICAL ABSTRACTS, vol. 83, no. 14, 6 October 1975 Columbus, Ohio, US; abstract no. 115921, T.TSUNODA ET AL.: "Naphthazolyphenyl azides" page 75; XP002097263 see abstract -& JP 50 040626 A (HODOGAYA CHEMICAL CO LTD) 14 April 1975 see example 5 ---	60,69,70
X	N. SOTOMAYOR ET AL. : "Bischler-Napieralsky cyclisation..." JOURNAL OF ORGANIC CHEMISTRY., vol. 61, no. 12, 1996, pages 4062-4072, XP002097262 EASTON US see page 4068, compound 28 ---	60,69, 76-78,84
X	CHEMICAL ABSTRACTS, vol. 114, no. 22, 3 June 1991 Columbus, Ohio, US; abstract no. 218012z, H.TAKAI: "Electrophotographic photoconductors" page 743; XP002097264 see abstract -& JP 02 050069 A (CANON K.K.) 22 February 1990 see pages 5,6 structures 2-1, 2-3, 2-7, 2-11, 2-12, 3-1 -----	60,69

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