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(54) **Title**: DIARYLAMINE-BASED FLUOROGENIC PROBES FOR DETECTION OF PEROXYNITRITE

(57) **Abstract**: Provided herein are improved fluorogenic compounds and probes that can be used as reagents for measuring, detecting and/or screening peroxynitrite. The fluorogenic compounds of the invention can produce fluorescence colors, such as green, yellow, red, or far-red. Also provided herein are fluorogenic compounds for selectively staining peroxynitrite in the mitochondria of living cells. Provided also herein are methods that can be used to measure, directly or indirectly, the presence and/or amount of peroxynitrite in chemical samples and biological samples such as cells and tissues in living organisms. Also provided are high- throughput screening methods for detecting or screening peroxynitrite or compounds that can increase or decrease the level of peroxynitrite in chemical and biological samples.

DIARYLAMINE-BASED FLUOROGENIC PROBES FOR DETECTION OF PEROXYNITRITE

DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 61/592,122, filed January 30, 2012, which is hereby incorporated by reference in its entirety.

BACKGROUND

Fluorescence technology is enjoying ever-increasing interest from chemistry to many areas of biology. In certain instances, fluorescent molecules are used to detect the presence of analytes in food and environmental samples. Some sensitive and quantitative fluorescence detection devices are ideal for *in vitro* biochemical assays such as DNA sequencing and blood glucose quantification. Moreover, certain fluorescent probes are indispensable for tracing molecular and physiological events in living cells. Finally, fluorescence measurements are often used in many high-throughput screenings.

The primary advantages of fluorescence technology over other types of optical measurements include sensitivity, simplicity, and a wealth of molecular information. Fluorescence measurements are highly sensitive because of the generally low level of fluorescence background in most chemical and biological samples. Along with the advances in fluorescence instrumentation such as confocal and multi-photo fluorescence microscopies, three-dimensional imagings of cellular events and biological species dynamics have become possible in real-time.

Particularly, fluorescence in biological sciences is generally used as a non-destructive way for tracking or analyzing biological molecules, such as proteins, metal ions, reactive oxygen species (ROS)/reactive nitrogen species (RNS), and so on, by recording or imaging the fluorescence emission of certain fluorescent probes for corresponding biological molecules at specific wavelengths where there is no cellular intrinsic fluorescence induced by the excitation light.

Among these intriguing biological molecules in living systems, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been receiving much attention from the scientific community in the development of fluorescent probes for their detection in biological samples. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generally known to scientists as very small inorganic or organic molecules with high reactivity in living systems. There are various forms of ROS and RNS, including free radicals such as superoxide radical, hydroxyl radical, nitric oxide, nitrogen dioxide, and organic peroxyl radical; as well as non-radical species such as hydrogen peroxide, singlet oxygen, ozone, nitrous acid, peroxynitrite, and hypochlorite. ROS and RNS are by-products of cellular respiration. Under normal conditions, ROS and RNS are present in very low levels and play important roles in cell signaling; while during oxidative stresses, ROS and RNS levels increase dramatically, which can cause serious damages to various biological molecules such as protein, lipids and DNA. The excessive generation of ROS and RNS has been implicated in a lot of human diseases, such as cardiovascular diseases, inflammatory diseases, metabolic diseases, cancer and central nervous system diseases. Therefore, there is a strong need for chemicals that can sensitively and selectively measure, detect or screen certain ROS and RNS to address their physiological roles both *in vitro* and *in vivo*.

Peroxynitrite has the strongest oxidizing power among the various forms of ROS and RNS, and their selective detections are highly desirable to clearly explain their critical roles in

living organisms. Peroxynitrite ($ONOO^-$) is a short-lived oxidant species that is formed *in vivo* by the diffusion-controlled reaction (k = 0.4–1.9×10^{10} $M^{-1}s^{-1}$) of nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) in one to one stoichiometry. The oxidant reactivity of peroxynitrite is highly pH-dependent and both peroxynitrite anion and its protonated form peroxynitrous acid can participate directly in one- and two-electron oxidation reactions with biomolecules. The pathological activity of $ONOO^-$ is also related to its reaction with the biologically ubiquitous CO_2, thereby producing the highly reactive radicals $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ in about 35% yield. As a result of this, peroxynitrite can nitrate tyrosine and oxidize proteins, lipids and iron and sulfur clusters of biological molecules. Like other oxidizing agents in living organisms, peroxynitrite and its protonated form have been associated with both beneficial and harmful effects. However, several studies have implicated that peroxynitrite contributes to tissue injury in a number of human diseases such as ischemic reperfusion injury, rheumatoid arthritis, septic shock, multiple sclerosis, atherosclerosis, stroke, inflammatory bowl disease, cancer, and several neurodegenerative diseases (MacMillan-Crow, L. A. et al., *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 11853–11858; Rodenas, J. et al., *Free Radical. Biol. & Med.* **2000**, *28*, 374; Cuzzocrea, S. et al., *Pharmacol Rev.* **2001**, *53*, 135–159; Szabo, C. *Toxicol. Lett.* **2003**, *140*, 105–112; White, C. R. et al., *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 1044–1048; Lipton, S. A. et al., *Nature* **1993**, *364*, 626–632; Pappolla, M. A. et al., *J. Neural Transm.* **2000**, *107*, 203–231; Beal, M. F., *Free Radical Biol. & Med.* **2002**, *32*, 797–803).

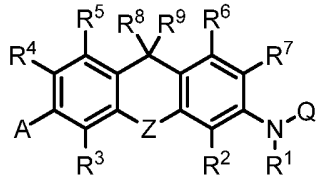
At present, peroxynitrite probes with green fluorescent color are available (U.S. Patent Application Serial No. 12/417,672); however, the existing green fluorescent probes exhibit limited intracellular retention in cell assays. In addition, peroxynitrite probes with other fluorescent colors or with the ability to localize in the desired intracellular compartment are rare. Long-wavelength fluorogenic probes, such as yellow, red, far-red, and near-infrared (NIR) fluorogenic probes, are more attractive and advantageous than green probes for providing

reliable imaging in biological samples, since they effectively avoid the interference from the auto-fluorescence of cells in the green region and possess longer excitation/emission wavelengths with deeper penetration into cells and tissues. Therefore, new generations of fluorescent probes with much more desirable and reliable detection and imaging of peroxynitrite are needed.

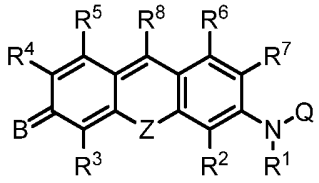
BRIEF SUMMARY

The subject invention provides improved fluorogenic or fluorescent compounds and probes for sensitive and specific detection of peroxynitrite. In one embodiment, provided herein are fluorogenic or fluorescent compounds that produce fluorescence colors such as green, yellow, red, or far-red. Also provided herein are fluorogenic or fluorescent compounds for selectively staining peroxynitrite in mitochondria of living cells.

In one aspect, the subject invention provides fluorogenic or fluorescent compounds represented by formula (I) or (II):



(I),



(II),

or a tautomer thereof;

wherein N is a nitrogen atom, and is linked to Q and R^1 through single covalent bonds;

R^1 is H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, carboxyalkyl, alkylamino, alkoxyamino, alkylamido, alkoxyamido, or acyl;

each of R^2, R^3, R^4, R^5, R^6 and R^7 is independently H, F, Cl, Br, I, CN, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, acylamino, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, nitro, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic acid, phosphate ester, sulfonic acid ($-SO_3H$), sulfonate ester, sulfonamide, $-C(=O)-P^1$ or $-C(=O)-M-P^2$;

each of P^1 and P^2 is independently hydrogen, halo, alkoxy, hydroxy, thiol, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl, arylalkyl, carbamate, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, alkylthio, heteroalkyl, alkyltriphenylphosphonium, or heterocyclyl having from 3 to 7 ring atoms; M is alkylene, alkenylene, alkynylene, arylene, aralkylene or alkarylene;

A is OR^{10} or $NR^{11}R^{12}$;

wherein R^{10} is H, alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, aryl, alkaryl, arylalkyl, carboxyalkyl, alkoxycarbonyl, acyl or aminocarbonyl;

wherein each of R^{11} and R^{12} is independently H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, acyl, carboxyalkyl, sulfoalkyl, a salt of carboxyalkyl, a salt of sulfoalkyl, or an ester or amide of carboxyalkyl or sulfoalkyl; or R^{11} in combination with R^{12} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by alkyl, carboxylic acid, a salt of

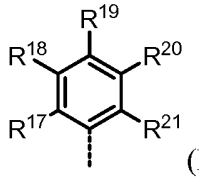
carboxylic acid, or a carboxylic acid ester of an alcohol; or R^{11} in combination with R^4, or R^{12} in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or further fused with an aryl or heteroaryl ring, and is optionally substituted by one or more alkyls, carboxylic acids, sulfonic acids ($-SO_3H$), or their salts, ester or amide derivatives;

B is O or $N^+R^{11}R^{12}$;

Z is O, S, NR^{13}, $CR^{13}R^{14}$, $SiR^{13}R^{14}$, $GeR^{13}R^{14}$, or $SnR^{13}R^{14}$;

wherein each of R^{13} and R^{14} is independently H, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic acid, phosphate ester, sulfonic acid ($-SO_3H$), sulfonate ester, sulfonamide, carboxylic acid, carboxylic ester, or carboxylic amide; or R^{13} in combination with R^{14} forms a saturated 5- or 6-membered heterocycle that is optionally substituted by alkyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol;

R^8 is H, CF_3, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol; or R^8 is a saturated or unsaturated alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$), wherein each of R^{15} and R^{16} represents a saturated or unsaturated, cyclic or acyclic alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, or alkyltriphenylphosphonium; or R^8 has the formula

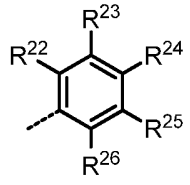


(III)

wherein each of R^{17}, R^{18}, R^{19}, R^{20} and R^{21} is independently H, F, Cl, Br, I, CN, nitro, a carboxylic acid, a salt of carboxylic acid, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), sulfonamide ($-SO_2NR^{15}R^{16}$), hydroxy, azide, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed alkylcarbonylalkyl such as trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$); or R^{17} and R^{18} together, R^{18} and R^{19} together, R^{19} and R^{20} together, or R^{20} and R^{21} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (III) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$);

R^9 is H, hydroxy, CN or alkoxy; or R^9 in combination with R^8 forms a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring; or R^9 in combination with R^{17} or R^{21} forms a 5- or 6-membered spirolactone or spirolactam ring or a 5- or 6-membered spirosultone or spirosultam ring that is optionally and independently substituted by H, F or CH_3; specifically, R^9, when taken in combination with R^8 forming a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring, is oxygen or substituted nitrogen; and

Q is substituted phenyl having formula (IV):



(IV),

wherein each of R^{22}, R^{23}, R^{24}, R^{25}, and R^{26} is independently H, hydroxy, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed alkylcarbonylalkyl such as trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$); or R^{22} and R^{23} together, R^{23} and R^{24} together, R^{24} and R^{25} together, or R^{25} and R^{26} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (IV) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$).

The subject invention also provides fluorogenic or fluorescent probe compositions, comprising a fluorogenic or fluorescent compound of the invention, and optionally, a carrier, solvent, an acid, a base, a buffer solution, or a combination thereof.

Also provided herein are methods for detecting the presence of, or measuring the level of, peroxynitrite in samples. In some embodiments, the methods comprise the steps of (a) contacting a fluorogenic compound or probe disclosed herein with a sample to form a fluorescent compound; and (b) determining or measuring fluorescent property of the fluorescent compound.

Also provided herein are high-throughput screening methods for detecting peroxynitrite in samples. In some embodiments, the high-throughput screening fluorogenic methods comprise the steps of (a) contacting a fluorogenic compound or probe disclosed herein with sample(s) to form a fluorescent compound; and (b) measuring fluorescence property of the fluorescent compound.

Also provided herein are high-throughput methods for screening one or more target compounds that can increase or decrease the level of peroxynitrite. In some embodiments, the high-throughput screening method for detecting peroxynitrite comprises the steps of: (a) contacting a fluorogenic compound or probe disclosed herein with samples to form one or more fluorescent compounds; and (b) measuring fluorescence property of the fluorescent compounds to determine the amount of peroxynitrite in the samples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts fluorescence spectra showing fluorescence intensities of compound **2** after treatment with different amounts of peroxynitrite. **Figure 1B** shows increases in fluorescence intensity of Compound **2** after treatment with different reactive oxygen species (ROS) and reactive nitrogen species (RNS). The spectra were acquired by dissolving Compound 2 in 0.1 M phosphate buffer at pH 7.4 to form a 1 μM solution, with excitation and emission spectra at 510 nm and 530 nm, respectively. The concentration of highly reactive oxygen species hydroxyl radical (•OH), hypochlorous acid ($^{-}$OCl), and peroxynitrite ($ONOO^{-}$) is 1 μM. The concentration of $^{1}O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 10 μM.

Figure 2A depicts fluorescence spectra showing fluorescence intensities of Compound **11** after treatment with different amounts of peroxynitrite. **Figure 2B** shows increases in fluorescence intensity of Compound **11** after treatment with different ROS and RNS. The spectra were acquired by dissolving Compound 7 in 0.1M phosphate buffer at pH 7.4 to form a 2 μM

solution, with excitation and emission at 547 nm and 570 nm, respectively. The concentration of highly reactive oxygen species hydroxyl radical (•OH), hypochlorous acid ($^-$OCl), and peroxynitrite($ONOO^-$) is 2 μM. The concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 20 μM.

Figure 3A depicts fluorescence spectra showing fluorescence intensities of Compound **22** after treatment with different amounts of peroxynitrite. **Figure 3B** shows increases in fluorescence intensity of Compound **22** after treatment with different ROS and RNS. The spectra were acquired by dissolving Compound **22** in 0.1M phosphate buffer at pH 7.4 to form a 5 μM solution, with excitation at 600 nm and emission at 617 nm, respectively. The concentration of highly reactive oxygen species hydroxyl radical (•OH), hypochlorous acid ($^-$OCl), and peroxynitrite ($ONOO^-$) is 5 μM. The concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 50 μM.

Figure 4A depicts fluorescence spectra showing fluorescence intensities of Compound **25** after treatment with different amounts of peroxynitrite. **Figure 4B** shows increases in fluorescence intensity of Compound **25** after treatment with different ROS and RNS. The spectra were acquired by dissolving Compound **25** in 0.1M phosphate buffer at pH 7.4 to form a 5 μM solution, with excitation at 650 nm and emission at 665 nm, respectively. The concentration of highly reactive oxygen species hydroxyl radical (•OH), hypochlorous acid ($^-$OCl), and peroxynitrite ($ONOO^-$) is 5 μM. The concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 50 μM.

Figure 5A depicts fluorescence spectra showing fluorescence intensities of Compound **30** after treatment with different amounts of peroxynitrite. **Figure 5B** shows increases in fluorescence intensity of Compound **30** at the emission maximum of 540 nm after treatment with different ROS and RNS. The spectra were acquired by dissolving Compound **30** in 0.1 M phosphate buffer at pH 7.4 and exciting at 515 nm. The concentration of highly reactive oxygen species hypochlorous acid ($^-$OCl) and peroxynitrite ($ONOO^-$) is 1 μM. The concentration of

hydroxyl radical (•OH) is 1 μM or 10 μM. The concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 10 μM or 100 μM. (see figure for exact concentrations of certain ROS and RNS)

Figure 6 shows fluorescent microscopy results of SH-SY5Y cells upon treatment with or without SIN-1, a peroxynitrite generator, using Compounds **6**, **7**, and **8**. SH-SY5Y cells were co-staining with different compounds with or without SIN-1 for 1 h, and then washed quickly with PBS for 3 times and maintained in non-phenol red medium. Left: no SIN-1 treatment; Right: with SIN-1 treatment.

Figure 7 shows fluorescent microscopy results of SH-SY5Y cells upon treatment with or without SIN-1, a peroxynitrite generator, using Compounds **14**. SH-SY5Y cells were co-staining with different compounds with or without SIN-1 for 1 h, and then washed quickly with PBS for 3 times and maintained in non-phenol red medium. Left: no SIN-1 treatment; Middel: with 1 mM SIN-1 treatment; Right: with 100 μM SIN-1 treatment.

Figure 8 shows fluorescent microscopy results of C17.2 cells upon treatment with SIN-1, a peroxynitrite generator, using Compounds **20** and **21**. The cells were incubated with Compound **20** or **21** at a concentration of 1 μM, and then treated with (Lower) or without (Upper) SIN-1.

Figure 9 shows fluorescent microscopy results of SH-SY5Y cells upon treatment with or without SIN-1, a peroxynitrite generator, using Compounds **24**. SH-SY5Y cells were co-staining with different compounds with or without SIN-1 for 1 h, and then washed quickly with PBS for 3 times and maintained in non-phenol red medium. Left: no SIN-1 treatment; Middle: with 100 μM SIN-1 treatment; Right: with 200 μM SIN-1 treatment.

Figure 10 shows fluorescent microscopy results of Raw 264.7 macrophages under the stimulation conditions. The macrophage cells were incubated with Compound **27** at a concentration of 500 nM. Upper: Control; Lower: The macrophages were stimulated with LPS

and IFN-γ for 14 hr. Left: Nuclear staining with Hoechst; Middle: Compound **27**; Right: Merged.

Figure 11 shows fluorescent microscopy results of C17.2 cells upon treated with SIN-1, a peroxynitrite generator, using Compounds **32**. The cells were incubated with Compound **32** at a concentration of 5 μM. The colocalization of red signal from Compound **32** and green signal from Mitotracker-Green indicates Compound **32** selectively localizes to mitochondria of cells.

Figure 12 shows a representative figure of screening drugs for scavenging peroxynitrite with Compound **14**. SH-SY5Y cells were seeded in 96 well black plates and incubated with Compound **14**. The cells were then treated with SIN-1 in the presence of different drug candidates. The fluorescence intensity for each well was recorded and used to determine the scavenging activity of the drug candidate.

Figure 13 shows fluorescent microscopy results of ex vivo rats brain slices upon treatment with SIN-1, a peroxynitrite generator, using Compounds **14**.

Figure 14 shows fluorescent microscopy results of liver sample sections from ethanol treated or non-treated mice (Ethanol group or Sham group, respectively) using Compounds **14**.

DEFINITIONS

To facilitate the understanding of the subject matter disclosed herein, a number of terms, abbreviations or other shorthand as used herein are defined below. Any term, abbreviation or shorthand not defined is understood to have the ordinary meaning used by a skilled artisan contemporaneous with the submission of this application.

"Amino" refers to a primary, secondary, or tertiary amine which may be optionally substituted. Specifically included are secondary or tertiary amine nitrogen atoms which are members of a heterocyclic ring. Also specifically included, for example, are secondary or tertiary amino groups substituted by an acyl moiety. Some non-limiting examples of an amino

group include –NR'R" wherein each of R' and R" is independently H, alkyl, aryl, aralkyl, alkaryl, cycloalkyl, acyl, heteroalkyl, heteroaryl or heterocycyl.

"Alkyl" refers to a fully saturated acyclic monovalent radical containing carbon and hydrogen, and which may be branched or a straight chain. In some embodiments, alkyl contains from about 1 to about 25 carbon atoms. Examples of alkyl groups include, but are not limited to, methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *t*-butyl, *n*-heptyl, *n*-hexyl, *n*-octyl, and *n*-decyl. "Lower alkyl" refers to an alkyl radical of one to six carbon atoms, as exemplified by methyl, ethyl, *n*-butyl, *i*-butyl, *t*-butyl, isoamyl, *n*-pentyl, and isopentyl.

"Heteroalkyl" refers to an alkyl group having one or more of the carbon atoms within the alkyl group substituted by a heteroatom such as O, S and N. In some embodiments, the heteroalkyl group comprises one or more O atoms. In other embodiments, the heteroalkyl group comprises one or more S atoms. In further embodiments, the heteroalkyl group comprises one or more aminylene groups. In certain embodiments, the heteroalkyl group comprises two or more O, S, aminylene, or a combination thereof.

"Alkenyl" or "alkenylene," respectively, refers to a monovalent or divalent hydrocarbyl radical which has at least one double bond. The alkenyl or alkenylene group may be cyclic, branched acyclic or straight acyclic. In some embodiments, the alkenyl or alkenylene group contains only one double bond. In other embodiments, the alkenyl or alkenylene group contains two or more double bonds. In further embodiments, the alkenyl or alkenylene group can be a lower alkenyl or alkenylene containing from two to eight carbon atoms in the principal chain. In further embodiments, the alkenyl or alkenylene group can have one double bond and up to 25 carbon atoms, as exemplified by ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, hexenyl, and the like.

"Alkynyl" or "alkynylene," respectively, refers to a monovalent or divalent hydrocarbyl radical which has at least a triple bond. In some embodiments, the alkynyl or alkynylene group

contains only one triple bond. In other embodiments, the alkynyl or alkynylene group contains two or more triple bonds. In further embodiments, the alkynyl or alkynylene group can be a lower alkynyl or alkynylene containing from two to eight carbon atoms in the principal chain. In further embodiments, the alkynyl or alkynylene group can have one triple bond and up to 20 carbon atoms, as exemplified by ethynyl, propynyl, isopropynyl, butynyl, isobutynyl, hexynyl, and the like.

"Aromatic" or "aromatic group" refers to aryl or heteroaryl.

"Aryl" refers to optionally substituted carbocyclic aromatic groups. In some embodiments, the aryl group includes a monocyclic or bicyclic group containing from 6 to 12 carbon atoms in the ring portion, such as phenyl, biphenyl, naphthyl, substituted phenyl, substituted biphenyl or substituted naphthyl. In other embodiments, the aryl group is phenyl or substituted phenyl.

"Aralkyl" refers to an alkyl group which is substituted with an aryl group. Some non-limiting examples of aralkyl include benzyl and phenethyl.

"Alkaryl" refers to an aryl group which is substituted with an alkyl group. Some non-limiting examples of alkaryl include methylphenyl and methylnaphthyl.

"Acyl" refers to a monovalent group of the formula −C(=O)H, −C(=O)−alkyl, −C(=O)−aryl, −C(=O)−aralkyl, or −C(=O)−alkaryl.

"Halogen" refers to fluorine, chlorine, bromine and iodine.

"Halo" refers to fluoro, chloro, bromo and iodo.

"Heteroatom" refers to atoms other than carbon and hydrogen.

"Heterocyclo" or "heterocyclyl" refers to optionally substituted, fully saturated or unsaturated, monocyclic or bicyclic, aromatic or nonaromatic groups having at least one heteroatom, such as O, S, N, B and P, in at least one ring. The aromatic heterocyclyl (*i.e.*, heteroaryl) group can have 1 or 2 oxygen atoms, 1 or 2 sulfur atoms, and/or 1 to 4 nitrogen

atoms in the ring, and may be bonded to the remainder of the molecule through a carbon or heteroatom. Some non-limiting examples of heteroaryl include furyl, thienyl, thiazolyl, pyridyl, oxazolyl, pyrrolyl, indolyl, quinolinyl, or isoquinolinyl and the like.

"Hydrocarbon" or "hydrocarbyl" refers to organic compounds or radicals consisting exclusively of the elements carbon and hydrogen. Hydrocarbyl includes alkyl, alkenyl, alkynyl, and aryl moieties. Hydrocarbyl also includes alkyl, alkenyl, alkynyl, and aryl moieties substituted with other aliphatic, cyclic or aryl hydrocarbon groups, such as alkaryl, alkenaryl and alkynaryl. In some embodiments, "hydrocarbon" or "hydrocarbyl" comprises 1 to 30 carbon atoms.

"Hydrocarbylene" refers to a divalent group formed by removing two hydrogen atoms from a hydrocarbon, the free valencies of which are not engaged in a double bond, *e.g.* arylene, alkylene, alkenylene, alkynylene, aralkylene or alkarylene.

"Substituted" as used herein to describe a compound or chemical moiety refers to that at least one hydrogen atom of that compound or chemical moiety is replaced with a second chemical moiety. Non-limiting examples of substituents are those found in the exemplary compounds and embodiments disclosed herein, as well as halogen; alkyl; heteroalkyl; alkenyl; alkynyl; aryl, heteroaryl, hydroxy; alkoxyl; amino; nitro; thiol; thioether; imine; cyano; amido; phosphonato; phosphine; carboxyl; thiocarbonyl; sulfonyl; sulfonamide; ketone; aldehyde; ester; oxo; haloalkyl (*e.g.*, trifluoromethyl); carbocyclic cycloalkyl, which can be monocyclic or fused or non-fused polycyclic (*e.g.*, cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl) or a heterocycloalkyl, which can be monocyclic or fused or non-fused polycyclic (*e.g.*, pyrrolidinyl, piperidinyl, piperazinyl, morpholinyl or thiazinyl); carbocyclic or heterocyclic, monocyclic or fused or non-fused polycyclic aryl (*e.g.*, phenyl, naphthyl, pyrrolyl, indolyl, furanyl, thiophenyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, triazolyl, tetrazolyl, pyrazolyl, pyridinyl, quinolinyl, isoquinolinyl, acridinyl, pyrazinyl, pyridazinyl, pyrimidinyl, benzimidazolyl, benzothiophenyl or

benzofuranyl); amino (primary, secondary or tertiary); o-lower alkyl; o-aryl, aryl; aryl-lower alkyl; $-CO_2CH_3$; $-CONH_2$; $-OCH_2CONH_2$; $-NH_2$; $-SO_2NH_2$; $-OCHF_2$; $-CF_3$; $-OCF_3$; –NH(alkyl); $–N(alkyl)_2$; –NH(aryl); –N(alkyl)(aryl); $–N(aryl)_2$; –CHO; –CO(alkyl); –CO(aryl); $–CO_2$(alkyl); and $–CO_2$(aryl); and such moieties can also be optionally substituted by a fused-ring structure or bridge, for example $–OCH_2O–$. These substituents can optionally be further substituted with a substituent selected from such groups. All chemical groups disclosed herein can be substituted, unless it is specified otherwise. For example, "substituted" alkyl, alkenyl, alkynyl, aryl, hydrocarbyl or heterocyclo moieties described herein are moieties which are substituted with a hydrocarbyl moiety, a substituted hydrocarbyl moiety, a heteroatom, or a heterocyclo. Further, substituents may include moieties in which a carbon atom is substituted with a heteroatom such as nitrogen, oxygen, silicon, phosphorus, boron, sulfur, or a halogen atom. These substituents may include halogen, heterocyclo, alkoxy, alkenoxy, alkynoxy, aryloxy, hydroxy, protected hydroxy, keto, acyl, acyloxy, nitro, amino, amido, cyano, thiol, ketals, acetals, esters and ethers.

"Fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. In some embodiments, the absorbed photon is in the ultraviolet range, and the emitted light is in the visible range.

"Green fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength that is within the range of about 520 nm to about 570 nm.

"Yellow fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength that is within the range of about 570 nm to about 590 nm.

"Orange fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength that is within the range of about 585 nm to about 620 nm.

"Red fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength that is within the range of about 620 nm to about 740 nm.

"Far-red fluorescence" refers to a luminescence where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength that is within the range of about 650 nm to about 740 nm.

"Fluorophore" refers to a small molecule or a part of a large molecule that can be excited by light to emit fluorescence. In some embodiments, fluorophores efficiently produce fluorescence upon excitation with light which has a wavelength from about 200 nanometers to about 1000 nanometers, or from about 500 nanometers to about 800 nanometers. The intensity and wavelength of the emitted radiation generally depend on both the fluorophore and the chemical environment of the fluorophore. A fluorophore may be selected from acridine orange, anthracene ring, allophycocyanin, BODIPY, cyanines, coumarin, Edans, Eosin, Erythrosin, fluorescamine, fluorescein, FAM (carboxyfluorescein), HEX (hexachlorofluorescein), JOE (6-carboxy-4',5'-dichloro-2',7'- dimethoxy-fluorescein), Oregon Green, phycocyanin, phycoerythrin, rhodamine, ROX (Carboxy-X-rhodamine), TAMRA (carboxytetramethylrhodamine), TET (tetrachloro-fluorescein), Texas red, tetramethylrhodamine, and xanthines. Other non-limiting examples can be found in *The Handbook: a Guide to Fluorogenic Probes and Labeling Technologies* (10th Edition, Molecular Probes, Eugene, Orgeon, 2006), which are incorporated herein by reference.

"Reactive group" or "Rg" refers to a group that is highly reactive toward an amine, a thio, an alcohol, an aldehyde or a ketone. Some non-limiting examples of a reactive group include

phosphoramidite, succinimidyl ester of a carboxylic acid, haloacetamide, hydrazine, isothiocyanate, maleimide, perfluorobenzamido, and azidoperfluorobenzamido.

"Conjugated substance" or "Cg" refers to a desired substance which needs to be conjugated and generally possess a suitable functional group for covalent reaction with a respective reactive group, Rg. Some non-limiting examples of conjugated substances include conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, amino acids, peptides, nucleotides, oligonucleotides, nucleic acid, carbohydrates, lipids, and the like.

"Reactive oxygen species" or ROS refer to oxygen-containing ions, free radicals as well as non-radical species. Some non-limiting examples of reactive oxygen species include $^1O_2$, $O_2^{\bullet-}$, $ROO^{\bullet}$, $^{\bullet}OH$, OCl^-, and H_2O_2.

"Reactive nitrogen species" or RNS refer to nitrogen-containing ions, free radicals as well as non-radical species. Some non-limiting examples of reactive nitrogen species include nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$).

"Fluorogenic probe" refers to a latent fluorogenic molecule, whose fluorescence stays in "off" state before reacting with the target and may switch to "on" state after reacting with the target.

"Peroxynitrite fluorogenic compound" refers to a compound that can react with peroxynitrite to produce a fluorescence signal. In certain embodiments, the peroxynitrite fluorogenic compounds of the invention substantially react with peroxynitrite.

"Peroxynitrite-specific fluorogenic compound" or "fluorogenic compound that specifically detects peroxynitrite" refers to a fluorogenic compound that reacts with peroxynitrite in a yield of about 10% higher than, about 15% higher than, about 20% higher than, about 25% higher than, about 30% higher than, about 35% higher than, about 40% higher than, about 45% higher than, about 50% higher than, about 55% higher than, about 60% higher than, about 65% higher than, about 70% higher than, about 75% higher than, about 80% higher than, about 85%

higher than, about 90% higher than, about 95% higher than, about 100% higher than, about 200% higher than, about 300% higher than, or about 500% higher than that of any other ROS and RNS.

"Reacting", "adding" or the like refers to contacting one reactant, reagent, solvent, catalyst, reactive group or the like with another reactant, reagent, solvent, catalyst, reactive group or the like. Reactants, reagents, solvents, catalysts, reactive group or the like can be added individually, simultaneously or separately and can be added in any order. They can be added in the presence or absence of heat and can optionally be added under an inert atmosphere. In some embodiments, "reacting" refers to *in situ* formation or intra-molecular reaction where the reactive groups are in the same molecule.

"Substantially react" refers to that at least a reactant of a reaction is consumed by an amount of more than about 75% by mole, by more than about 80% by mole, by more than about 85% by mole, or by more than about 90% by mole. In some embodiments, "substantially react" refers to that the reactant is consumed by more than about 95% by mole. In other embodiments, "substantially react" refers to that the reactant is consumed by more than about 97% by mole. In further embodiments, "substantially react" refers to that the reactant is consumed by more than about 99% by mole.

"High-throughput method" refers to a method that can autonomously process or evaluate a large number of samples. In some embodiments, informatics systems can be used and implemented in the high-throughput method. The informatics systems can provide the software control of the physical devices used in the high-throughput method, as well as organize and store electronic data generated by the high-throughput method.

DETAILED DESCRIPTION

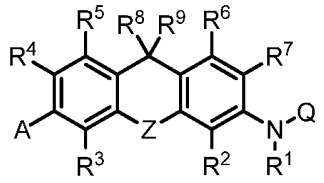
The subject invention provides a class of fluorogenic or fluorescent compounds and probes for sensitive and specific detection of peroxynitrite. Exemplary fluorogenic compounds and probes of the invention utilize an *N*-dearylation reaction between the diarylamine-caged fluorogenic compounds with peroxynitrite to achieve high sensitivity and selectivity for detecting peroxynitrite in aqueous solution over other reactive oxygen and nitrogen species (ROS/RNS). Exemplary fluorogenic compounds include compounds that produce fluorescence colors such as green, yellow, red, or far-red. Also provided herein are fluorogenic compounds for selectively staining peroxynitrite in mitochondria of living cells.

The fluorogenic compounds of the subject invention can be used to measure, directly or indirectly, the amount of peroxynitrite in both chemical and biological samples such as cells and tissues in living organisms, and therefore serve as powerful tools for interrogating the physiological and pathological roles of cellular peroxynitrite.

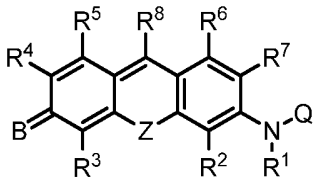
Compounds

General aspects

In one aspect, the subject invention provides fluorogenic or fluorescent compounds. In one embodiment, the fluorogenic or fluorescent compounds of the invention are represented by formula (I) or (II):



(I),



(II),

or a tautomer thereof;

wherein N is a nitrogen atom, and is linked to Q and R^1 through single covalent bonds;

R^1 is H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, carboxyalkyl, alkylamino, alkoxyamino, alkylamido, alkoxyamido, or acyl;

each of R^2, R^3, R^4, R^5, R^6 and R^7 is independently H, F, Cl, Br, I, CN, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, acylamino, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, nitro, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic acid, phosphate ester, sulfonic acid (-SO_3H), sulfonate ester, sulfonamide, $-C(=O)-P^1$ or $-C(=O)-M-P^2$;

each of P^1 and P^2 is independently hydrogen, halo, alkoxy, hydroxy, thiol, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl, arylalkyl, carbamate, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, alkylthio, heteroalkyl, alkyltriphenylphosphonium, or heterocyclyl having from 3 to 7 ring atoms; M is alkylene, alkenylene, alkynylene, arylene, aralkylene or alkarylene;

A is OR^{10} or $NR^{11}R^{12}$;

wherein R^{10} is H, alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, aryl, alkaryl, arylalkyl, carboxyalkyl, alkoxycarbonyl, acyl or aminocarbonyl;

wherein each of R^{11} and R^{12} is independently H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, acyl, carboxyalkyl, sulfoalkyl, a salt of carboxyalkyl, a salt of sulfoalkyl, or an ester or amide of carboxyalkyl or sulfoalkyl; or R^{11} in combination with R^{12} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by alkyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol; or R^{11} in combination with R^4, or R^{12} in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or further fused with an aryl or heteroaryl ring, and is optionally substituted by one or more alkyls, carboxylic acids, sulfonic acids ($-SO_3H$), or their salts, ester or amide derivatives;

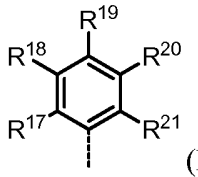
B is O or $N^+R^{11}R^{12}$;

Z is O, S, NR^{13}, $CR^{13}R^{14}$, $SiR^{13}R^{14}$, $GeR^{13}R^{14}$, or $SnR^{13}R^{14}$;

wherein each of R^{13} and R^{14} is independently H, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic acid, phosphate ester, sulfonic acid ($-SO_3H$), sulfonate ester, sulfonamide, carboxylic acid, carboxylic ester, or carboxylic amide; or R^{13} in combination with R^{14} forms a saturated 5- or 6-membered heterocycle that is optionally substituted by alkyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol;

R^8 is H, CF_3, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol; or R^8 is a saturated or unsaturated alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$), wherein each of R^{15} and R^{16} represents

a saturated or unsaturated, cyclic or acyclic alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, or alkyltriphenylphosphonium; or R^8 has the formula



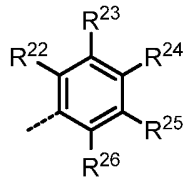
(III),

wherein each of R^{17}, R^{18}, R^{19}, R^{20} and R^{21} is independently H, F, Cl, Br, I, CN, nitro, a carboxylic acid, a salt of carboxylic acid, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), sulfonamide ($-SO_2NR^{15}R^{16}$), hydroxy, azide, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed alkylcarbonylalkyl such as trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$); or R^{17} and R^{18} together, R^{18} and R^{19} together, R^{19} and R^{20} together, or R^{20} and R^{21} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (III) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$);

R^9 is H, hydroxy, CN or alkoxy; or R^8 in combination with R^9 forms a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring; or R^9 in combination with

R^{17} or R^{21} forms a 5- or 6-membered spirolactone or spirolactam ring or a 5- or 6-membered spirosultone or spirosultam ring that is optionally and independently substituted by H, F or CH_3; specifically, R^9, when taken in combination with R^8 forming a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring, is oxygen or substituted nitrogen; and

Q is substituted phenyl having formula (IV):



(IV),

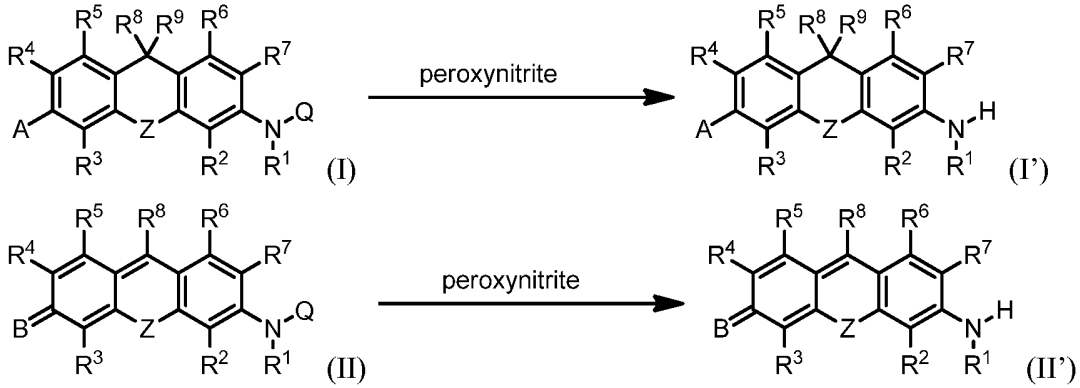
wherein each of R^{22}, R^{23}, R^{24}, R^{25}, and R^{26} is independently H, hydroxy, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed alkylcarbonylalkyl such as trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$); or R^{22} and R^{23} together, R^{23} and R^{24} together, R^{24} and R^{25} together, or R^{25} and R^{26} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (IV) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$).

In certain embodiments, Q is substituted phenyl, which can be oxidized by certain reactive oxygen or nitrogen species, such as peroxynitrite and hypochlorous acid, to release

highly fluorescent fluorophores. In one embodiment, one of R^{22}, R^{24}, or R^{26} is such a group that can react with peroxynitrite effectively and selectively. In certain specific embodiments, one of R^{22}, R^{24}, or R^{26} is OR^{27}, $CH_2CH_2COR^{28}$, or $NR^{29}R^{30}$; wherein R^{27} is hydrogen or a group selected from alkyl, alkoxyalkyl, alkanoyl, and polyether; R^{28} is an electron-withdrawing group selected from CF_3, halogen-substituted lower alkyl (*e.g.*, CF_nH_{3-n}, wherein n is 1, 2, or 3), or (C=O)–O–W_1, wherein W_1 is a group selected from alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl or arylalkyl; R^{29} and R^{30} are independently hydrogen or a group selected from hydrogen or a group selected from alkyl, alkenyl, alkynyl, alkoxyalkyl, alkanoyl, alkenoyl, alkynoyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl, arylalkyl, aryloyl, or polyether. Preferably, R^{24} is a group that can react with peroxynitrite effectively and selectively, such as OR^{27}, $CH_2CH_2COR^{28}$, or $NR^{29}R^{30}$. In a preferred embodiment, R^{24} is $CH_2CH_2CF_3$, $CH_2CH_2COCOOMe$, or OH.

In a preferred embodiment, R^1 of formula (I) or (II) is CH_3.

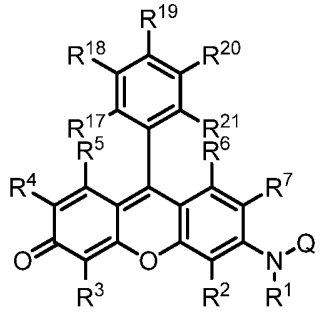
In one embodiment, the fluorogenic or fluorescent compounds of the invention substantially react with peroxynitrite to generate highly fluorescent *N*-dearylated product (I') or (II') shown as follows, along with increase of fluorescence.



(I) (I') (II) (II')

Green fluorescent probes with improved intracellular retention

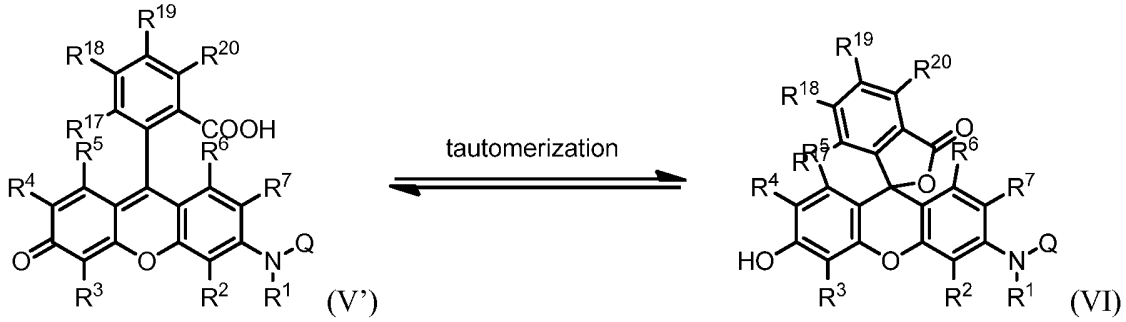
In certain embodiments, the subject invention provides green fluorogenic or fluorescent compounds with improved intracellular retention, retained sensitivity and selectivity for peroxynitrite detection. In specific embodiments, the green fluorogenic or fluorescent probes with improved intracellular retention provided by the subject invention have the following formula (V)



(V)

wherein R^1 – R^7, R^{17} – R^{21}, and Q are defined as in the formula of (I) or (II). In certain embodiments, at least one of R^{17}, R^{18}, R^{19}, and R^{20} is a carboxyl group. In certain embodiments, R^{21} is H, CH_3, OMe, or COOH. In certain embodiments, the carboxyl group(s) on the top phenyl ring of formula (V) is further conjugated with iminodialkylcarboxylic acid(s) ($HN((CH_2)_nCOOH)_2$, n = 1, 2, or 3) through amide bond(s).

In certain embodiments, when R^{21} of formula (V) is COOH, the compound has a formula of (V'), and a tautomerization exists between formula (V') and formula (VI) as shown below.



(V') (VI)

The definitions of substituents (R^1 – R^7, R^{17} – R^{20}, and Q) in formula (V') and (VI) are the same as those of formula (V).

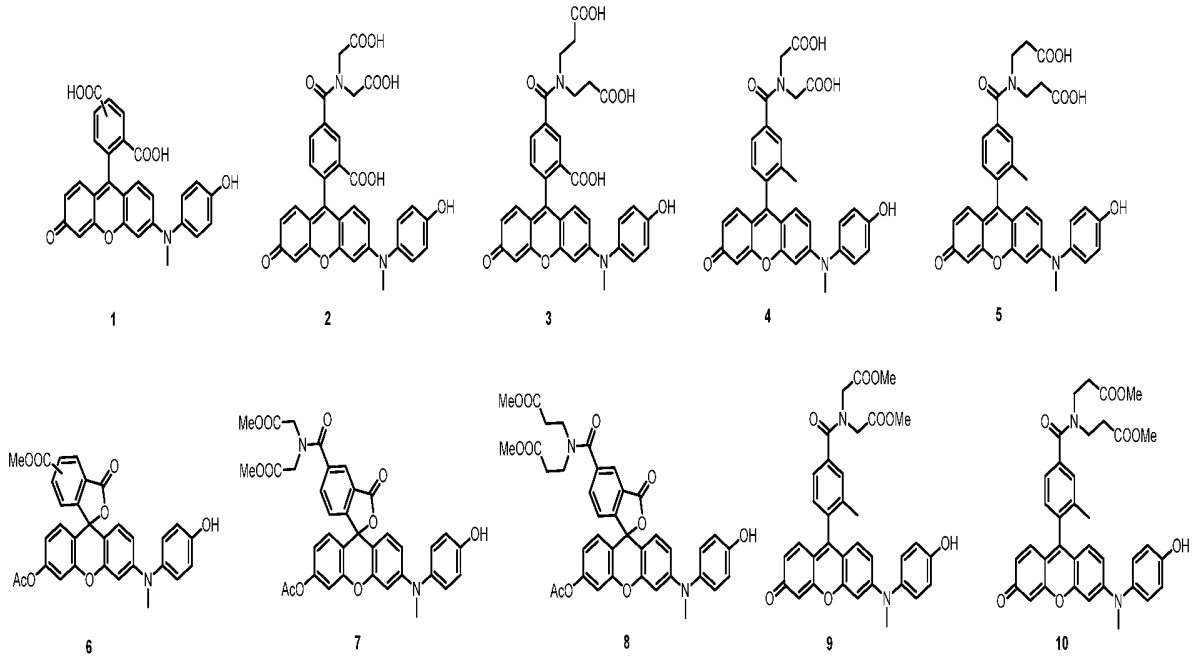
In certain embodiments, the free carboxyl groups in formula (V), (V') and (VI) are optionally esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability. In certain embodiments, the free phenolic groups in formula (V), (V') and (VI) are optionally acylated with acetyl, propionyl, or butyryl groups, or protected with acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

In certain embodiments, R^2 – R^7 of formula (V), (V'), and (VI) are independently H. In certain embodiments, R^4 and R^7 of formula (V), (V'), and (VI) are F or Cl.

In certain embodiments, R^{19} of formula (V), (V'), and (VI) is a carboxyl or a carboxylic methyl or ethyl ester. In certain embodiments, R^{19} of formula (V), (V'), and (VI) is a carboxyl further conjugated with an iminodialkylcarboxylic acid ($HN((CH_2)_nCOOH)_2$, n = 1, 2, or 3) or a dimethyl or diethyl iminodialkylcarboxylate through amide bond.

In specific embodiments, exemplified species of green fluorogenic compounds **1-10** are shown in Scheme 1.

Scheme 1 - Green Fluorogenic Compounds for Peroxynitrite Detection



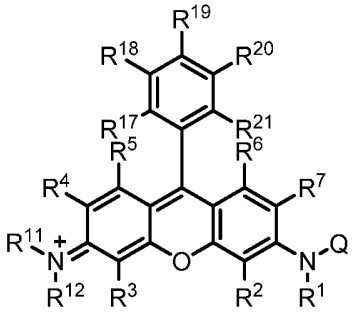
In one specific embodiment, the subject invention provides green fluorogenic compounds for detection of peroxynitrite in chemical (non-biological) systems, wherein the compounds comprise one or more free carboxylic acid groups. In another specific embodiment, the subject invention provides green fluorogenic compounds for detection of peroxynitrite in *in vitro* or *in vivo* biological assays, wherein the compounds comprise one or more ester derivatives of carboxylic acid groups.

For instance, Compounds **1-5**, which comprise free carboxylic acids, are preferably used in chemical, non-biological systems, while their corresponding ester derivatives **6-10** are preferably used for biological assays.

Yellow fluorescent probes and its mitochondrial-targeting analogs

In certain embodiments, the subject invention provides yellow fluorogenic compounds for peroxynitrite detection.

In specific embodiments, the fluorogenic or fluorescent probes with yellow fluorescence color provided by the subject invention have the following formula (VII)

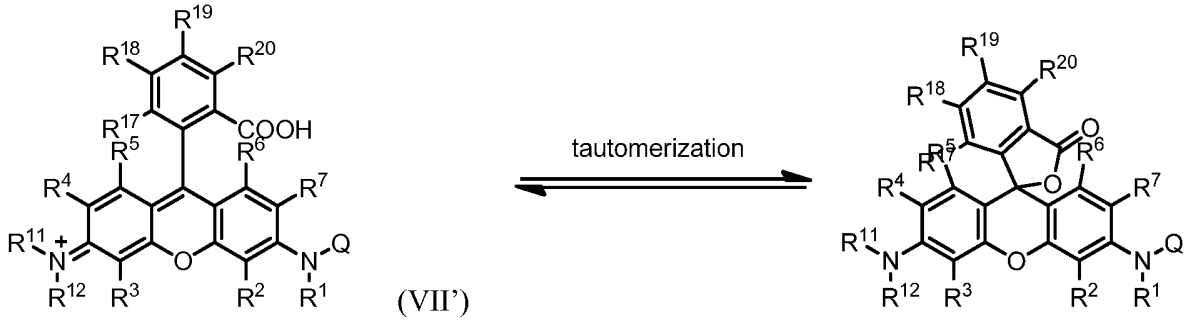


(VII)

wherein $R^1 - R^7$, $R^{11} - R^{12}$, $R^{17} - R^{21}$, and Q are defined as in formula (I) or (II).

In certain embodiments, R^{21} is H, CH_3, OMe, or COOH.

In certain embodiments, when R^{21} of formula (VII) is COOH, the compound has a formula of (VII'), and a tautomerization exists between formula (VII') and formula (VIII) as shown below.



(VII') (VIII)

The definitions of substituents ($R^1 - R^7$, $R^{11} - R^{12}$, $R^{17} - R^{20}$, and Q) in formula (VII') and (VIII) are the same as those of formula (VII).

In certain embodiments, R^{11} in combination with R^4, or R^{12} in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or can be further fused with an aryl or heteroaryl ring, and can be optionally substituted by one or more alkyls, carboxylic acids, sulfonic acids ($-SO_3H$), or their salts, ester or amide derivatives.

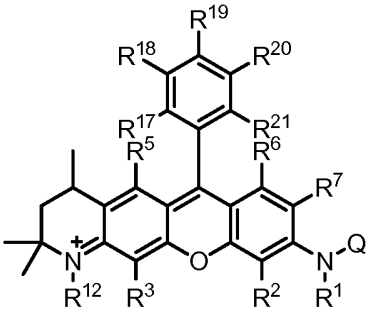
In certain embodiments, the free carboxyl groups in formula (VII), (VII') and (VIII) are optionally esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

In certain embodiments, the fluorogenic or fluorescent probes with yellow fluorescence color provided by the subject invention having the formula (VII) can selectively localize to mitochondria of living cells wherein the net charges of the probes having formula (VII) are positive. In these embodiments, R^{21} of formula (VII) is preferably H, CH_3, or OMe.

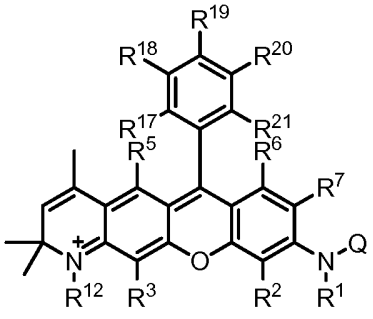
In certain embodiments, when the net charges of the probes having formula (VII) are positive, the positive charges of the probes are balanced by the presence of biologically compatible counterions presented by the symbol Ω. Biologically compatible counterions are well-known in the art, and are herein referred to anions not toxic and deleterious on biomolecules. Non-limiting examples of Ω include chloride, bromide, iodide, sulfate, alkanesulfonate, arylsulfonate, phosphate, perchlorate, tetrafluoroborate, tetraphenylboride, nitrate and anions of aromatic or aliphatic carboxylic acids. Preferred counterions Ω used herein are chloride, iodide, and perchlorate.

In certain embodiments, the mitochondrial-localizing probes having formula (VII) can irreversibly stain mitochondria of living cells wherein at least one of R^{17} – R^{20} is an alkylating group (AG). AG is such a reactive site which can react, either directly or through the catalysis of an enzyme, with intracellular nucleophiles, such as glutathione or a cysteine-containing protein to form macromolecular conjugates. Preferably, AG has the formula of $CR^{31}R^{32}X$, wherein R^{31} and R^{32} are independently H and CH_3, and X is Cl, Br, or I.

In certain embodiments, the fluorogenic or fluorescent probes with yellow fluorescence color provided by the subject invention have the following formula (IX) or (X)



(IX)



(X)

wherein $R^1 - R^3$, $R^5 - R^7$, $R^{11} - R^{12}$, $R^{17} - R^{21}$, and Q are defined as in formula (I) or (II).

In certain embodiments, R^{12} in formula (IX) and (X) is a C_{1-10} alkyl or alkene. In certain embodiments, R^{12} in formula (IX) and (X) is a C_{1-10} alkyl or alkene substituted with a carboxyl group at the terminal position. In preferred embodiments, R^{12} is ethyl, carboxylmethyl, carboxylethyl, or carboxylpropyl. In certain embodiments, the terminal carboxyl groups in R^{12} of formula (IX) and (X) are esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

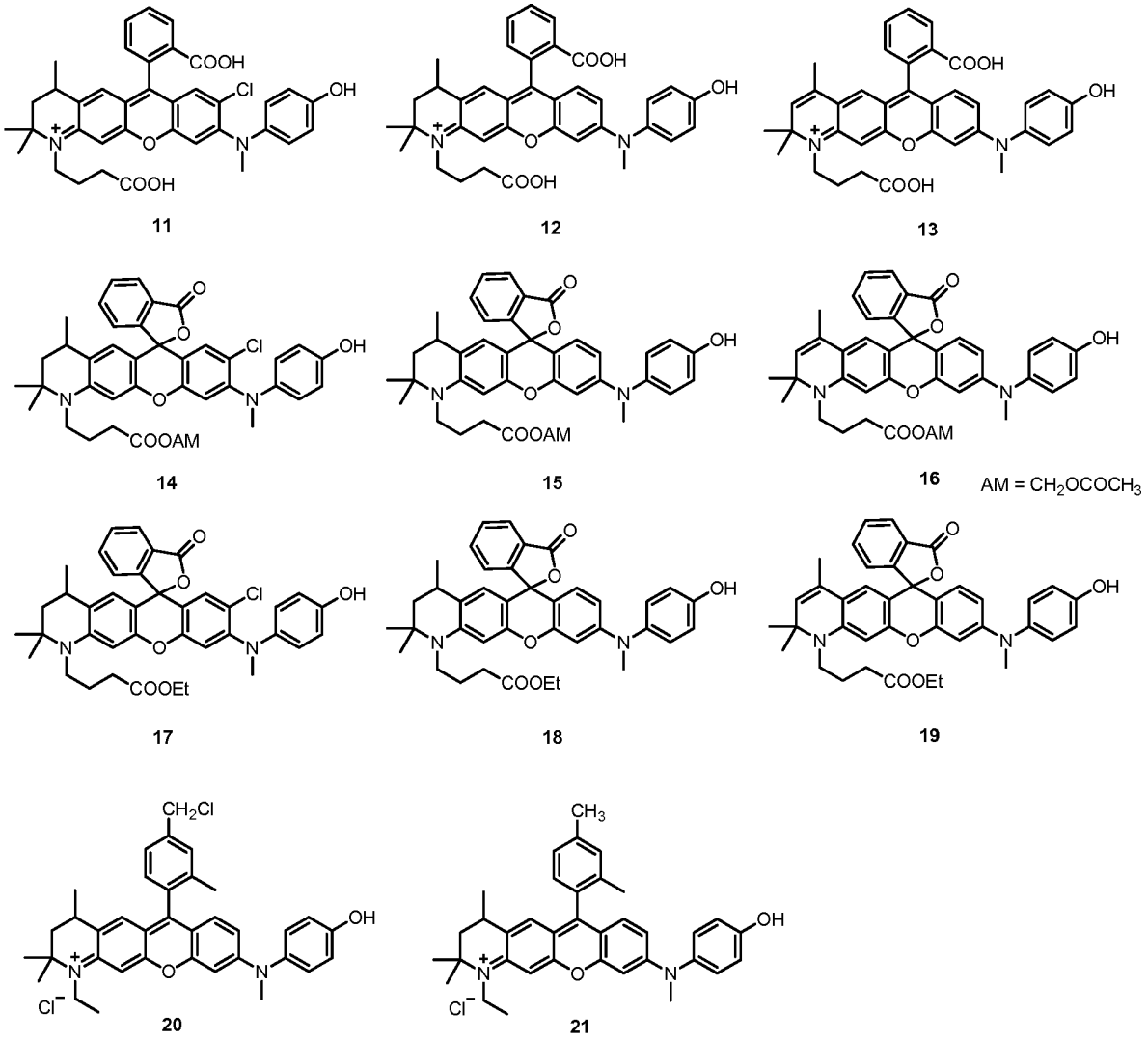
In certain embodiments, R^2, R^3, R^5, R^6, and R^7 in formula (IX) and (X) are independently H. In certain embodiments, R^7 in formula (IX) and (X) is F or Cl.

In certain embodiments, R^{21} in formula (IX) and (X) is COOH, H, CH_3, or OMe.

In certain embodiments, R^{17}, R^{18}, R^{19}, and R^{20} in formula (IX) and (X) are independently H. In certain embodiments, at least one of R^{17}, R^{18}, R^{19}, and R^{20} in formula (IX) and (X) is an alkylating group, preferably, chloromethyl (CH_2Cl).

In specific embodiments, exemplified species of yellow fluorogenic compounds **11-21** are shown in Scheme 2.

Scheme 2 - Yellow Fluorogenic Compounds for Peroxynitrite Detection



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The Compounds **11-13** react with peroxynitrite to give strong yellow fluorescence signals with emission maxima at about 570 nm, and exhibit high selectivity towards peroxynitrite over other ROS and RNS in chemical (non-biological) systems.

In one specific embodiment, the subject invention provides yellow fluorogenic compounds for detection of peroxynitrite in chemical (non-biological) systems, wherein the compounds comprise one or more free carboxylic acid groups. In another specific embodiment, the subject invention provides yellow fluorogenic compounds for detection of peroxynitrite in *in vitro* or *in vivo* biological assays, wherein the compounds comprise one or more lactone and ester derivatives of carboxylic acid groups.

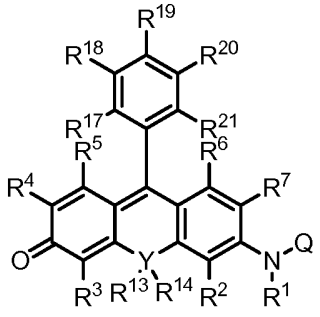
In still another specific embodiment, the subject invention provides yellow fluorogenic compounds with selective localization in mitochondria of living cells for detection of peroxynitrite in *in vitro* or *in vivo* biological assays, wherein the compounds comprise at least one positive net charge.

For instance, Compounds **11-13** are preferably used for detection of peroxynitrite in chemical, non-biological systems; while the corresponding lactone and ester derivatives **14-19** are preferably used for biological assays. The positively charged fluorogenic probes **20-21** are used for detection of peroxynitrite in both non-biological and biological systems. When applied to biological systems for detecting peroxynitrite, the positively charged fluorogenic probes **20-21** are selectively localized to mitochondria of living cells.

Red fluorescent probes

In certain embodiments, the subject invention provides red fluorogenic compounds for peroxynitrite detection. The red florescent compounds, which are based on *Si*-fluorescein scaffold, react with peroxynitrite effectively to provide strong red fluorescence signals with emission maxima at about 620 nm. The red fluorogenic compounds also exhibit high selectivity towards peroxynitrite over other ROS and RNS.

In specific embodiments, the fluorogenic or fluorescent probes with red fluorescence color provided by the subject invention have the following formula (XI)

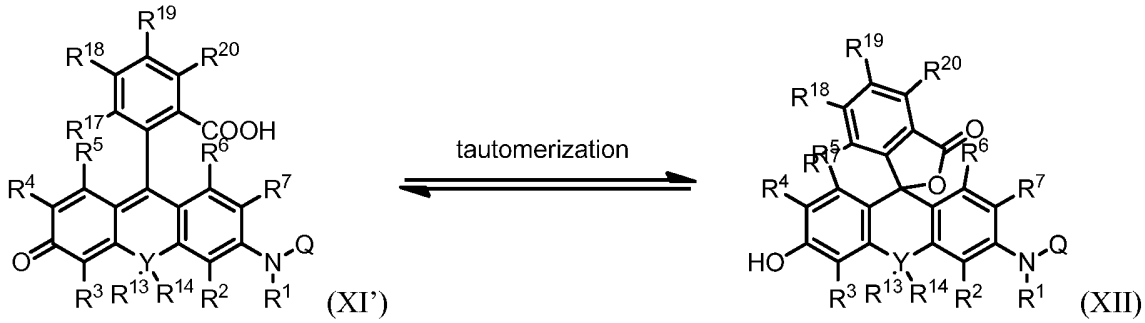


(XI)

wherein R^1 – R^7, R^{13} – R^{14}, R^{17} – R^{21}, and Q are defined as in formula (I) or (II); and wherein in certain embodiments, Y is Si, Ge, or Sn. Preferably, R^{13} and R^{14} are independently CH_3, or phenyl.

In certain embodiments, at least one of R^{17}, R^{18}, R^{19}, and R^{20} is a carboxyl group. In certain embodiments, R^{21} is H, CH_3, OMe, or COOH. In certain embodiments, the carboxyl group(s) on the top phenyl ring of formula (XI) is further conjugated with iminodialkylcarboxylic acid(s) ($HN((CH_2)_nCOOH)_2$, n = 1, 2, or 3) through amide bond(s).

In certain embodiments, when R^{21} of formula (XI) is COOH, the compound has a formula of (XI'), and a tautomerization exists between formula (XI') and formula (XII) as shown below.



(XI') (XII)

The definitions of substituents (R^1 – R^7, R^{13} – R^{14}, R^{17} – R^{20}, Y, and Q) in formula (XI') and (XII) are the same as those of formula (XI).

In certain embodiments, the free carboxyl groups in formula (XI), (XI') and (XII) are optionally esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively

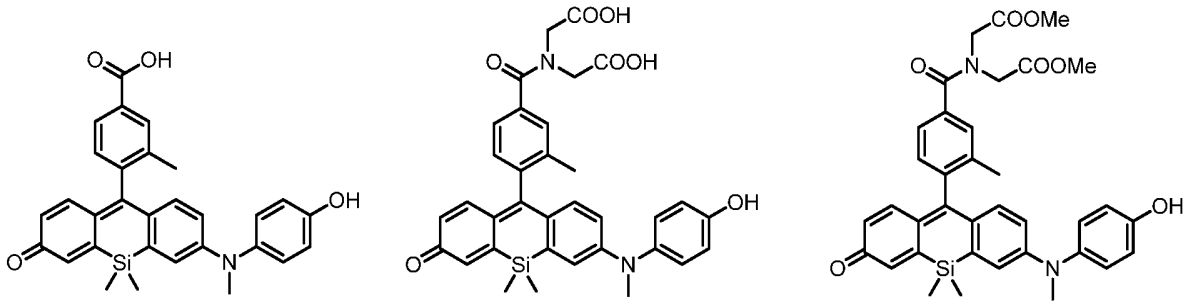
charged fluorescent probes with cell membrane permeability. In certain embodiments, the free phenolic groups in formula (XI), (XI') and (XII) are optionally acylated with acetyl, propionyl, or butyryl groups, or protected with acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

In certain embodiments, $R^2 - R^7$ of formula (XI), (XI'), and (XII) are independently H. In certain embodiments, R^4 and R^7 of formula (XI), (XI'), and (XII) are F or Cl.

In certain embodiments, R^{19} of formula (XI), (XI'), and (XII) is a carboxyl or a carboxylic methyl or ethyl ester. In certain embodiments, R^{19} of formula (XI), (XI'), and (XII) is a carboxyl further conjugated with an iminodialkylcarboxylic acid ($HN((CH_2)_nCOOH)_2$, n = 1, 2, or 3) or a dimethyl or diethyl iminodialkylcarboxylate through amide bond.

In specific embodiments, exemplified species of red fluorogenic compounds **22-24** are shown in Scheme 3.

Scheme 3 - Red Fluorogenic Compounds for Peroxynitrite Detection



22 **23** **24**

In one specific embodiment, the subject invention provides red fluorogenic compounds for detection of peroxynitrite in chemical (non-biological) systems, wherein the compounds comprise one or more free carboxylic acid groups. In another specific embodiment, the subject invention provides red fluorogenic compounds for detection of peroxynitrite in *in vitro* or *in vivo*

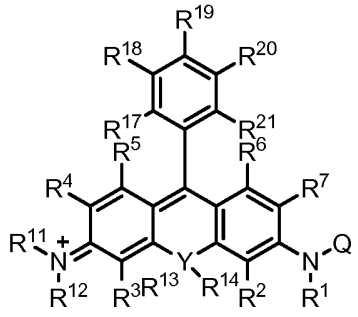
biological assays, wherein the compounds comprise one or more ester derivatives of carboxylic acid groups.

For instance, Compounds **22-23**, which comprise free carboxylic acids, are preferably used in chemical, non-biological systems, while their corresponding ester derivatives **24** are preferably used for biological assays.

Far-red fluorescent probes and its mitochondrial-targeting analogs

In certain embodiments, the subject invention provides far-red fluorogenic compounds for peroxynitrite detection.

In specific embodiments, the fluorogenic or fluorescent probes with far-red fluorescence color provided by the subject invention have the following formula (XIII)

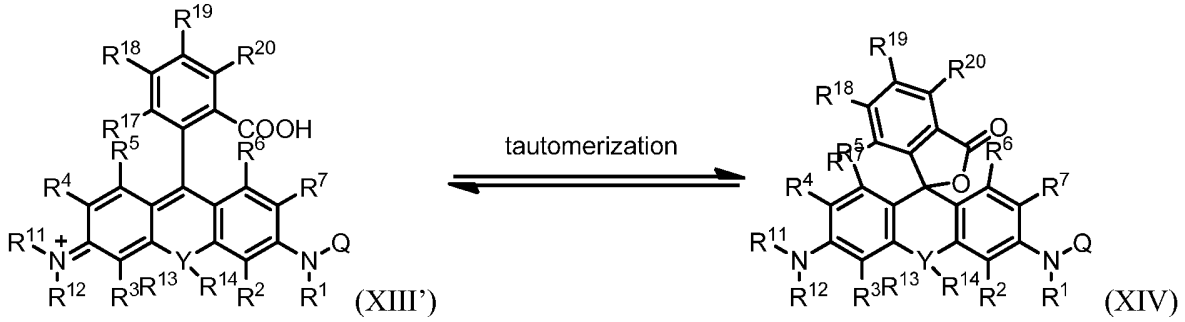


(XIII)

wherein R^1 – R^7, R^{11} – R^{14}, R^{17} – R^{21}, and Q are defined as in formula (I) or (II); and wherein in certain embodiments, Y is Si, Ge, or Sn. Preferably, R^{13} and R^{14} are independently CH_3, or phenyl.

In certain embodiments, R^{21} is H, CH_3, OMe, or COOH.

In certain embodiments, when R^{21} of formula (XIII) is COOH, the compound has a formula of (XIII'), and a tautomerization exists between formula (XIII') and formula (XIV) as shown below.



(XIII') (XIV)

The definitions of substituents ($R^1 - R^7$, $R^{11} - R^{14}$, $R^{17} - R^{20}$, Y, and Q) in formula (XIII') and (XIV) are the same as those of formula (XIII).

In certain embodiments, R^{11} in combination with R^4, or R^{12} in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or can be further fused with an aryl or heteroaryl ring, and can be optionally substituted by one or more alkyls, carboxylic acids, sulfonic acids ($-SO_3H$), or their salts, ester or amide derivatives.

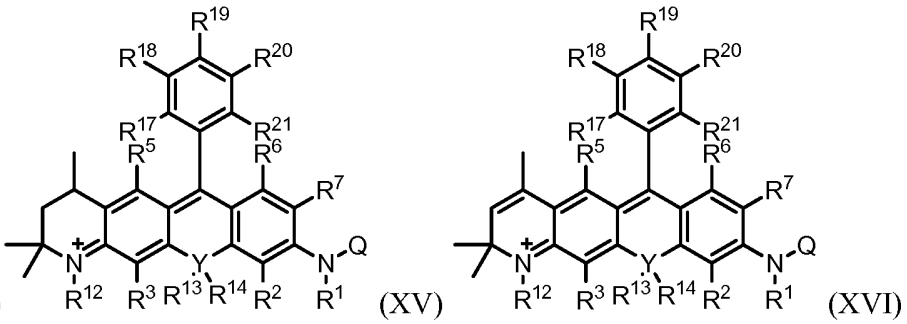
In certain embodiments, the free carboxyl groups in formula (XIII), (XIII') and (XIV) are optionally esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

In certain embodiments, the fluorogenic or fluorescent probes with far-red fluorescence color provided by the subject invention having the formula (XIII) can selectively localize to mitochondria of living cells when the net charges of the probes having formula (XIII) are positive. In further embodiments, R^{21} of formula (XIII) is preferably H, CH_3, or OMe. In certain embodiments, when the net charges of the probes having formula (XII) are positive, the positive charges of the probes are balanced by the presence of biologically compatible counterions presented by the symbol Ω. Biologically compatible counterions are well-known in the art, and are herein referred to anions not toxic and deleterious on biomolecules. Non-limiting examples of Ω include chloride, bromide, iodide, sulfate, alkanesulfonate, arylsulfonate, phosphate,

perchlorate, tetrafluoroborate, tetraphenylboride, nitrate and anions of aromatic or aliphatic carboxylic acids. Preferred counterions Ω used herein are chloride, iodide, and perchlorate.

In certain embodiments, the mitochondrial-localizing probes having formula (XIII) can irreversibly stain mitochondria of living cells wherein at least one of R^{17} – R^{20} is an alkylating group (AG). AG is such a reactive site which can react, either directly or through the catalysis of an enzyme, with intracellular nucleophiles, such as glutathione or a cysteine-containing protein to form macromolecular conjugates. Preferably, AG has the formula $CR^{31}R^{32}X$, wherein R^{31} and R^{32} are independently H and CH_3, and X is Cl, Br, or I.

In certain embodiments, the fluorogenic or fluorescent probes with far-red fluorescence color provided by the subject invention have the following formula (XV) or



(XVI) (XV) (XVI)

wherein R^1 – R^3, R^5 – R^7, R^{13} – R^{14}, R^{17} – R^{21}, and Q are defined as in formula (I) or (II), and wherein in certain embodiments, Y is Si, Ge, or Sn.

In certain embodiments, R^{12} in formula (XV) and (XVI) is a $C_{1\text{-}10}$ alkyl or alkene. In certain embodiments, R^{12} in formula (XV) and (XVI) is a $C_{1\text{-}10}$ alkyl or alkene substituted with a carboxyl group at the terminal position. In preferred embodiments, R^{12} is ethyl, carboxylmethyl, carboxylethyl, or carboxylpropyl. In certain embodiments, the terminal carboxyl groups in R^{12} of formula (XV) and (XVI) are esterified with methyl, ethyl, or acetoxymethyl (AM) groups to render the negatively charged fluorescent probes with cell membrane permeability.

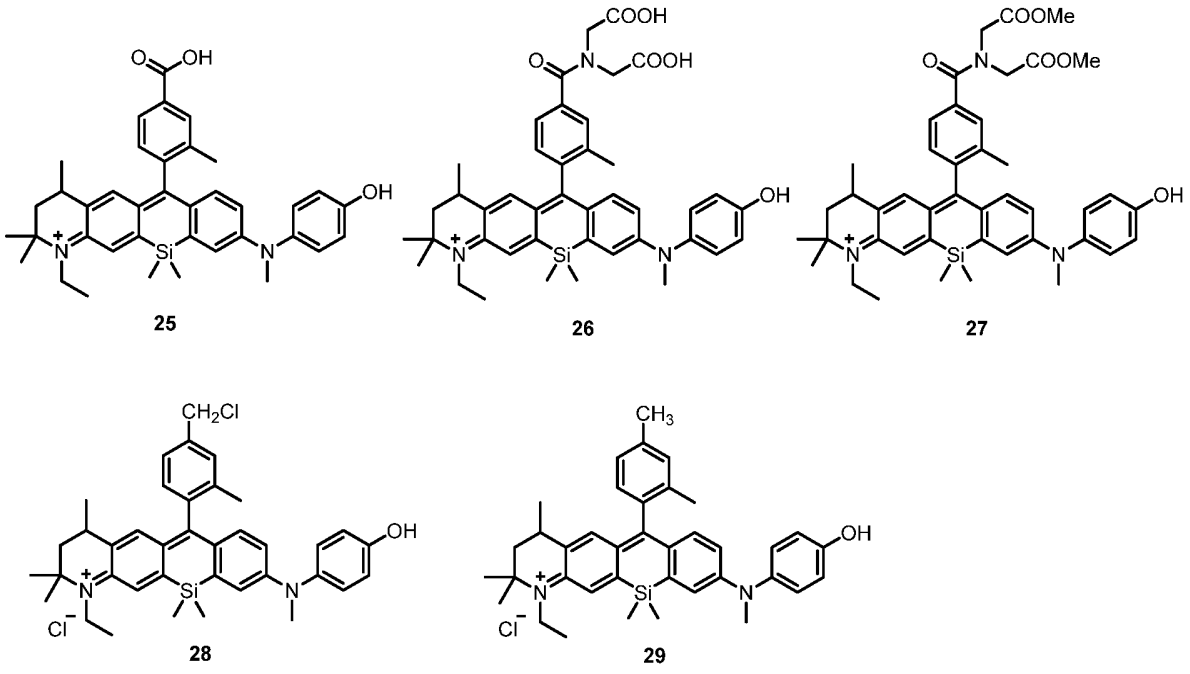
In certain embodiments, R^2, R^3, R^5, R^6, and R^7 in formula (XV) and (XVI) are independently H. In certain embodiments, R^7 in formula (XV) and (XVI) is F or Cl.

In certain embodiments, R^{21} in formula (XV) and (XVI) is COOH, H, CH_3, or OMe.

In certain embodiments, R^{17}, R^{18}, R^{19}, and R^{20} in formula (XV) and (XVI) are independently H. In certain embodiments, at least one of R^{17}, R^{18}, R^{19}, and R^{20} in formula (XV) and (XVI) is an alkylating group, preferably, chloromethyl (CH_2Cl).

In specific embodiments, exemplified species of far-red fluorogenic compounds **25-29** are shown in Scheme 4.

Scheme 4 - Far-red Fluorogenic Compounds for Peroxynitrite Detection



25 **26** **27**

28 **29**

In one specific embodiment, the subject invention provides far-red fluorogenic compounds for detection of peroxynitrite in chemical (non-biological) systems, wherein the compounds comprise one or more free carboxylic acid groups. In another specific embodiment, the subject invention provides far-red fluorogenic compounds for detection of peroxynitrite in *in*

vitro or *in vivo* biological assays, wherein the compounds comprise one or more lactone and ester derivatives of carboxylic acid groups.

In still another specific embodiment, the subject invention provides far-red fluorogenic compounds with selective localization in mitochondria of living cells for detection of peroxynitrite in *in vitro* or *in vivo* biological assays, wherein the compounds comprise at least one positive net charge.

For instance, the fluorogenic compounds **25-26** shown in Scheme 4 can be used in chemical (non-biological) systems for detection of peroxynitrite. In another specific embodiment, the positively charged fluorogenic compounds **27-29** can be used for detection of peroxynitrite in *in vitro* or *in vivo* biological assays. In still another specific embodiment, the subject invention provides far-red fluorogenic compounds **28-29** with selective localization in mitochondria of living cells for detection of peroxynitrite in *in vitro* or *in vivo* biological assays, wherein the compounds comprise at least one positive net charge after entering into the cells.

Mitochondrial-targeting fluorescent probes

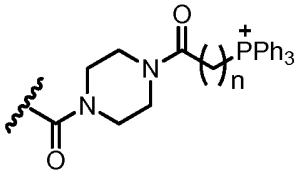
Mitochondria are the primary generators and targets of reactive oxygen species (ROS) including peroxynitrite. Development of mitochondrial-targeting fluorogenic probes for peroxynitrite detection is therefore important for elucidating and understanding the generation, metabolism, and biological effects of peroxynitrite. In addition, mitochondrial-targeting probes facilitate the accumulation of probes in mitochondria, and therefore efficiently avoid the probe leakage problem.

One method for targeting molecules to mitochondria of living cells is to conjugate the molecules with triphenylphosphonium (TPP) head groups, which possess one positive charge and large hydrophobic surface area. The resulting conjugates can be attracted by the negative

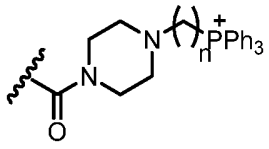
potential across the inner mitochondrial membrane, and therefore be accumulated several-hundred folds into mitochondria.

In certain embodiments, the subject invention provides fluorogenic compounds for targeting mitochondria and simultaneously detecting peroxynitrite. The mitochondrial-targeted compounds exhibit retained sensitivity and selectivity for peroxynitrite detection, and selectively stain peroxynitrite in mitochondria of living cells.

In certain embodiments, the above stated fluorogenic probes for detection of peroxynitrite can be made to selectively target mitochondria of living cells by conjugating the probes with positively charged triphenylphosphonium moieties at the free carboxyl groups of the probes through simple amide bond linkage. In certain embodiments, the linkages between the probes and the triphenylphosphonium moieties have the following formula (XVII) or (XVIII)



(XVII)

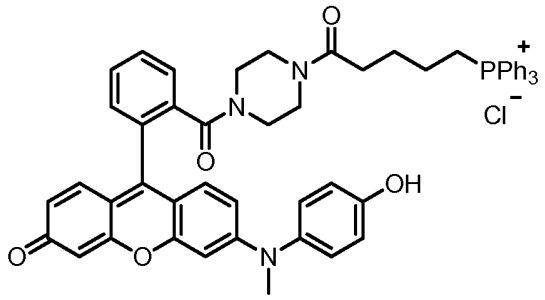


(XVIII)

wherein n = 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In certain embodiments, the triphenylphosphonium moieties can be conjugated to the fluorogenic probes at any free carboxyl group of the probes. In specific embodiments, some non-limiting examples of mitochondrial-targeting fluorogenic compounds **30-33** for detection of peroxynitrite are shown in Scheme 5.

Scheme 5 - Mitochondrial-Targeting Fluorogenic Compounds for Peroxynitrite Detection



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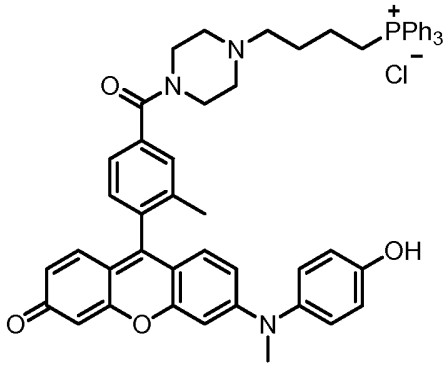
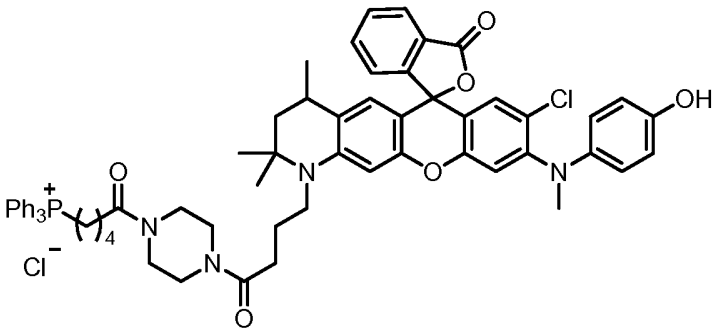
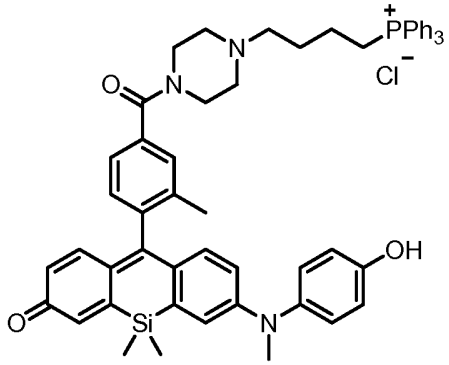


31



32



33

In one embodiment, the subject invention does not encompass compounds or fluorogenic compounds and probes that are described in U.S. Patent Application Serial No. 12/417,672.

Probe conjugates

In some embodiments, at least one of the groups of the compounds of formula (I) or (II) is substituted by a reactive group (Rg) or a conjugated group (Cg), wherein Rg or Cg is optionally attached to the aromatic amine compounds disclosed herein through a linkage group, –L–. In other embodiments, at least one of the groups of the compounds disclosed herein is substituted by an –L–Rg or –L–Cg group.

In some embodiments, L is or comprises a bond or a linking group such as O, S, an aminylene group (e.g., an NR group where R is H, an alkyl group, an alkenyl group, an alkynyl group, a carboxyl group, an acyl group, an aromatic group, or a heterocyclic group), a sulfonyl group, an organic linking group, or a combination thereof. The organic linking group disclosed herein may be a divalent linking organic group connecting any of two fragments.

Some non-limiting examples of the divalent organic linking group include a carbonyl group, an alkylene group, an arylene group, a divalent heterocyclic group, and combinations thereof. Another non-limiting example of the divalent organic linking group includes a $-(CH_2)_m-$ group, where m is an integer between 1 and 50, inclusive, and one or more of the methylene groups is optionally replaced by O, S, N, C, B, Si, P, C=O, O=S=O, a heterocyclic group, an aromatic group, an NR_a group, a CR_b group, a CR_cR_d group, a SiR_eR_f group, a BR_g group, or a $P(=O)R_h$ group, where R_a, R_b, R_c, R_d, R_e, R_f, R_g, and R_h are, each independently, a bond, H, a hydroxyl group, a thiol group, a carboxyl group, an amino group, a halogen, an acyl group, an alkoxy group, an alkylsulfanyl group, an alkenyl group, such as a vinyl group, an allyl group, and a 2-phenylethenyl group, an alkynyl group, a heterocyclic group, an aromatic group, a part of a ring group, such as cycloalkyl groups, heterocyclic groups, and a benzo group, or an alkyl group where one or more of the hydrogens of the alkyl group is optionally replaced by an aromatic group, a hydroxyl group, a thiol group, a carboxyl group, an amino group, or a halogen. A non-limiting example of the aminylene group includes an NR group where R is H, an alkyl group, an alkenyl group, an alkynyl group, an acyl group, an aromatic group, and a heterocyclic group.

In certain embodiments, the organic linking group may have a valence of 3 or more and, therefore, may link any of 3 or more fragments. A non-limiting example of an organic linking group having a valence of 3 is a trivalent organic linking group created by replacing a methylene group in the $-(CH_2)_m-$ group with a CR_b group. Another non-limiting example of an organic

linking group having a valence of 4 is a tetravalent organic linking group created by replacing a methylene group in the $-(CH_2)_m-$ group with a carbon atom.

Another non-limiting example of an organic linking group having a valence of 3 is a trivalent organic linking group created by replacing a methylene group in the $-(CH_2)_m-$ group with N, P, or B. A further non-limiting example of an organic linking group having a valence of 4 is a tetravalent organic linking group created by replacing two methylene groups in the $-(CH_2)_m-$ group with two CR_b groups. Based on the disclosure herein, a person skill in the art may create an organic linking group having a valence greater than 2 by replacing at least one methylene group in the $-(CH_2)_m-$ group with at least an atom or a group having a valence of 3 or more, such as N, P, B, C, Si, a CR_b group, an aromatic group having a valence greater than 2, and a heterocyclic group having a valence greater than 2.

In other embodiments of interest, the organic linking group may comprise at least an unsaturated bond, such as a $-CR_b=N-$ bond, a double bond or a triple bond. A non-limiting example of an organic linking group having a double bond is an unsaturated organic linking group created by replacing two adjacent methylene groups in the $-(CH_2)_m-$ group with two CR_b groups. The double bond is located between the two adjacent CR_b groups. Another non-limiting example of an organic linking group having a triple bond is an unsaturated organic linking group created by replacing two adjacent methylene groups in the $-(CH_2)_m-$ group with two carbon atoms respectively. The triple bond is located between the two adjacent carbon atoms. Another non-limiting example of an organic linking group having a $-CR_b=N-$ bond is an unsaturated organic linking group created by replacing two adjacent methylene groups in the $-(CH_2)_m-$ group with one CR_b group and an N atom. Based on the disclosure herein, a person skilled in the art may create an organic linking group having at least an unsaturated bond by replacing at least one pair of adjacent methylene groups in the $-(CH_2)_m-$ group, each independently, with an atom or a

group selected from the group consisting of N, P, B, C, Si, a CR_b group, an aromatic group having a valence greater than 2, and a heterocyclic group having a valence greater than 2.

The compounds having a reactive group (Rg) may comprise a wide variety of organic or inorganic substances that contain or are modified to contain at least one functional group with suitable reactivity toward the Rg group which result in chemical attachment of the reactive group (Rg), represented by –Cv-Rg. In some embodiments, the reactive group (Rg) and functional group are respectively an electrophile and a nucleophile that can react to generate a covalent linkage. The conjugation reaction between the reactive group (Rg) and functional group at the conjugated substance (Cg) results in one or more atoms of the reactive group (Rg) to be incorporated into the linkage, Cv, which attaches the compound with reactive group (Rg) to the conjugated substance (Cg). Some non-limiting examples of the reactive group (Rg) and the respective functional group are listed in **Table 1**. The tabulation is not meant to be inclusive of chemical reactivity since with the appropriate choice of solvent, co-solvent, stoichiometric ratio, temperature, pressure, reaction time, pH, catalyst and the like, other functional groups can be made to react with the reactive sites disclosed herein whereas the functional groups disclosed herein can be made to react with other reactive sites. Some non-limiting examples of suitable reactive groups (Rg) include acrylamide, acyl azide, acyl halide, nitrile, aldehyde, ketone, alkyl halide, alkyl sulfonate, anhydride, aryl halide, alkyne, alcohol, amine, carboxylic acid, carbodiimide, diazoalkane, epoxide, haloacetamide, hydroxylamine, hydrazine, imido ester, isothiocyanate, maleimide, sulfonate ester or sulfonyl halide.

Table 1.

Reactive group (Electrophile)	Functional Group (Nucleophile)	Resulting Linkage

activated esters (succinimidyl esters)	amines/anilines	amides
acrylamides	thiols	thioethers
acyl azides	amines/anilines	amides
acyl halides	amines/anilines	amides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	amides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones
aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	amides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
alkynes	azides	triazoles
alcohols	acid derivatives	esters
amines	carboxylic acids	amides
amines	halides	alkyl amines
amines	aldehydes/	imines

	ketones	
carboxylic acids	amines/anilines	amides
carboxylic acids	alcohols	esters
carboxylic acids	hydrazines	hydrazides
carbodiimides	carboxylic acids	*N*-acylureas or anhydrides
diazoalkanes	carboxylic acids	esters
epoxides	thiols	thioesters
haloacetamides	thiols	thioethers
hydroxylamines	aldehydes/ ketones	oximes
hydrazines	aldehydes/ ketones	hydrazones
imido esters	amines/anilines	amidines
isothiocyanates	amines/anilines	thioureas
isothiocyanates	alcohols/phenols	isourethanes
maleimides	thiols	thioethers
maleimides	amines	amines
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioesters
sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

The reactive group in the compounds disclosed herein is useful for the preparation of any conjugated substance that bears a suitable functional group for covalent linkage of the two. Some non-limiting examples of suitable conjugates include conjugates of antigens, steroids,

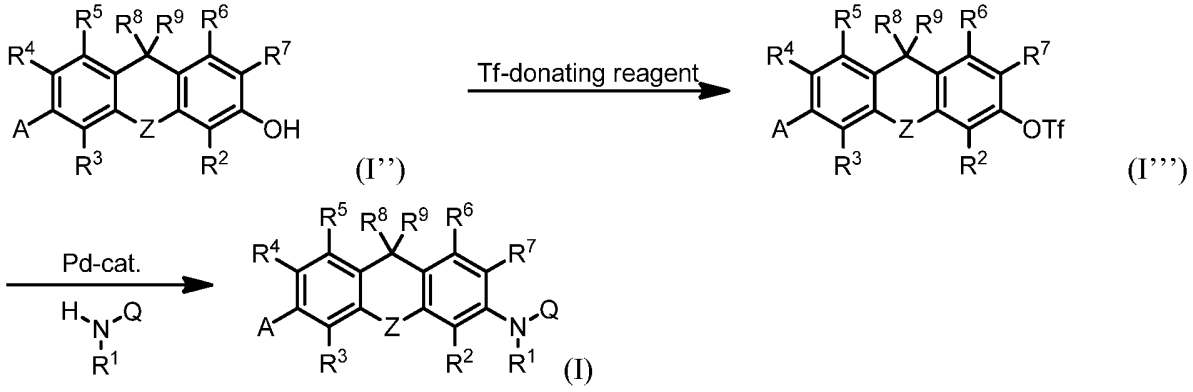
vitamins, drugs, haptens, metabolites, toxins, amino acids, peptides, nucleotides, oligonucleotides, nucleic acid, carbohydrates, lipids, and so on. Choice of the reactive group used to attach the compounds disclosed herein to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the substances include, but are not limited to, amines, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines or a combination of these groups.

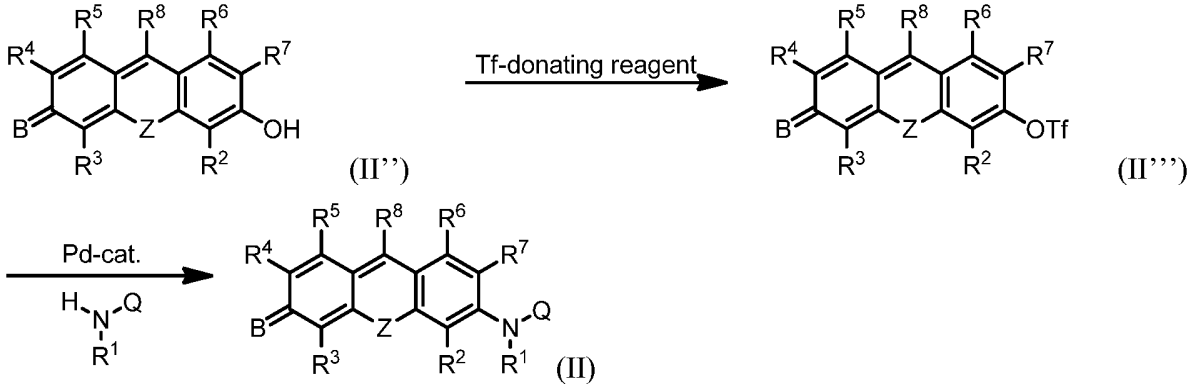
Synthesis of Compounds

The fluorogenic or fluorescent compounds of the invention may be made by one skilled in the art with known organic syntheses as well as various general or specific synthetic procedures disclosed herein and in U.S. Patent Applications US 8,148,423 and US 8,114,904, which are herein incorporated by reference in its entirety.

Generally, the key steps for the synthesis of the subject compounds (I) and (II) include activation of phenol, typically triflation, and subsequent amination as shown in Scheme 6 below.

Scheme 6 – General Synthetic Strategy for Compounds (I) and (II)





(II'') (II''') (II)

Where R^1 – R^9, A, B, Z, and Q are defined as above; Tf is triflyl; Pd-cat. is palladium-ligand catalysis system for C-N bond formation. Firstly, the phenolic OH group of (I'') or (II'') was activated by reacting with triflyl-donating reagent, typically triflic anhydride, to form a triflate group. Then the triflate group subsequently underwent cross-coupling reaction with an amine having formula HNR^1Q in the presence of a catalyst, such as a Pd catalyst, to form the subject compound having formula (I) or (II).

Uses of the Probes for Sensitive and Specific Detection of Peroxynitrite

The subject invention also provides use of the compounds as fluorogenic probes for detecting, measuring, and screening peroxynitrite *in vitro* and/or *in vivo*. In one embodiment, the subject invention specifically detects peroxynitrite with respect to any other reactive oxygen species and reactive nitrogen species.

In one embodiment, the fluorogenic or fluorescent compounds of the invention sensitively detect peroxynitrite present in aqueous samples at a concentration of lower than 10 μM, or any concentration lower than 10 μM, such as, lower than 8 μM, lower than 6 μM, lower than 4 μM, lower than 2 μM, lower than 1.6 μM, lower than 1.2 μM, lower than 0.8 μM, lower than 0.4 μM, lower than 0.2 μM, lower than 0.1 μM, lower than 0.05 μM, or lower than 0.01 μM,

In certain embodiments, the subject invention provides fluorogenic probe compositions, comprising a fluorogenic or fluorescent compound of the invention, and optionally, a carrier, solvent, an acid, a base, a buffer solution, or a combination thereof.

The fluorogenic or fluorescent compounds and probe compositions can be formulated into reagent compositions for measuring, directly or indirectly, peroxynitrite in chemical or biological samples. In a specific embodiment, the fluorogenic or fluorescent compounds and probes are formulated into a fluorogenic cell assay kit.

Also provided herein are methods for detecting the presence of, or measuring the level of, peroxynitrite in a sample. In some embodiments, the methods comprise the steps of (a) contacting a fluorogenic compound or probe disclosed herein with a sample to form a fluorescent compound; and (b) measuring fluorescence property of the fluorescent compound. In some embodiments, the fluorescence properties are measured with methods disclosed herein or any method known to a person skilled in the art.

Also provided herein are high-throughput screening fluorogenic methods for detecting peroxynitrite in samples. In some embodiments, the high-throughput screening fluorogenic methods comprise the steps of (a) contacting a fluorogenic compound or probe disclosed herein with samples to form one or more fluorescent compounds; and (b) measuring fluorescence property of the fluorescent compounds.

Also provided herein are high-throughput methods for screening one or more target compounds that can increase or decrease the level of peroxynitrite. In some embodiments, the high-throughput screening method for detecting peroxynitrite comprises the steps of: (a) contacting a fluorogenic compound or probe disclosed herein with the samples to form one or more fluorescent compounds; and (b) measuring fluorescence property of the fluorescent compounds to determine the amount of peroxynitrite in the samples.

Suitable samples include, but are not limited to, chemical (non-biological) samples and biological samples. Suitable biological samples include, but are not limited to, samples containing unicellular or unicellular organisms, microorganisms, cells, tissues, and organs of living organisms, preferably, of animals including humans.

In some embodiments, the high-throughput methods comprise the steps of (a) contacting a fluorogenic compound or probe disclosed herein with one or more target compounds to form one or more fluorescent compounds; and (b) measuring fluorescence properties of the fluorescent compounds to determine the target compounds quantitatively or qualitatively. In other embodiments, the fluorescence properties are measured with methods disclosed herein or any method known to a person skilled in the art.

In some embodiments, informatics systems can be used and implemented in the high-throughput methods disclosed herein. In other embodiments, the informatics systems provide the software control of the physical devices used in the high-throughput method. In other embodiments, the informatics systems organize electronic data generated by the high-throughput methods. In further embodiments, the informatics systems store electronic data generated by the high-throughput methods.

In certain embodiments, mitochondrial-targeting fluorogenic compounds are utilized for selectively staining peroxynitrite in the mitochondria of cells. In addition, the methods for detecting, measuring, and/or screening peroxynitrite can be performed *in vitro* or *in vivo* for studying physiological effects of peroxynitrite.

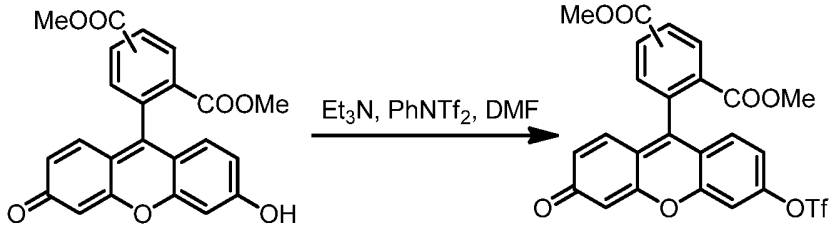
The applications of fluorogenic compounds also include various well-documented uses such as calorimetric labels for a conjugated substance, or in Fluorescence Resonance Energy Transfer (FRET) technology. Some non-limiting examples of such applications are described in U.S. Patent No. 6,399,392; and *The Handbook: a Guide to Fluorescent Probes and Labeling*

Technologies, 10th Edition, Molecular Probes, 2006, both of which are incorporated herein by reference.

EXAMPLES

Following are examples that illustrate embodiments for practicing the invention. The detailed disclosure falls within the scope of, and serve to exemplify, the synthetic schemes or procedures disclosed herein which form part of this disclosure. These examples, figures and schemes are presented for illustrative purposes only and are not intended to limit the scope of this disclosure. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

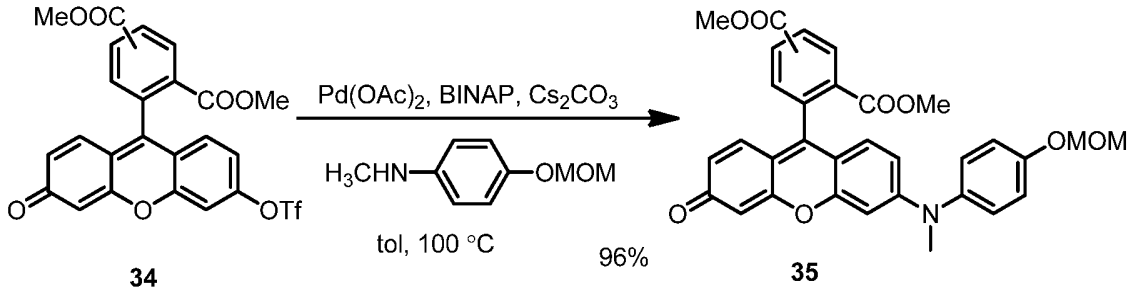
EXAMPLE 1 – SYNTHESIS OF GREEN FLUOROGENIC COMPOUNDS 1, 2, AND 10



34

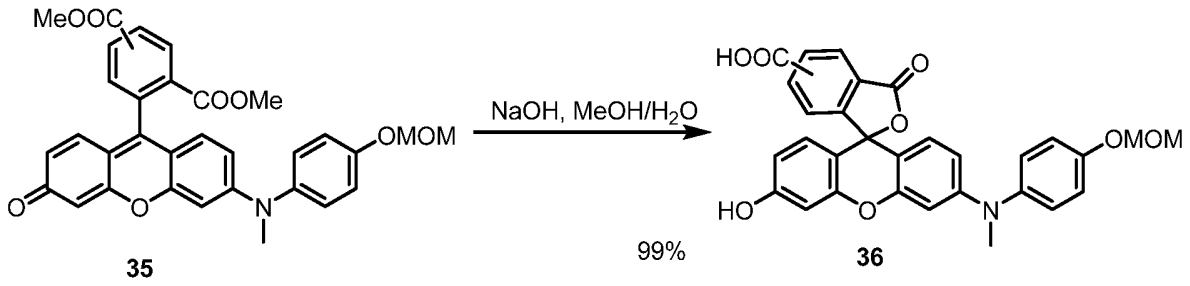
To a solution of the starting phenol (4.3 g, 10.6 mmol) in DMF (30 mL) were added Et_3N (7.5 mL, 53.1 mmol) and *N*-phenyl-bis(trifluoromethanesulfonimide) (4 g, 11.7 mmol) under Ar at room temperature. The mixture was stirred overnight and then diluted with ethyl acetate (300 mL). The organic solution was washed with HCl solution, water, and dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give **34** (5.4 g, 95% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.94 (d, J = 1.5 Hz, 1H), 8.45 (dd, J = 7.9, 1.5 Hz, 1H), 8.41 – 8.34 (m, 2H), 8.03 (d, J = 0.7 Hz, 1H), 7.50 (d, J = 7.9 Hz, 1H), 7.44 (d, J = 1.8 Hz, 1H), 7.43 (d, J = 1.8 Hz, 1H), 7.10 (dd, J = 8.8, 1.8 Hz, 1H), 7.08

(dd, J = 8.8, 1.8 Hz, 1H), 7.03 (d, J = 8.8 Hz, 1H), 7.00 (d, J = 8.8 Hz, 1H), 6.85 (d, J = 9.8 Hz, 1H), 6.82 (d, J = 9.8 Hz, 1H), 6.54 (dd, J = 9.8, 1.8 Hz, 1H), 6.52 (dd, J = 9.8, 1.8 Hz, 1H), 6.42 (d, J = 1.8 Hz, 1H), 6.41 (d, J = 1.8 Hz, 1H), 4.05 (s, 3H), 3.98 (s, 2H), 3.76 (s, 3H), 3.74 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.66, 185.64, 165.16, 165.01, 164.67, 164.55, 157.81, 157.73, 152.36, 152.31, 151.18, 146.14, 146.06, 138.04, 134.27, 133.98, 133.75, 133.71, 132.38, 132.16, 131.59, 131.45, 131.08, 130.98, 130.64, 129.90, 129.82, 129.07, 128.95, 120.92, 120.73, 120.61, 118.57 (q, $J_{C\text{-}F}$ = 319.0 Hz), 117.41, 110.35, 110.32, 106.76, 106.73, 52.78, 52.77, 52.74, 52.70; ^{19}F NMR (376 MHz, $CDCl_3$) δ -72.62, -72.63; LRMS (EI) *m/z* (%): 536 (M^+, 60), 404 (100); HRMS (EI): calcd for $C_{24}H_{15}F_3O_9S$ (M^+), 536.0389; found, 536.0385.



An oven-dried Schlenk tube was charged with $Pd(OAc)_2$ (135 mg, 0.60 mmol), BINAP (751 mg, 1.21 mmol) and Cs_2CO_3 (1.44 g, 4.42 mmol), and flushed with Ar gas for 5 min. A solution of **34** (2.16 g, 4.02 mmol) and 4-(methoxymethoxy)-*N*-methylaniline (705 mg, 4.22 momol) in toluene (20 mL) was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 °C for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2 (3 × 30 mL). The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give **35** (2.13 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$ with 10% CD_3OD) δ 8.86 (d, J = 1.4 Hz, 1H), 8.36 (dd, J = 7.9, 1.4 Hz, 1H), 8.29 (s, 2H), 7.97 (s, 1H), 7.42 (d, J = 7.9 Hz, 1H), 7.16 (d, J = 8.9 Hz, 4H), 7.12 (d, J = 8.9 Hz, 4H), 6.82 – 6.68 (m, 6H), 6.58 – 6.47 (m, 6H), 5.21 (s, 4H), 4.03 (s, 3H), 3.95 (s, 3H), 3.69 (s, 3H),

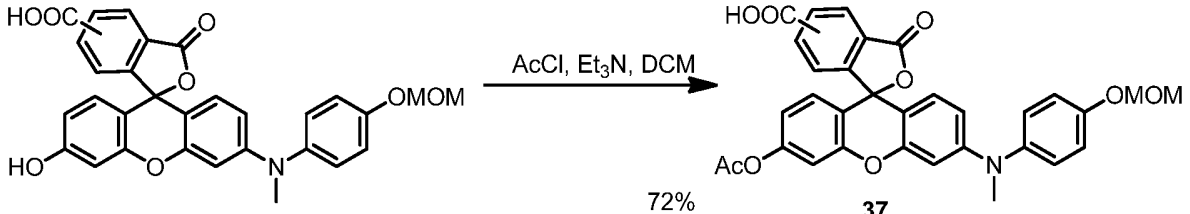
3.67 (s, 3H), 3.51 (s, 6H), 3.41 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$ with 10% CD_3OD) δ 184.99, 165.47, 165.33, 165.03, 164.83, 159.19, 159.13, 156.07, 155.11, 155.03, 154.39, 151.11, 151.05, 139.52, 139.16, 134.90, 133.98, 133.61, 133.12, 131.99, 131.50, 131.35, 131.10, 130.93, 130.73, 130.40, 130.03, 129.92, 128.61, 128.48, 128.04, 127.96, 117.71, 115.22, 114.92, 112.27, 112.24, 111.81, 111.56, 105.11, 105.08, 98.52, 98.48, 94.41, 56.06, 52.70, 52.66, 52.59, 40.67; LRMS (EI) *m/z* (%): 553 (M^+, 79), 508 (100); HRMS (EI): calcd for $C_{32}H_{27}NO_8$ (M^+), 553.1737; found, 553.1734.



35 **36**

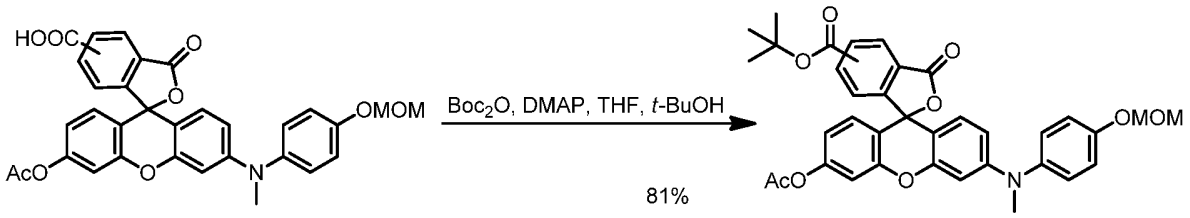
To a solution of **35** (2.13 g, 3.85 mmol) in MeOH (30 mL) was added a solution of NaOH (1.54 g, 38.5 mmol) in H_2O (15 mL) at room temperature. The resulting solution was stirred at room temperature for 2 hr, and then concentrated *in vacuo*. The residue was acidified with concentrated HCl. The precipitates were collected by filtration, and dried under reduced pressure to provide the product **36** as a red solid (2.0 g, 99% yield). The crude product was generally pure enough for the next step, and could also be purified by silica gel column chromatography. ^{1}H NMR (400 MHz, CD_3OD) δ 8.62 (d, J = 0.9 Hz, 1H), 8.33 (dd, J = 8.0, 0.9 Hz, 1H), 8.29 (dd, J = 8.0, 1.3 Hz, 1H), 8.11 (d, J = 8.0 Hz, 1H), 7.76 (s, 1H), 7.28 (d, J = 8.0 Hz, 1H), 7.14 (d, J = 8.9 Hz, 4H), 7.08 (d, J = 8.9 Hz, 4H), 6.76 – 6.67 (m, 4H), 6.67 – 6.55 (m, 6H), 6.51 – 6.44 (m, 2H), 5.18 (s, 4H), 3.45 (s, 6H), 3.33 (s, 3H), 3.32 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 168.81, 166.95, 166.85, 163.42, 162.87, 155.67, 155.58, 154.27, 153.98, 153.92, 153.54, 153.14, 152.35, 147.48, 140.89, 140.71, 136.83, 135.06, 133.16, 132.23, 130.72, 129.39,

129.24, 128.83, 128.69, 128.49, 127.96, 127.47, 127.09, 126.44, 126.22, 125.44, 117.31, 114.20, 113.84, 112.22, 111.85, 111.16, 110.66, 108.95, 108.27, 102.28, 99.29, 99.18, 94.21, 54.82, 39.72, 39.62; LRMS (FAB) *m/z* (%): 526 (M^+, 15).



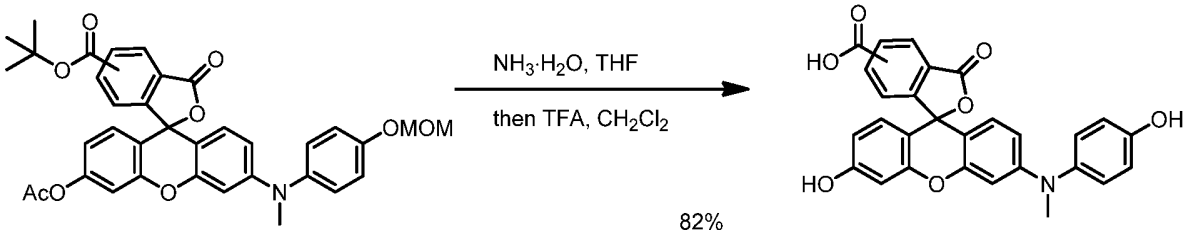
36 **37**

To a suspension of **36** (2.0 g, 3.73 mmol) in CH_2Cl_2 (50 mL) were added Et_3N (2.6 mL, 18.7 mmol) and acetyl chloride (0.53 mL, 7.46 mmol) successively at 0 °C under Ar. The resulting solution was stirred at room temperature overnight. The reaction mixture was quenched with water and diluted with ethyl acetate. The organic solution was washed with diluted HCl solution and brine, dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica gel column chromatography to give the product **37** (1.55 g, 72% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 10.27 (s, 2H), 8.74 (s, 1H), 8.36 (dd, *J* = 8.1, 1.3 Hz, 1H), 8.32 (dd, *J* = 8.0, 1.1 Hz, 1H), 8.10 (d, *J* = 8.0 Hz, 1H), 7.91 (s, 1H), 7.28 (d, *J* = 8.1 Hz, 1H), 7.13 – 7.09 (m, 4H), 7.08 – 7.02 (m, 6H), 6.77 (s, 4H), 6.56 (d, *J* = 2.5 Hz, 1H), 6.55 (d, *J* = 2.5 Hz, 1H), 6.52 (d, *J* = 2.9 Hz, 1H), 6.50 (d, *J* = 2.9 Hz, 1H), 6.41 – 6.36 (m, 2H), 5.17 (s, 4H), 3.49 (s, 6H), 3.26 (s, 6H), 2.29 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.85, 169.73, 169.21, 169.18, 168.67, 168.57, 157.76, 155.28, 155.25, 153.34, 152.38, 152.28, 152.22, 151.94, 141.71, 141.66, 136.65, 135.88, 131.67, 131.05, 129.12, 128.45, 128.03, 127.56, 126.21, 125.42, 124.73, 117.74, 117.48, 116.33, 116.26, 115.30, 111.47, 111.42, 110.61, 106.33, 106.22, 100.55, 94.77, 84.12, 83.81, 56.25, 40.60, 21.31.



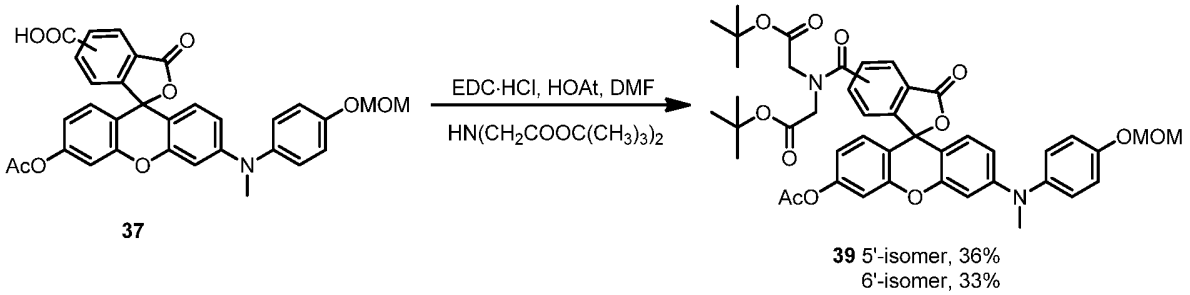
37 38

To a solution of **37** (170 mg, 0.3 mmol) in anhydrous THF (5 mL) and *t*-BuOH (1 mL) was added DMAP (96 mg, 0.45 mmol), followed by addition of Boc_2O (0.34 mL, 1.5 mmol) dropwise at 0 °C under Ar. The resulting mixture was heated to reflux for 2 hr. After cooled to room temperature, the mixture was diluted with ethyl acetate (50 mL), washed with diluted HCl, H_2O, and brine. The organic solution was dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica gel column chromatography to give the 1:1 ratio of 5'-isomer and 6'-isomer **38**.



38 1

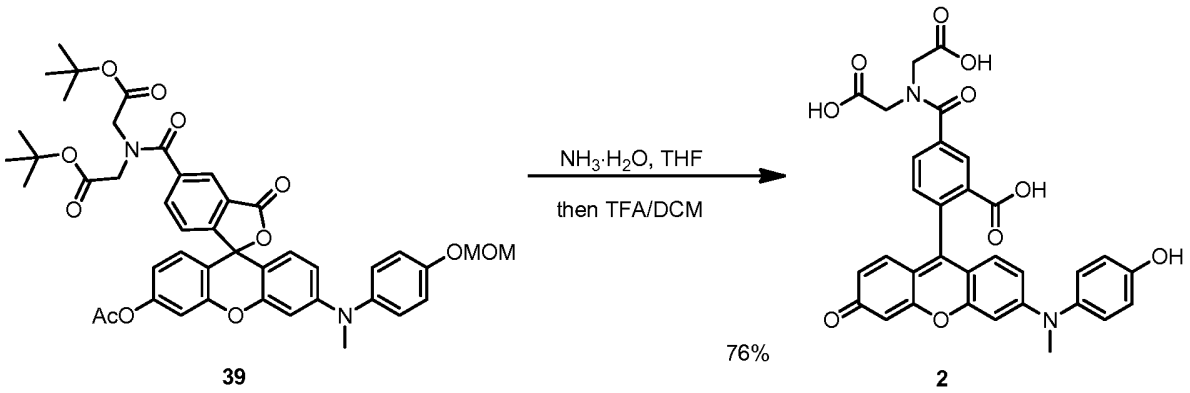
To a solution of **38** (50 mg, 0.08 mmol) in THF (2 mL) was added ammonia solution (28%, 5 drops) dropwise. The reaction was stirred at room temperature for half an hour, and then acidified with diluted HCl. The reaction mixture was extracted with ethyl acetate. The organic solution was dried over anhydrous sodium sulfate, and concentrated. The resulting residue was re-dissolved in DCM (2 mL), and treated with TFA (2 mL) at room temperature for 2 hr. The mixture was concentrated, and then diluted with saturated $NaHCO_3$. The mixture was extracted with chloroform with 10% isopropanol three times. The organic solutions were combined, concentrated, and then purified by silica gel column chromatography to give the product **1** (1:1 ratio of 5'-isomer and 6'-isomer, 32 mg, 0.067 mmol, 82% yield).



37

39 5'-isomer, 36%
6'-isomer, 33%

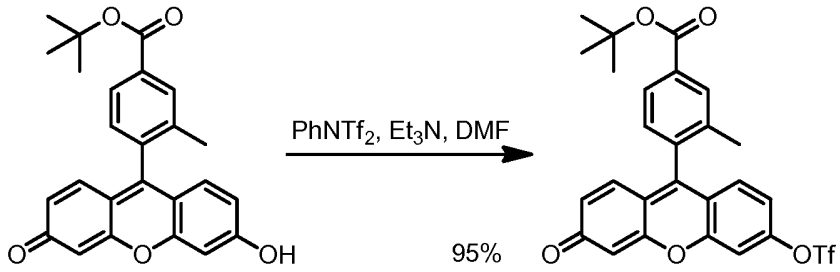
To a solution of **37** (405 mg, 0.71 mmol) in DMF were added di-*t*-butyl iminodiacetate (524 mg, 2.14 mmol), 1-hydroxy-7-azabenzotriazole (HOAt) (116 mg, 0.86 mmol), and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) (164 mg, 0.86 mmol) successively under Ar. The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ solution followed by 0.1 N HCl and brine. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the 5'-isomer **39** (203 mg, 0.26 mmol, 36% yield) and 6'-isomer (186 mg, 0.23 mmol, 33% yield) separately.



39 76% **2**

To a solution of **39** (25 mg, 0.031 mmol) in THF (2 mL) was added ammonia solution (28%, 5 drops). The reaction was stirred at room temperature for half an hour, and then acidified with diluted HCl. The reaction mixture was extracted with ethyl acetate. The organic solution was dried over anhydrous sodium sulfate, and concentrated. The resulting residue was re-dissolved in DCM (2 mL), and treated with TFA (2 mL) at room temperature for 2 hr. The

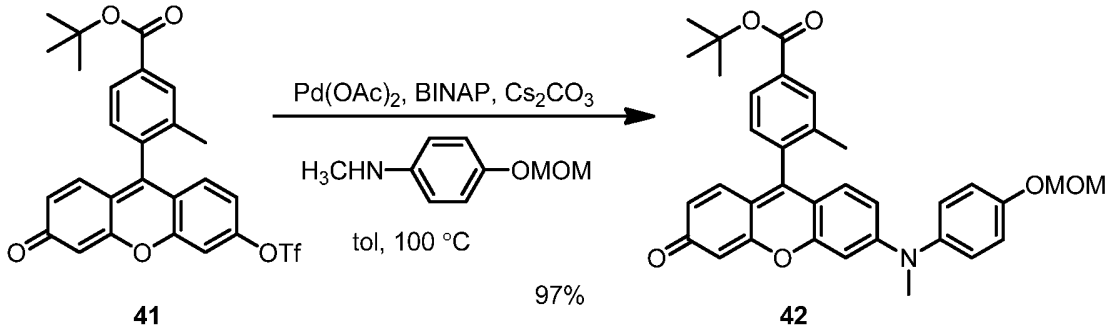
solution was concentrated, and then diluted with saturated $NaHCO_3$. The mixture was extracted with chloroform with 10% isopropanol three times. The organic solutions were combined, concentrated, and then purified by silica gel column chromatography to give the product **2** (14 mg, 0.023 mmol, 76% yield). ^1H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 7.86 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 7.8 Hz, 1H), 7.15 – 7.09 (m, 3H), 7.04 (d, J = 9.4 Hz, 1H), 7.00 (d, J = 2.2 Hz, 1H), 6.93 – 6.85 (m, 4H), 6.79 (dd, J = 9.4, 2.2 Hz, 1H), 4.35 (s, 2H), 4.23 (s, 2H), 3.51 (s, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 173.09, 172.99, 172.58, 168.33, 168.02, 158.91, 158.70, 158.63, 157.77, 142.16, 138.73, 138.33, 132.77, 132.22, 131.88, 130.38, 129.19, 128.99, 118.08, 117.86, 117.06, 115.28, 114.61, 103.65, 99.30, 53.74, 42.18; LRMS (FAB) *m/z* (%): 596 (M^+, 3); HRMS (FAB): calcd for $C_{32}H_{24}N_2O_{10}$ (M^+), 596.1431; found, 596.1433.



40 **41**

To a solution of **40** (500 mg, 1.24 mmol) in DMF (10 mL) were added Et_3N (0.52 mL, 3.72 mmol) and *N*-phenyl-bis(trifluoromethanesulfonimide) (487 mg, 1.36 mmol) under Ar at room temperature. The mixture was stirred overnight and then diluted with ethyl acetate (50 mL). The organic solution was washed with HCl solution, water, and dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give the product **41** (630 mg, 95% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 8.04 (d, J = 7.9 Hz, 1H), 7.45 (s, 1H), 7.31 (d, J = 7.9 Hz, 1H), 7.12 (s, 2H), 6.94 (d, J = 9.8 Hz, 1H), 6.57 (dd, J = 9.8, 1.5 Hz, 1H), 6.43 (d, J = 1.5 Hz, 1H), 2.19 (s, 3H), 1.66 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.72, 164.89, 157.69, 152.76, 151.50, 145.41, 136.63, 135.63,

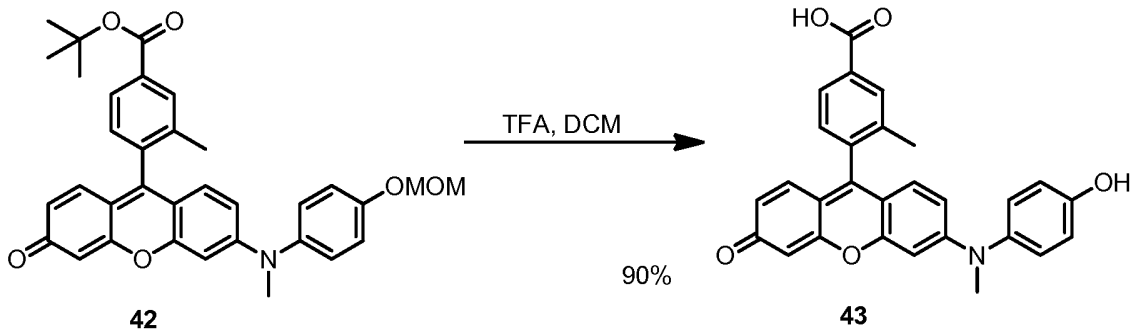
133.61, 131.77, 131.69, 130.13, 129.50, 129.28, 127.43, 121.60, 120.20, 117.64, 116.87 (q, J_{C-F} = 320.1 Hz), 110.46, 106.88, 81.68, 28.10, 19.60; ^{19}F NMR (376 MHz, $CDCl_3$) δ -72.63; LRMS (EI) *m/z* (%): 534 (M^+, 71), 478 (100), 345 (94); HRMS (EI): calcd for $C_{26}H_{21}F_3O_7S$ (M^+), 534.0960; found, 534.0965.



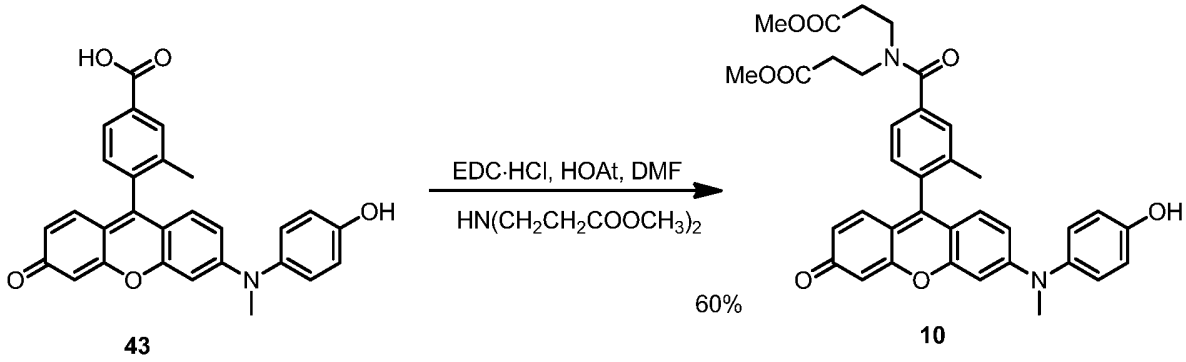
41 **42**

An oven-dried Schlenk tube was charged with $Pd(OAc)_2$ (24 mg, 0.11 mmol), BINAP (134 mg, 0.21 mmol) and Cs_2CO_3 (257 mg, 0.79 mmol), and flushed with Ar gas for 5 min. A solution of **41** (383 mg, 0.72 mmol) and 4-(methoxymethoxy)-*N*-methylaniline (126 mg, 0.75 momol) in toluene (10 mL) was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 °C for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2 (3 × 20 mL). The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give the product **42** (386 mg, 0.70 mmol, 97% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (s, 1H), 7.90 (d, *J* = 8.0 Hz, 1H), 7.18 (d, *J* = 8.0 Hz, 1H), 7.14 (d, *J* = 8.8 Hz, 2H), 7.09 (d, *J* = 8.8 Hz, 2H), 6.85 (d, *J* = 9.4 Hz, 1H), 6.75 (d, *J* = 9.2 Hz, 1H), 6.67 (d, *J* = 2.1 Hz, 1H), 6.57 (dd, *J* = 9.2, 2.1 Hz, 1H), 6.50 (dd, *J* = 9.4, 1.5 Hz, 1H), 6.36 (d, *J* = 1.5 Hz, 1H), 5.17 (s, 2H), 3.43 (s, 3H), 3.39 (s, 3H), 2.00 (s, 3H), 1.60 (s, 9H); ^{13}C NMR (101 MHz, CD_3OD) δ 185.82, 166.46, 160.83, 157.80, 157.20, 156.95, 154.25, 140.56, 138.12, 137.75, 134.39, 132.32, 131.90, 130.52, 130.35, 129.14, 128.02, 127.96, 118.93, 115.57, 114.57, 112.49, 105.51, 99.19, 95.55, 82.70, 56.36, 41.41, 28.45, 19.67.

60

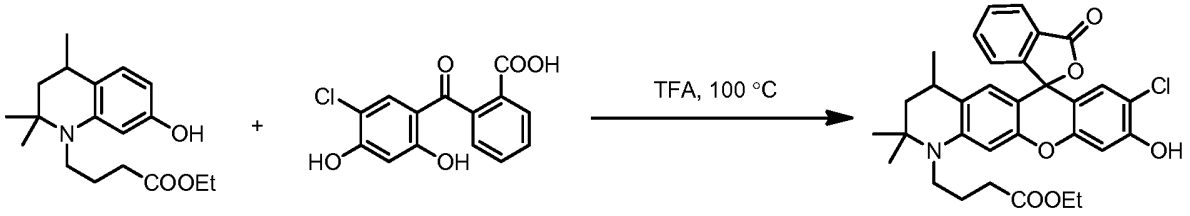


To a solution of **42** (320 mg, 0.58 mmol) in CH_2Cl_2 (3 mL) was added TFA (3 mL). The resulting solution was stirred at room temperature for 2 hr. The solution was concentrated, and then diluted with saturated $NaHCO_3$. The mixture was extracted with chloroform with 10% isopropanol three times. The organic solutions were combined, dried over anhydrous sodium sulfate, and then concentrated. The residue was purified by silica gel column chromatography to give the product **43** (236 mg, 0.52 mmol, 90% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.16 (s, 1H), 8.10 (d, J = 7.9 Hz, 1H), 7.41 (d, J = 7.9 Hz, 1H), 7.31 (d, J = 9.1 Hz, 1H), 7.24 (d, J = 9.1 Hz, 1H), 7.22 – 7.18 (m, 3H), 7.11 – 7.05 (m, 2H), 7.02 (d, J = 10.0 Hz, 1H), 6.96 (d, J = 8.7 Hz, 2H), 3.66 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 170.34, 168.94, 161.12, 161.06, 160.57, 159.31, 159.22, 137.94, 137.28, 136.96, 134.04, 133.00, 132.91, 132.71, 130.48, 128.60, 128.48, 119.64, 119.51, 118.13, 117.37, 116.17, 103.69, 98.81, 42.74, 19.63.



To a solution of **43** (140 mg, 0.31 mmol) in DMF (4 mL) were added dimethyl 3,3'-iminodipropanoate (176 mg, 0.93 mmol), 1-hydroxy-7-azabenzotriazole (HOAt) (70 mg, 0.47 mmol), and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) (99 mg, 0.47 mmol) successively under Ar. The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ solution followed by 0.1 N HCl and brine. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the product **10** (116 mg, 0.19 mmol, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$ with 10% CD_3OD) δ 7.40 (s, 1H), 7.36 (d, J = 7.7 Hz, 1H), 7.22 (d, J = 7.7 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.99 – 6.91 (m, 3H), 6.87 (d, J = 9.2 Hz, 1H), 6.66 – 6.57 (m, 3H), 6.55 (s, 1H), 3.74 – 3.68 (m, 10H), 3.41 (s, 3H), 2.79 (br, 2H), 2.63 (br, 2H), 2.10 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$ with 10% CD_3OD) δ 172.47, 171.73, 171.27, 159.54, 156.82, 155.69, 155.47, 151.62, 137.23, 137.12, 134.47, 130.70, 129.52, 129.40, 128.74, 128.07, 127.74, 124.14, 117.12, 115.12, 112.39, 111.38, 105.01, 98.58, 52.07, 45.80, 41.70, 40.91, 33.59, 32.45, 19.65.

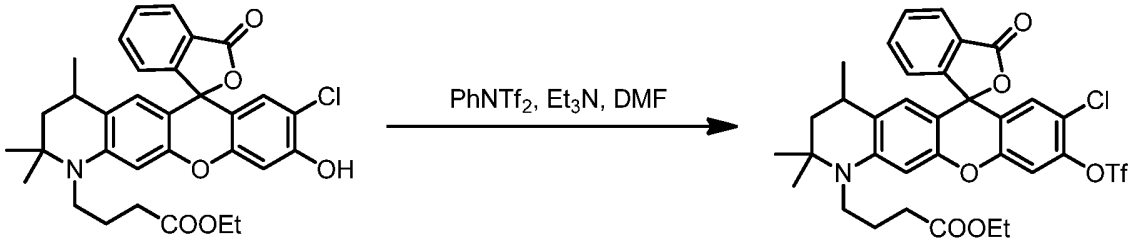
EXAMPLE 2 – SYNTHESIS OF YELLOW FLUOROGENIC COMPOUND 11



44

The suspension of the starting materials in TFA was heated to 100 °C for 4 hr in a sealed-tube. The resulting red solution was then concentrated in vacuum and azeotroped with toluene three times to provide the crude product of **44**, which was purified by silica gel column chromatography to give the pure product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, J = 6.9 Hz, 1H),

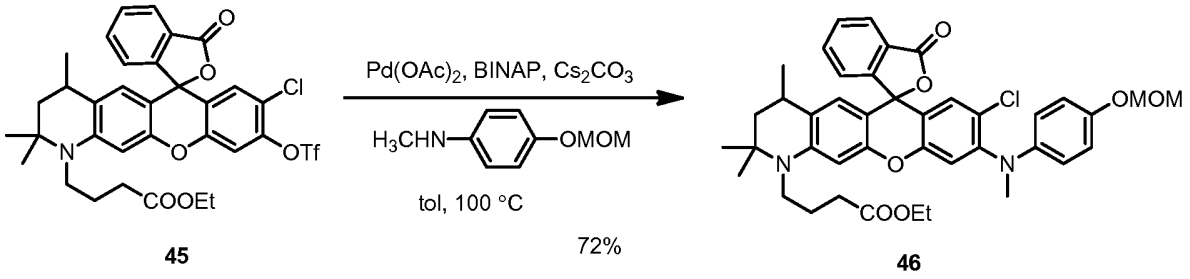
7.68 – 7.61 (m, 2H), 7.22 – 7.18 (m, 1H), 6.86 (s, 1H), 6.79 (s, 0.5 × 1H), 6.77 (s, 0.5 × 1H), 6.56 (s, 1H), 6.48 (s, 1H), 4.22 (br, 1H), 4.20 (q, J = 7.0 Hz, 2H), 3.55 – 3.43 (m, 1H), 3.30 – 3.17 (m, 1H), 2.80 – 2.60 (m, 1H), 2.45 (t, J = 6.5 Hz, 2H), 2.05 – 1.88 (m, 2H), 1.69 (d, J = 12.9 Hz, 1H), 1.49 (dt, J = 20.1, 12.9 Hz, 1H), 1.35 (s, 3H), 1.31 (t, J = 7.0 Hz, 3H), 1.21 (s, 3H), 1.07 (d, J = 6.3 Hz, 0.5 × 3H), 0.99 (d, J = 6.3 Hz, 0.5 × 3H).



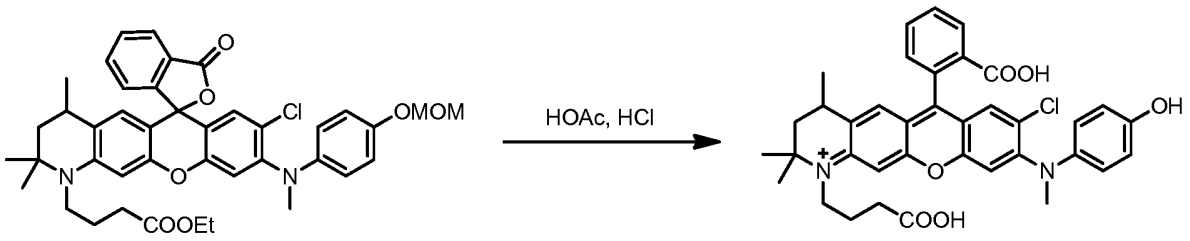
44 90% **45**

To a solution of **44** in DMF were added Et_3N and *N*-phenyl-bis(trifluoromethanesulfonimide) under argon at room temperature. The mixture was stirred overnight and then diluted with ethyl acetate. The organic solution was washed with HCl solution, water, and dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give the product **45** (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 7.2 Hz, 1H), 7.77 – 7.63 (m, 2H), 7.33 (s, 0.5 × 1H), 7.32 (s, 0.5 × 1H), 7.23 (d, J = 7.2 Hz, 0.5 × 1H), 7.21 (d, J = 7.2 Hz, 0.5 × 1H), 6.90 (s, 1H), 6.44 (s, 0.5 × 1H), 6.43 (s, 0.5 × 1H), 6.40 (s, 1H), 4.22 (t, J = 7.1 Hz, 2H), 3.52 – 3.36 (m, 1H), 3.25 – 3.10 (m, 1H), 2.78 – 2.57 (m, 1H), 2.44 (t, J = 6.8 Hz, 2H), 2.05 – 1.96 (m, 2H), 1.74 – 1.64 (m, 1H), 1.45 (dd, J = 24.7, 12.6 Hz, 1H), 1.35 – 1.30 (m, 6H), 1.17 (s, 3H), 1.06 (d, J = 6.6 Hz, 0.5 × 3H), 0.96 (d, J = 6.6 Hz, 0.5 × 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.20, 173.18, 169.11, 169.08, 152.27, 152.05, 151.06, 150.99, 150.69, 150.57, 147.57, 147.37, 145.89, 145.85, 135.43, 135.36, 130.28, 130.23, 130.15, 130.11, 129.69, 126.85, 126.61, 126.54, 126.11, 125.35, 124.68, 124.11, 123.99, 121.43, 121.36, 120.91, 118.67 (q, $J_{C\text{-}F}$ = 318.92 Hz), 112.13, 103.61, 103.60, 97.79, 97.75, 82.73, 82.58, 60.70, 55.19, 55.05, 46.38, 46.29, 44.71, 44.51, 31.67, 31.65, 29.49,

29.36, 26.91, 26.75, 25.74, 25.29, 23.52, 23.36, 19.68, 19.55, 14.34; ^{19}F NMR (376 MHz, $CDCl_3$) δ -73.17.



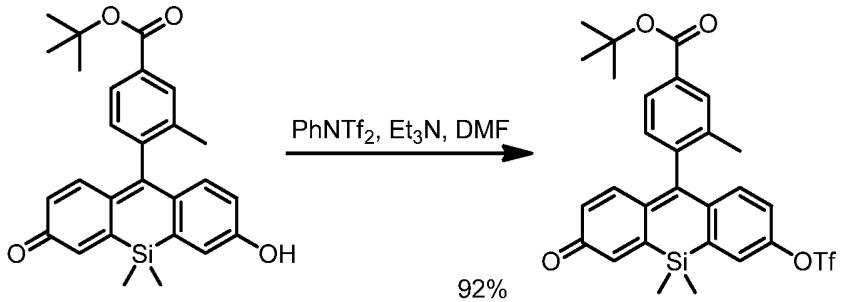
An oven-dried Schlenk tube was charged with $Pd(OAc)_2$, BINAP and Cs_2CO_3, and flushed with Ar gas for 5 min. A solution of **45** and 4-(methoxymethoxy)-*N*-methylaniline in toluene was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 °C for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2. The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give the product **46** (72% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, J = 7.5 Hz, 1H), 7.72 – 7.62 (m, 2H), 7.26 (d, J = 7.5 Hz, 1H), 7.11 (s, 1H), 6.94 (d, J = 7.7 Hz, 2H), 6.76 (s, 1H), 6.73 (d, J = 7.7 Hz, 2H), 6.38 (s, 2H), 5.11 (s, 2H), 4.19 (q, J = 6.9 Hz, 2H), 3.47 (s, 3H), 3.45 – 3.35 (m, 1H), 3.24 (s, 3H), 3.20 – 3.10 (m, 1H), 2.78 – 2.58 (m, 1H), 2.40 (t, J = 6.5 Hz, 2H), 2.05 – 1.80 (m, 2H), 1.67 (d, J = 12.7 Hz, 1H), 1.45 (dd, J = 25.7, 12.7 Hz, 1H), 1.34 – 1.25 (m, 6H), 1.16 (s, 3H), 1.05 (d, J = 6.1 Hz, 1.5 × 3H), 0.95 (d, J = 6.1 Hz, 1.5 × 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.14, 173.11, 169.37, 152.47, 152.31, 151.33, 151.28, 151.22, 151.06, 150.97, 148.22, 148.16, 147.22, 147.00, 143.75, 134.98, 134.92, 129.79, 129.74, 129.66, 127.19, 127.02, 125.76, 125.37, 125.27, 125.05, 124.74, 124.11, 124.00, 118.27, 118.23, 117.24, 116.93, 116.84, 115.05, 115.02, 104.29, 97.76, 97.71, 95.13, 83.93, 83.76, 60.56, 55.85, 55.02, 54.86, 46.45, 46.36, 44.59, 44.36, 40.54, 40.52, 31.61, 31.59, 29.45, 29.30, 26.82, 26.64, 25.66, 25.14, 23.51, 23.32, 19.68, 19.48, 14.26.



46 **11**

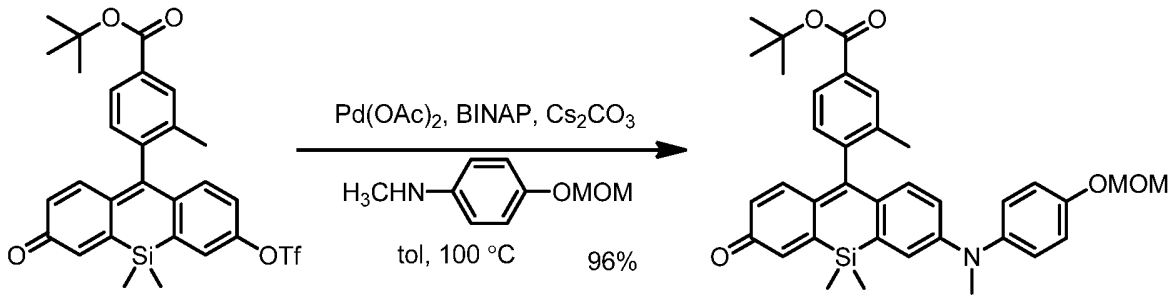
To a solution of **46** in acetic acid was slowly added aqueous HCl solution. The mixture was heated at 100 °C for 2 hr. After cooled to room temperature, the reaction mixture was carefully basified with satd. $NaHCO_3$, and then extracted with CH_2Cl_2 containing 10% isopropanol three times. The organic solutions were combined, dried over anhydrous sodium sulfate, and concentrated. The residue was purified with silica gel column chromatography to give the product **11**. ^1H NMR (400 MHz, CD_3OD) δ 8.38 – 8.33 (m, 1H), 7.89 (t, J = 7.4 Hz, 1H), 7.83 (t, J = 7.4 Hz, 1H), 7.49 (s, 0.5 × 1H), 7.48 (s, 0.5 × 1H), 7.45 (d, J = 7.4 Hz, 1H), 7.31 (s, 1H), 7.10 (s, 0.5 × 1H), 7.06 (s, 0.5 × 1H), 7.02 – 6.92 (m, 3H), 6.76 (d, J = 8.7 Hz, 2H), 3.89 – 3.77 (m, 1H), 3.74 – 3.62 (m, 1H), 3.53 (s, 0.5 × 3H), 3.52 (s, 0.5 × 3H), 2.97 – 2.85 (m, 1H), 2.60 – 2.56 (m, 2H), 2.10 – 2.00 (m, 2H), 1.96 (dd, J = 13.6, 4.0 Hz, 1H), 1.61 (td, J = 13.6, 5.0 Hz, 1H), 1.54 (s, 3H), 1.43 (s, 0.5 × 3H), 1.42 (s, 0.5 × 3H), 1.13 (d, J = 3.4 Hz, 0.5 × 3H), 1.12 (d, J = 3.4 Hz, 0.5 × 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 176.56, 168.26, 168.21, 159.26, 159.17, 157.60, 157.57, 156.92, 156.89, 156.07, 155.93, 155.37, 155.33, 141.29, 135.10, 134.91, 134.78, 134.76, 134.13, 134.10, 132.51, 132.41, 132.23, 131.88, 131.71, 131.56, 131.33, 126.85, 126.82, 126.73, 125.88, 117.56, 117.49, 117.14, 117.05, 117.00, 107.62, 107.60, 98.46, 98.41, 60.51, 60.48, 47.26, 45.27, 45.25, 44.68, 44.67, 31.33, 29.19, 29.16, 28.03, 25.98, 23.94, 19.07, 18.97.

EXAMPLE 3 – SYNTHESIS OF RED FLUOROGENIC COMPOUND 22



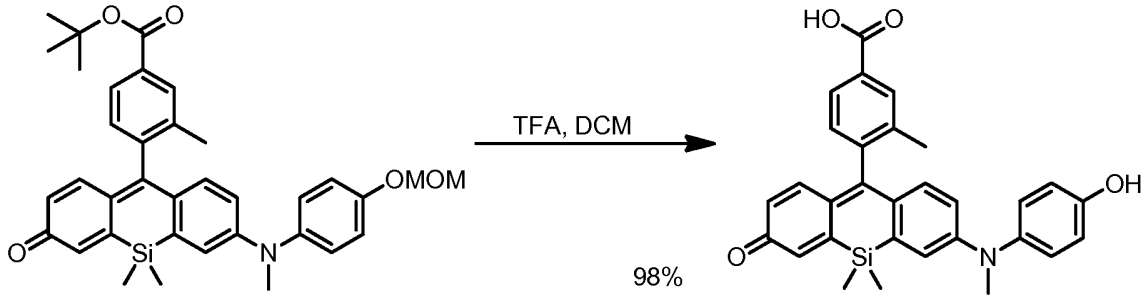
47 **48**

To a solution of **47** in DMF were added Et_3N and *N*-phenyl-bis(trifluoromethanesulfonimide) under Ar at room temperature. The mixture was stirred overnight and then diluted with ethyl acetate. The organic solution was washed with HCl solution, water, and dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give the product **48** (92% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 1H), 7.92 (d, J = 7.9 Hz, 1H), 7.50 (d, J = 2.8 Hz, 1H), 7.17 (d, J = 7.9 Hz, 1H), 7.08 (dd, J = 9.0, 2.8 Hz, 1H), 6.90 – 6.87 (m, 2H), 6.84 (d, J = 2.1 Hz, 1H), 6.21 (dd, J = 10.2, 2.1 Hz, 1H), 2.09 (s, 3H), 1.59 (s, 9H), 0.52 (s, 3H), 0.50 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.13, 165.18, 151.50, 149.68, 145.80, 142.75, 141.29, 140.70, 140.30, 138.15, 136.47, 134.31, 132.49, 131.45, 130.89, 129.45, 129.33, 129.03, 127.25, 126.59, 123.02, 122.76, 118.67 (q, J_{C-F} = 322.1 Hz), 81.45, 28.14, 19.50, -1.48, -1.74; ^{19}F NMR (376 MHz, $CDCl_3$) δ -72.82.



48 **49**

An oven-dried Schlenk tube was charged with $Pd(OAc)_2$, BINAP and Cs_2CO_3, and flushed with Ar gas for 5 min. A solution of **48** and 4-(methoxymethoxy)-*N*-methylaniline in toluene was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 °C for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2. The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give the product **49** (96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H), 7.91 (d, *J* = 7.9 Hz, 1H), 7.17 (d, *J* = 7.8 Hz, 1H), 7.13 (d, *J* = 8.9 Hz, 2H), 7.08 (d, *J* = 8.9 Hz, 2H), 6.99 (d, *J* = 2.7 Hz, 1H), 6.85 (d, *J* = 2.0 Hz, 1H), 6.84 (d, *J* = 10.0 Hz, 1H), 6.63 (d, *J* = 9.2 Hz, 1H), 6.44 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.22 (dd, *J* = 10.0, 2.0 Hz, 1H), 5.19 (s, 2H), 3.50 (s, 3H), 3.37 (s, 3H), 2.13 (s, 3H), 1.63 (s, 9H), 0.45 (s, 3H), 0.44 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.20, 165.54, 156.25, 155.65, 150.09, 146.82, 144.26, 141.14, 140.68, 140.30, 136.51, 136.23, 134.97, 131.79, 130.99, 129.43, 128.80, 127.90, 127.02, 126.76, 126.66, 119.14, 117.62, 114.32, 94.61, 81.29, 56.16, 40.35, 28.26, 19.48, -0.97, -1.21.

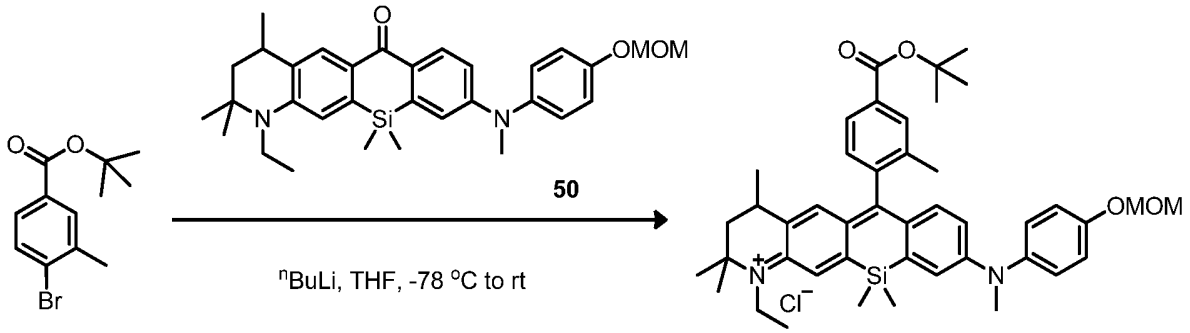


49 **22**

To a solution of **49** in CH_2Cl_2 was added TFA slowly at room temperature. The mixture was stirred for 2 hr and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give the product **22** in 98% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 8.07 (s, 1H), 8.03 (d, *J* = 7.9 Hz, 1H), 7.36 (d, *J* = 2.2 Hz, 1H), 7.30 – 7.27 (m, 2H), 7.22 (d, *J* = 8.8 Hz, 2H), 7.16 (d, *J* = 9.9 Hz, 1H), 7.05 (d, *J* = 9.1 Hz, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.78 (dd,

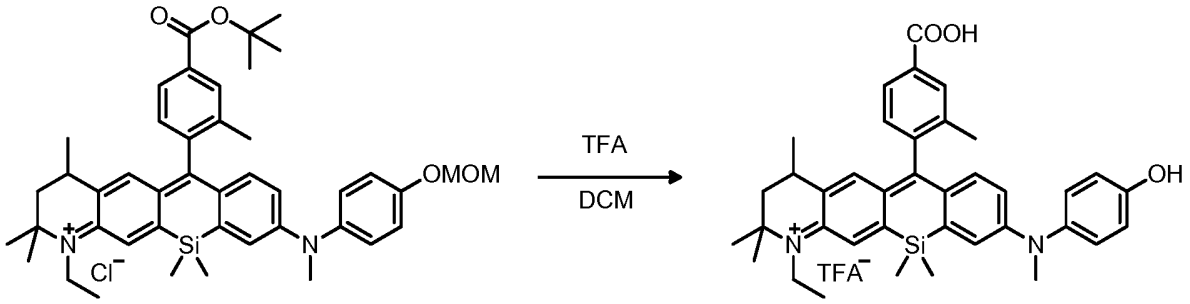
J = 9.9, 2.2 Hz, 1H), 6.75 (dd, J = 9.1, 2.6 Hz, 1H), 3.74 (s, 3H), 2.11 (s, 3H), 0.53 (s, 3H), 0.51 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 170.30, 169.09, 167.89, 159.75, 157.82, 152.92, 147.96, 144.45, 144.35, 141.90, 137.61, 136.37, 132.87, 132.59, 131.45, 130.50, 130.08, 128.26, 128.14, 126.12, 125.77, 120.01, 118.52, 117.96, 43.17, 19.50, -1.64, -1.89.

EXAMPLE 4 – SYNTHESIS OF FAR RED FLUOROGENIC COMPOUNDS 25 AND 27



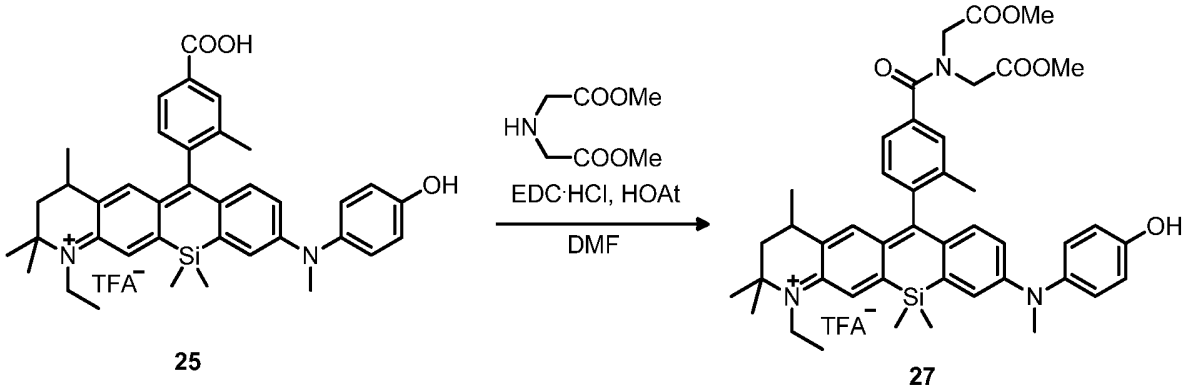
To a solution of t-butyl 4-bromo-3-methylbenzoate (585 mg, 2.16 mmol) in dry THF (10 mL) at -78 °C, was added *n*-BuLi (1.46 mL, 2.38 mmol) dropwise under argon atmosphere. Then HMPA (73 μL, 0.431 mmol) was added after 30 min. And a solution of **50** (226 mg, 0.431 mmol) in dry THF (2 mL) was added 5 min later. The resulting mixture was stirred at -78 °C to room temperature for 12 h. The reaction was quenched with 3N HCl for 10 min and extracted with DCM for 3 times. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the product **51** (307 mg, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H), 7.81 (d, J = 7.8 Hz, 1H), 7.07 – 7.03 (m, 4H), 7.01 – 6.99 (m, 3H), 6.75 (s, 1H), 6.72 (d, J = 9.5 Hz, 1H), 6.38 (dd, J = 9.5, 2.6 Hz, 1H), 5.08 (s, 2H), 3.80 – 3.72 (m, 2H), 3.52 (s, 3H), 3.37 (s, 3H), 3.31 – 3.28 (m, 1H), 2.57 – 2.50 (m, 1H), 1.99 (s, 3H), 1.73 (dd, J = 13.6, 4.3 Hz, 1H), 1.51 (s, 9H), 1.48 – 1.43 (m, 2H), 1.40 (s, 3H), 1.31 – 1.26 (m, 6H), 1.03 – 1.01 (m, 3H), 0.83 – 0.81 (m, 3H), 0.46 (d, J = 2.2 Hz, 3H), 0.45 (d, J = 2.4 Hz,

3H) ; ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.11, 164.99, 156.49, 153.20, 153.16, 151.91, 151.86, 148.19, 148.06, 146.14, 146.34, 142.61, 139.48, 139.39, 138.02, 136.53, 136.43, 135.92, 135.77, 132.17, 132.14, 130.91, 130.70, 130.54, 130.47, 128.91, 128.76, 127.81, 127.78, 127.46, 127.01, 126.90, 126.49, 126.32, 122.31, 121.12, 121.06, 117.61, 115.02, 94.29, 81.39, 59.12, 59.03, 56.00, 44.30, 41.36, 41.16, 29.43, 28.67, 27.98, 26.23, 26.17, 26.11, 26.06, 19.35, 19.28, 18.53, 18.33, 14.81, -0.85, -1.13, -1.43, -1.76.



51 **25**

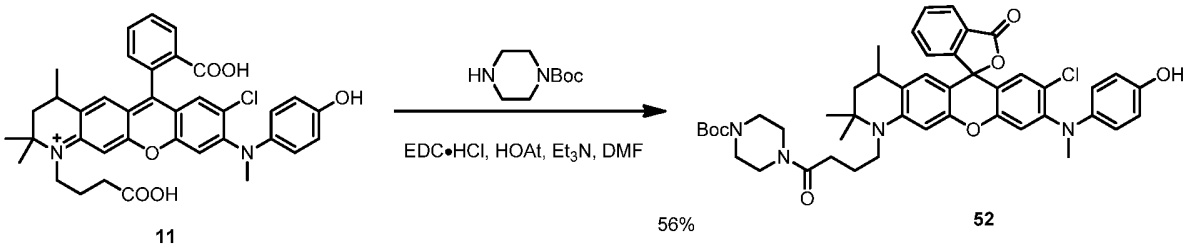
To a solution of **51** (306 mg, 2.16 mmol) in DCM at 0 °C was added TFA dropwise. The resulting mixture was stirred at room temperature for 3 h. The reaction mixture was then concentrated and azeotroped with toluene for 3 times, and purified by silica gel column chromatography to give the product **25** (236 mg, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.97 (d, *J* = 7.9 Hz, 1H), 7.30 (s, 1H), 7.21 – 7.19 (m, 2H), 7.11 – 7.08 (m, 2H), 6.92 – 6.87 (m, 4H), 6.58 (dd, *J* = 9.5, 2.4 Hz, 1H), 3.92 (br, 1H), 3.90 (br, 1H), 3.52 (s, 3H), 2.75 (br, 1H), 2.09 (s, 3H), 1.87 (dd, *J* = 13.5, 4.2 Hz, 1H), 1.50 (s, 3H), 1.54 – 1.48 (m, 1H), 1.40 – 1.37 (m, 6H), 0.90 (m, 6H), 0.53 (d, *J* = 2.9 Hz, 3H), 0.51 (t, *J* = 3.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.98, 168.13, 168.09, 158.63, 155.21, 155.18, 153.41, 153.35, 149.09, 148.98, 147.65, 147.56, 144.76, 140.81, 140.75, 137.73, 137.55, 137.31, 132.51, 132.45, 132.30, 132.26, 130.59, 130.35, 128.82, 128.75, 128.27, 128.18, 127.95, 123.75, 122.85, 122.80, 117.86, 116.09, 60.52, 60.44, 45.55, 41.78, 41.68, 28.91, 28.86, 27.47, 27.43, 26.25, 19.51, 19.47, 18.83, 18.66, 15.15, -1.13, -1.37, -1.63, -1.89.



25 **27**

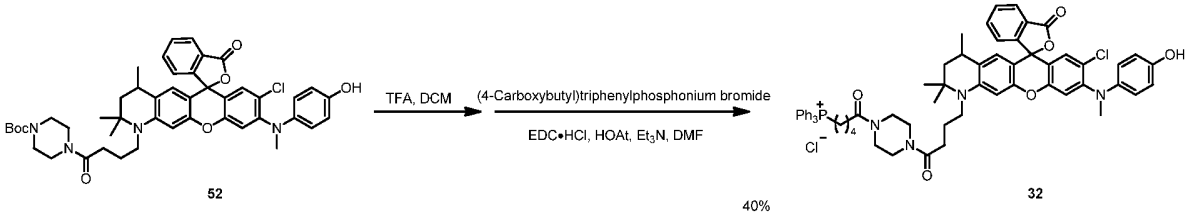
To a solution of **25** (116 mg, 0.162 mmol) and dimethyl 3,3'-iminodipropanoate (52 mg, 0.324 mmol) in anhydrous DMF (2 mL) at room temperature was added 1-hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.194 mmol), and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (44 mg, 0.227 mmol) successively under argon atmosphere. The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$, H_2O and brine. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the product **27** (122 mg, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.25 (br, 1H), 7.49 (d, J = 10.2 Hz, 1H), 7.42 (dd, J = 8.1, 3.9 Hz, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.16 (dd, J = 7.2, 5.7 Hz, 1H), 7.07 – 7.05 (m, 2H), 6.99 – 6.97 (m, 3H), 6.81 (d, J = 6.9 Hz, 1H), 6.61 (d, J = 8.4 Hz, 1H), 4.45 (s, 2H), 4.40 (s, 2H), 3.85 – 3.65 (m, 1H), 3.82 (s, 3H), 3.79 (s, 3H), 3.55 (s, 3H), 3.70 – 3.50 (m, 1H), 2.10 – 2.09 (m, 3H), 1.85 (dd, J = 13.5, 3.8 Hz, 1H), 1.61 – 1.58 (m, 1H), 1.56 (s, 3H), 1.43 – 1.38 (m, 6H), 0.94 (t, J = 5.4 Hz, 3H), 0.50 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.57, 169.52, 169.32, 166.83, 158.61, 154.05, 154.02, 151.45, 151.41, 147.59, 147.46, 146.59, 140.82, 140.54, 136.74, 136.22, 136.17, 135.14, 135.12, 134.66, 130.33, 130.22, 129.27, 128.88, 128.57, 127.64, 127.58, 127.10, 126.98, 126.62, 124.15, 123.74, 122.41, 121.58, 117.85, 114.84, 58.84, 58.78, 52.69, 52.36, 51.68, 47.56, 44.55, 41.65, 40.84, 28.88, 26.36, 26.31, 26.25, 19.51, 19.40, 18.56, 18.35, 14.84, -0.81, -1.04, -1.39, -1.65.

EXAMPLE 5 – SYNTHESIS OF MITOCHONDRIAL-TARGETING FLUOROGENIC COMPOUND 25



11 56% 52

To a solution of **11** in DMF were added triethylamine, 1-hydroxy-7-azabenzotriazole (HOAt), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl), and *N*-methylpiperazine successively under Ar. The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ solution followed by 0.1 N HCl and brine. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the product **52** (56% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (d, J = 7.6 Hz, 1H), 7.71 (t, J = 7.4 Hz, 1H), 7.64 (t, J = 7.4 Hz, 1H), 7.24 (d, J = 7.6 Hz, 1H), 7.02 (s, 1H), 6.81 – 6.72 (m, 5H), 6.38 (s, 2H), 3.67 – 3.62 (m, 3H), 3.46 – 3.42 (m, 7H), 3.23 (s, 3H), 2.71 – 2.62 (m, 1H), 2.42 (s, 2H), 2.17 – 2.04 (m, 3H), 1.68 – 1.64 (m, 10H), 1.31 (s, 3H), 1.25 (s, 3H), 1.05 (d, J = 6.6 Hz, 0.5 × 3H), 0.95 (d, J = 6.6 Hz, 0.5 × 3H).



52 40% 32

To a solution of **52** in CH_2Cl_2 was added TFA slowly at room temperature. The mixture was stirred for 2 hr and then concentrated *in vacuo*. The residue was redissolved in anhydrous

DMF. To this solution were added triethylamine, 1-hydroxy-7-azabenzotriazole (HOAt), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl), and (4-Carboxybutyl)triphenylphosphonium bromide successively under Ar. The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ solution followed by 0.1 N HCl and brine. The extracts was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give the product **32** (40% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 8.32 (d, J = 7.3 Hz, 1H), 7.90 – 7.86 (m, 5H), 7.82 – 7.75 (m, 12H), 7.49 – 7.48 (m, 1H), 7.44 – 7.41 (m, 2H), 7.07 – 7.02 (m, 1H), 6.96 – 6.93 (m, 3H), 6.75 (d, J = 8.6 Hz, 2H), 3.68 – 3.67 (m, 1H), 3.63 – 3.56 (m, 9H), 3.50 – 3.43 (m, 5H), 2.89 – 2.88 (m, 1H), 2.67 (t, J = 6.7 Hz, 2H), 2.51 (t, J = 6.7 Hz, 2H), 2.11 – 2.02 (m, 2H), 1.85 (dd, J = 14.5, 5.3 Hz, 1H), 1.76 – 1.73 (m, 2H), 1.63 – 1.60 (m, 2H), 1.58 – 1.56 (m, 1H), 1.54 (s, 3H), 1.42 – 1.41 (m, 3H), 1.27 – 1.10 (m, 3H).

EXAMPLE 6 – SENSITIVE AND SPECIFIC DETECTION OF PEROXYNITRITE WITH GREEN FLUOROGENIC COMPOUND 2

This Example shows that green fluorogenic Compound 2 sensitively and selectively detects peroxynitrite. Specifically, Compound 2 is dissolved in 0.1 M phosphate buffer at pH 7.4 to form a 1 μM solution, with excitation and emission spectra at 510 nm and 530 nm, respectively. The 1 μM solution of Compound 2 is treated with peroxynitrite at various concentrations. **Figure 1A** shows that the florescence intensity of Compound 2 increases with increasing concentration of peroxynitrite.

The reactivity of Compound 2 is compared toward different reactive oxygen species (ROS) and reactive nitrogen species (RNS). Specifically, the 1 μM solution of compound 2 is treated with various ROS and RNS. The concentration of highly reactive oxygen species (hydroxyl radical (•OH), hypochlorous acid ($^-$OCl), and peroxynitrite ($ONOO^-$)) is 1 μM. The

concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 10 μM. **Figure 1B** shows that treatment with peroxynitrite results in a much higher increase in fluorescence intensity of Compound 2 than treatment with other ROS and RNS.

EXAMPLE 7 - SENSITIVE AND SPECIFIC DETECTION OF PEROXYNITRITE WITH YELLOW FLUOROGENIC COMPOUND 11

This Example shows that yellow fluorogenic Compound 11 sensitively and selectively detects peroxynitrite. Specifically, Compound 11 is dissolved in 0.1 M phosphate buffer at pH 7.4 to form a 2 μM solution, with excitation and emission spectra at 547 nm and 570 nm, respectively. The 2 μM solution of Compound 11 is treated with peroxynitrite at various concentrations. **Figure 2A** shows that the florescence intensity of Compound 11 increases with increasing concentration of peroxynitrite.

The reactivity of Compound 11 is compared with different reactive oxygen species (ROS) and reactive nitrogen species (RNS). Specifically, the 2 μM solution of compound 11 is treated with various ROS and RNS. The concentration of highly reactive oxygen species (hydroxyl radical (•OH), hypochlorous acid ($^-$OCl), and peroxynitrite ($ONOO^-$)) is 2 μM. The concentration of $^1O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 20 μM. **Figure 2B** shows that treatment with peroxynitrite results in a much higher increase in fluorescence intensity of Compound 11 than treatment with other ROS and RNS.

EXAMPLE 8 - SENSITIVE AND SPECIFIC DETECTION OF PEROXYNITRITE WITH RED FLUOROGENIC COMPOUND 22

This Example shows that red fluorogenic Compound **22** sensitively and selectively detects peroxynitrite. Specifically, Compound **22** is dissolved in 0.1 M phosphate buffer at pH 7.4 to form a 5 μM solution, with excitation and emission spectra at 600 nm and 617 nm,

respectively. The 5 μM solution of Compound **22** is treated with peroxynitrite at various concentrations. **Figure 3A** shows that the florescence intensity of Compound **22** increases with increasing concentration of peroxynitrite.

The reactivity of Compound 22 is compared with different reactive oxygen species (ROS) and reactive nitrogen species (RNS). Specifically, the 5 μM solution of compound 22 is treated with various ROS and RNS. The concentration of highly reactive oxygen species (hydroxyl radical (•OH), hypochlorous acid ($^{-}$OCl), and peroxynitrite ($ONOO^{-}$)) is 5 μM. The concentration of $^{1}O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 50 μM. **Figure 3B** shows that treatment with peroxynitrite results in a much higher increase in fluorescence intensity of Compound 22 than treatment with other ROS and RNS.

EXAMPLE 9 - SENSITIVE AND SPECIFIC DETECTION OF PEROXYNITRITE WITH DEEP RED FLUOROGENIC COMPOUND **25**

This Example shows that deep red fluorogenic Compound **25** sensitively and selectively detects peroxynitrite. Specifically, Compound **25** is dissolved in 0.1 M phosphate buffer at pH 7.4 to form a 5 μM solution, with excitation and emission spectra at 650 nm and 665 nm, respectively. The 5 μM solution of Compound **25** is treated with peroxynitrite at various concentrations. **Figure 4A** shows that the florescence intensity of Compound **25** increases with increasing concentration of peroxynitrite.

The reactivity of Compound **25** is compared with different reactive oxygen species (ROS) and reactive nitrogen species (RNS). Specifically, the 5 μM solution of compound **25** is treated with various ROS and RNS. The concentration of highly reactive oxygen species (hydroxyl radical (•OH), hypochlorous acid ($^{-}$OCl), and peroxynitrite ($ONOO^{-}$)) is 5 μM. The concentration of $^{1}O_2$, $O_2^{\bullet-}$, NO, ROO• and H_2O_2 is 50 μM. **Figure 4B** shows that treatment with

peroxynitrite results in a much higher increase in fluorescence intensity of Compound **25** than treatment with other ROS and RNS.

EXAMPLE 10 - SENSITIVE AND SPECIFIC DETECTION OF PEROXYNITRITE WITH MITOCHONDRIAL-TARGETING FLUOROGENIC COMPOUND **30**

This Example shows that mitochondrial-targeting fluorogenic Compound **30** sensitively and selectively detects peroxynitrite. Specifically, Compound **30** is dissolved in 0.1 M phosphate buffer at pH 7.4 to form a 1 μM solution and excited at 515 nm. The 1 μM solution of Compound **30** is treated with peroxynitrite at various concentrations. **Figure 5A** shows that the florescence intensity of Compound **30** increases with increasing concentration of peroxynitrite.

The reactivity of Compound **30** is compared with different reactive oxygen species (ROS) and reactive nitrogen species (RNS). Specifically, the 1 μM solution of compound **30** is treated with various ROS and RNS. **Figure 5B** shows that treatment with peroxynitrite results in a much higher increase in fluorescence intensity of Compound **22** than treatment with other ROS and RNS.

EXAMPLE 11 – APPLICATION OF SUBJECT COMPOUNDS IN CELL ASSAY

Human SH-SY5Y neuroblastoma cells (ATCC, USA) were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (PS, Gibco) and 1% L-glutamine (Gibco). Mouse C17.2 neural progenitor cells (ATCC, USA) were maintained in high glucose Dulbecco's Modified Eagle Medium supplemented with 8% fetal bovine serum (Gibco), 4% horse serum (Gibco), 1% penicillin/streptomycin and 1% L-glutamine. Mouse RAW264.7 macrophage cells (ATCC, USA) were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone)

supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine.

Generally, cells were grown to confluence prior to experiment. Cells were incubated with corresponding compounds (see Figures 6 – 11) for 1 hr and then washed three times with PBS buffer. Only very weak fluorescence was observed in the absence of stimulants, such as SIN-1 or LPS (lipopolysaccharide)/IFN-γ (Interferon-γ). The fluorescence of compounds was strongly induced after treatment with stimulants, such as SIN-1 or LPS/ IFN-γ. The results are shown in Figures 6 – 11, which strongly suggest that the subject compounds are suitable for the detection of peroxynitrite in living cells.

EXAMPLE 12 – SCREENING PEROXYNITRITE SCAVENGERS BY COMPOUND **14** BASED PLATFORM

SH-SY5Y cells were seeded at a density of 5×10^4 cells per well onto 96-well plates and incubated at 37℃ under 5% CO_2 atmosphere in DMEM medium supplemented with 10% FBS, 1% PS plus 1% L-glutamine. Cells were subjected to serum free medium containing 20 μM Compound **14** in the next day. Cells were then treated with or without different concentration of drug candidates (10 μM, 100 μM) for 10 min, followed by adding SIN-1 to final concentration of 1 mM for 2 h. The plates were then subjected to spectrofluorometer (Lambda55, PerkinElmer) at an excitation wavelength of 543 nm and emission wavelength of 567 nm. The group that cells were treated with neither drug candidates nor SIN-1 was considered as "Ctrl" group; the group that cells were treated with SIN-1 but without drug candidates was considered as "Ctrl+SIN-1" group; and the group that cells were treated with both drug candidates and SIN-1 was considered as "drug+SIN-1" group. The scavenging activity of drug candidates was calculated by $\{[(A_{ctrl+SIN-1}-A_{ctrl})-(A_{drug+SIN-1}-A_{ctrl})]/(A_{ctrl+SIN-1}-A_{ctrl})\}*100\%$. A representative figure of the screening results using Compound **14** based platform is shown in Figure 12.

EXAMPLE 13 – APPLICATION OF COMPOUND **14** FOR DETECTING PEROXYNITRITE IN BRAIN SLICES *EX VIVO*

SD rats were decapitated and the skulls were quickly opened. After removal of the frontal and occipital poles (including the cerebellum), the isolated brain was immediately placed into ice-cold ACSF (Artificial cerebrospinal fluid) saturated with oxygen. After dissection of the rat brain, the specimens were placed into ACSF (saturated with 95% O_2 to 5% CO_2). The specimens were sliced in 300 μm thick sections on a NVSL/NVSLM1 tissue slicer (World Precision Instruments Inc., USA). Slices were collected and placed in 6-well culture dishes and maintained with 1 ml culture medium consisting of 50% minimum essential medium, 24% horse serum and 25% HBSS, 1% penicillin-streptomycin (all from Invitrogen) and supplemented with 36 mM glucose, and 25 mM Hepes (Sigma, St. Louis, MO, USA) (pH 7.2). After 1 day in culture, culture medium was replaced with fresh medium containing no antibiotics. After 5 days cluture, slices were pre-staining with 10 μM Compound **14** for 30 min and then washed out with new medium. Slides were then treated with or without SIN-1 (200 μM) and FeTMPyP (50 μM), a peroxynitrite decomposer, and monitored by fluorescence microscopy. The results are shown in Figure 13, indicating the potential application for *ex vivo* experimental systems of Compound **14**.

EXAMPLE 14 – APPLICATION OF COMPOUND **14** FOR DETECTING ENDOGENOUS PEROXYNITRITE FORMATION IN ISCHEMIC BRAIN TISSUES

C57 mice (8 weeks) were fasted for 6 hour before experiments. After fasting, the ethanol group mice were given 50% (vol/vol) ethanol at a total accumulative dosage of 5g/kg body weight by 3 equally divided gavages in 20 minute intervals. After 6 hours of fasting, the ethanol group mice were given 50% (vol/vol) ethanol at a total accumulative dosage of 5g/kg body weight by 3 equally divided gavages in 20 minute intervals. Sham mice group were received the same volume of water. After 3h treated with etnanol, mice were anesthetized and live in situ

reperfusion with COMPOUND **14** (1μM, 2ml/min, total 25ml). Fresh liver sample sectioned into 15 μM cryosection slices. After washed with PBS for 5 min and then incubated with DAPI for 10 min, the sections were monitored by epifluorescence microscopy. The results are shown in Figure 14. Strong fluorescence signal from Compound **14** was observed in samples from alcohol treated mice, indicating that peroxynitrite was produced in acute alcohol induced injury of liver.

All references, including publications, patent applications and patents, cited herein are hereby incorporated by reference to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and was set forth in its entirety herein.

The terms “a” and “an” and “the” and similar referents as used in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Unless otherwise stated, all exact values provided herein are representative of corresponding approximate values (e.g., all exact exemplary values provided with respect to a particular factor or measurement can be considered to also provide a corresponding approximate measurement, modified by "about," where appropriate).

The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise indicated. No language in the specification should be construed as indicating any element is essential to the practice of the invention unless as much is explicitly stated.

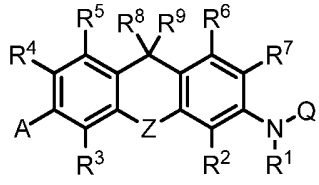
The description herein of any aspect or embodiment of the invention using terms such as "comprising", "having", "including" or "containing" with reference to an element or elements is intended to provide support for a similar aspect or embodiment of the invention that "consists of", "consists essentially of", or "substantially comprises" that particular element or elements, unless otherwise stated or clearly contradicted by context (e.g., a composition described herein as comprising a particular element should be understood as also describing a composition consisting of that element, unless otherwise stated or clearly contradicted by context).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

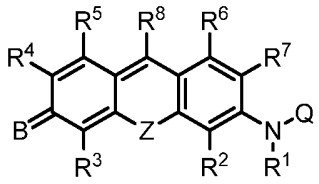
CLAIMS

We claim:

1. A compound of formula (I) or (II):



(I),



(II),

or a tautomer thereof;

wherein N is a nitrogen atom, and is linked to Q and R^1 through single covalent bonds;

R^1 is H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, carboxyalkyl, alkylamino, alkoxyamino, alkylamido, alkoxyamido, or acyl;

each of R^2, R^3, R^4, R^5, R^6 and R^7 is independently H, F, Cl, Br, I, CN, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, acylamino, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, nitro, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic

acid, phosphate ester, sulfonic acid (-SO_3H), sulfonate ester, sulfonamide, $-C(=O)-P^1$ or $-C(=O)-M-P^2$;

each of P^1 and P^2 is independently hydrogen, halo, alkoxy, hydroxy, thiol, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl, arylalkyl, carbamate, amino, alkylamino, arylamino, dialkylamino, alkylarylamino, diarylamino, alkylthio, heteroalkyl, alkyltriphenylphosphonium, or heterocyclyl having from 3 to 7 ring atoms; M is alkylene, alkenylene, alkynylene, arylene, aralkylene or alkarylene;

A is OR^{10} or $NR^{11}R^{12}$;

wherein R^{10} is H, alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, aryl, alkaryl, arylalkyl, carboxyalkyl, alkoxycarbonyl, acyl or aminocarbonyl;

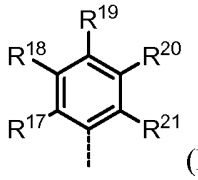
wherein each of R^{11} and R^{12} is independently H, alkyl, halogenated alkyl, alkenyl, alkynyl, alkoxyalkyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aminoalkyl, arylalkyl, alkyloxy, acyl, carboxyalkyl, sulfoalkyl, a salt of carboxyalkyl, a salt of sulfoalkyl, or an ester or amide of carboxyalkyl or sulfoalkyl; or R^{11} in combination with R^{12} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by alkyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol; or R^{11} in combination with R^4, or R^{12} in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or further fused with an aryl or heteroaryl ring, and is optionally substituted by one or more alkyls, carboxylic acids, sulfonic acids (-SO_3H), or their salts, ester or amide derivatives;

B is O or $N^+R^{11}R^{12}$;

Z is O, S, NR^{13}, $CR^{13}R^{14}$, $SiR^{13}R^{14}$, $GeR^{13}R^{14}$, or $SnR^{13}R^{14}$;

wherein each of R^{13} and R^{14} is independently H, alkyl, halogenated alkyl, heteroalkyl, alkenyl, alkynyl, aralkyl, aryl, alkaryl, heterocyclyl, cycloalkyl, cycloalkenyl, cycloalkynyl, hydroxyalkyl, aminoalkyl, hydroxy, thiol, thioalkyl, alkoxy, alkylthio, alkoxyalkyl, aryloxy, arylalkoxy, acyloxy, carbamoyl, trifluoromethyl, phenoxy, benzyloxy, phosphonic acid, phosphate ester, sulfonic acid ($-SO_3H$), sulfonate ester, sulfonamide, carboxylic acid, carboxylic ester, or carboxylic amide; or R^{13} in combination with R^{14} forms a saturated 5- or 6-membered heterocycle that is optionally substituted by alkyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol;

R^8 is H, CF_3, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of an alcohol; or R^8 is a saturated or unsaturated alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$), wherein each of R^{15} and R^{16} represents a saturated or unsaturated, cyclic or acyclic alkyl that is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, or alkyltriphenylphosphonium; or R^8 has the formula



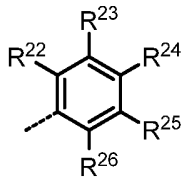
(III),

wherein each of R^{17}, R^{18}, R^{19}, R^{20} and R^{21} is independently H, F, Cl, Br, I, CN, nitro, a carboxylic acid, a salt of carboxylic acid, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), sulfonamide ($-SO_2NR^{15}R^{16}$), hydroxy, azide, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed alkylcarbonylalkyl such as

trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid (-SO_3H), sulfonate ester (-SO_3R^{15}), or sulfonamide (-$SO_2NR^{15}R^{16}$); or R^{17} and R^{18} together, R^{18} and R^{19} together, R^{19} and R^{20} together, or R^{20} and R^{21} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (III) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid (-SO_3H), sulfonate ester (-SO_3R^{15}), or sulfonamide (-$SO_2NR^{15}R^{16}$);

R^9 is H, hydroxy, CN or alkoxy; or R^8 in combination with R^9 forms a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring; or R^9 in combination with R^{17} or R^{21} forms a 5- or 6-membered spirolactone or spirolactam ring or a 5- or 6-membered spirosultone or spirosultam ring that is optionally and independently substituted by H, F or CH_3; and

Q is a substituted phenyl represented by formula (IV):



(IV),

wherein each of R^{22}, R^{23}, R^{24}, R^{25}, and R^{26} is independently H, hydroxy, alkyl, alkenyl, alkynyl, heteroalkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkylaryl, arylalkyl, heterocyclyl, alkoxy, alkoxyalkyl, alkoxyalkoxy, acyl, alkylcarbonylalkyl, halogentaed

alkylcarbonylalkyl such as trifluoromethylcarbonylalkyl, aminoalkyl, carboxyalkyl, thiol, alkylthio, amino, alkylamino, dialkylamino, alkoxycarbonyl, alkoxycarbonylalkyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, or arylcarboxamido, the alkyl or aryl of which is optionally substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$); or R^{22} and R^{23} together, R^{23} and R^{24} together, R^{24} and R^{25} together, or R^{25} and R^{26} together form a part of a 5- or 6-membered cycloalkyl, heterocyclyl, aryl or heteroaryl ring fused with the phenyl ring of formula (IV) that is optionally further substituted by one or more F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of an alcohol, amino, alkylamino, dialkylamino, alkoxy, thiol, alkylthio, alkyltriphenylphosphonium, sulfonic acid ($-SO_3H$), sulfonate ester ($-SO_3R^{15}$), or sulfonamide ($-SO_2NR^{15}R^{16}$).

2. The compound of claim 1, wherein R^9 when taken in combination with R^8 forms a 5-membered spirolactone or spirolactam ring or a 5-membered spirosultam ring, and R^9 is oxygen or substituted nitrogen.

3. The compound of claim 1, wherein Q of formula (I) or (II) is substituted phenyl represented by formula (IV).

4. The compound of claim 3, wherein R^{24} is a group that reacts with peroxynitrite.

5. The compound of claim 4, wherein R^{24} is OR^{27}, $CH_2CH_2COR^{28}$, or $NR^{29}R^{30}$, wherein R^{27} is hydrogen or a group selected from alkyl, alkoxyalkyl, alkanoyl, or polyether; R^{28} is an

electron-withdrawing group selected from CF_3, halogen-substituted lower alkyl, or (C=O)–O–W_1, wherein W_1 is a group selected from alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl or arylalkyl; R^{29} and R^{30} are independently hydrogen or a group selected from hydrogen or a group selected from alkyl, alkenyl, alkynyl, alkoxyalkyl, alkanoyl, alkenoyl, alkynoyl, cycloalkyl, cycloalkenyl, cycloalkynyl, aryl, alkaryl, arylalkyl, aryloyl, or polyether.

6. The compound of claim 1, wherein R^1 of formula (I) or (II) is CH_3.

7. The compound of claim 1, wherein R^8 is formula (III).

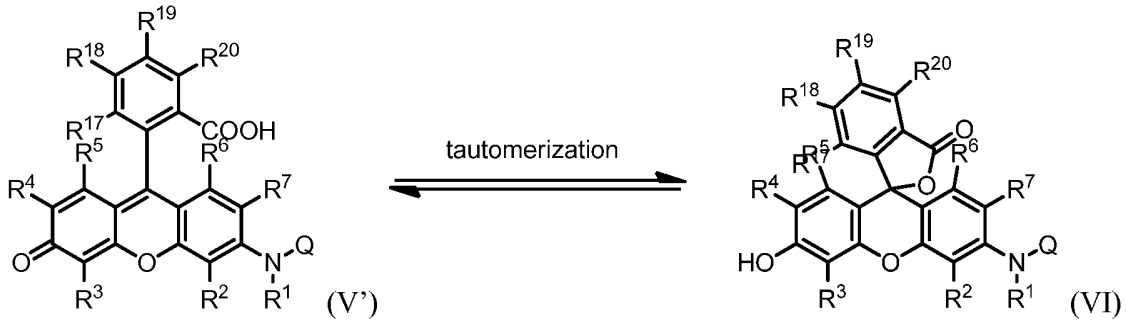
8. The compound of claim 1, wherein the compound has a structure of formula (II), or a tautomer thereof, and wherein B is O, Z is O, and R^8 is formula (III).

9. The compound of claim 8, wherein at least one of R^{17}, R^{18}, R^{19}, and R^{20} is a carboxyl group.

10. The compound of claim 8, wherein R^{21} is H, CH_3, OMe, or COOH.

11. The compound of claim 8, wherein the R^8 group of formula (III) comprises one or more carboxyl groups, wherein at least one carboxyl group is further conjugated with an iminodialkylcarboxylate having a structure of $(HN((CH_2)_nCOOH)_2$ wherein n = 1, 2, or 3.

12. The compound of claim 8, wherein the compound has a structure of (V') or its tautomer (VI)

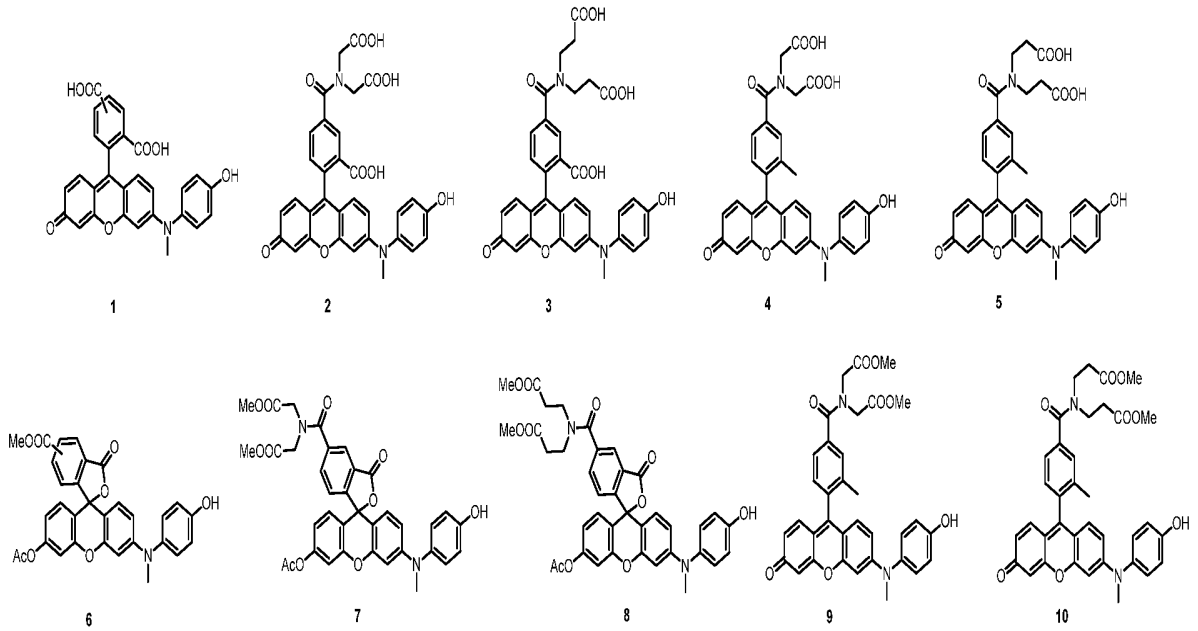


(V') (VI)

13. The compound of claim 8, wherein the COOH group is esterified with a methyl, ethyl, or acetoxymethyl (AM) group.

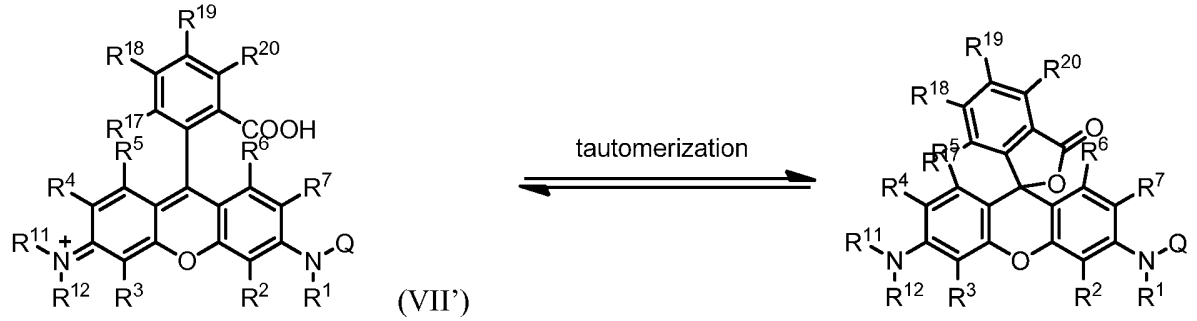
14. The compound of claim 8, wherein the phenolic group is acylated with acetyl, propionyl, or butyryl groups, or is protected with acetoxymethyl (AM) groups.

15. The compound of claim 8, wherein the compound has one of formulae 1-10:



16. The compound of claim 1, wherein the compound has a structure of formula (II), or a tautomer thereof, and wherein B is $N^{+}R^{11}R^{12}$, Z is O, and R^{8} is formula (III).

17. The compound of claim 16, wherein the compound has a structure of (VII') or its tautomer (VIII)



(VII')

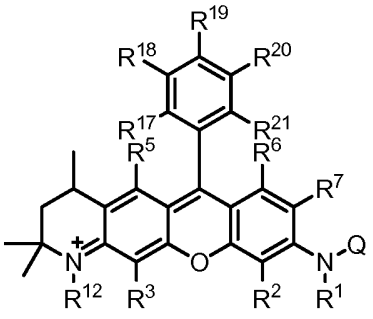
(VIII).

18. The compound of claim 17, wherein the COOH group is esterified with a methyl, ethyl, or acetoxymethyl (AM) group.

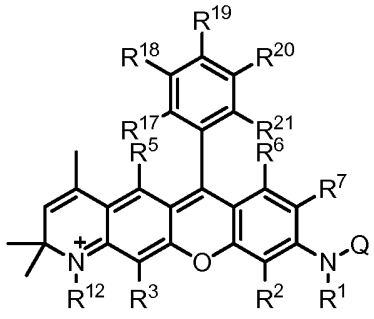
19. The compound of claim 17, wherein at least one of R^{17} – R^{20} is an alkylating group.

20. The compound of claim 19, wherein the alkylating group has a formula of $CR^{31}R^{32}X$, wherein R^{31} and R^{32} are independently H and CH_3, and X is Cl, Br, or I.

21. The compound of claim 16, wherein the compound has a structure of formula (IX) or (X):



(IX)



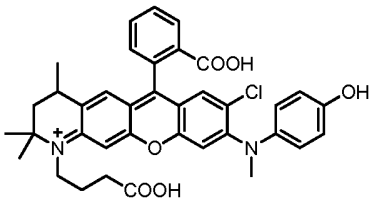
(X).

22. The compound of claim 21, wherein R^{12} in formula (IX) or (X) is a $C_{1\text{-}10}$ alkyl or alkene.

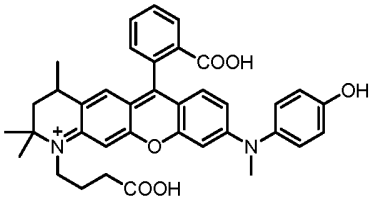
23. The compound of claim 21, wherein R^{12} in formula (IX) or (X) is a $C_{1\text{-}10}$ alkyl or alkene substituted with a carboxyl group at the terminal position.

24. The compound of claim 21, wherein R^{12} is ethyl, carboxylmethyl, carboxylethyl, or carboxylpropyl.

25. The compound of claim 16, wherein the compound has one of formulae 11-21:



11



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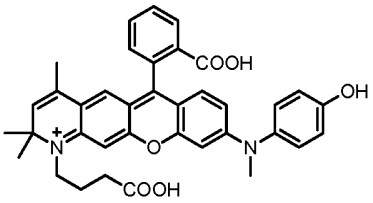

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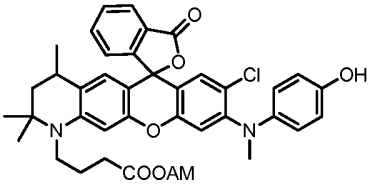

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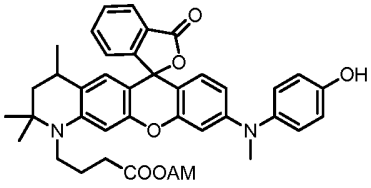

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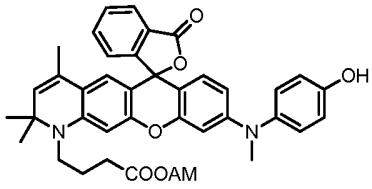
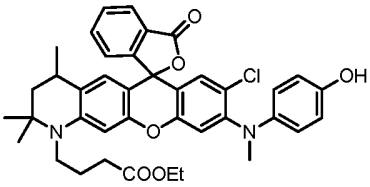
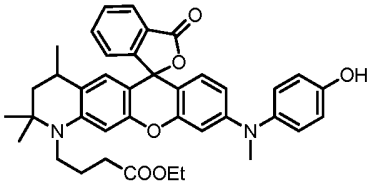


16 AM = CH_2OCOCH_3



17



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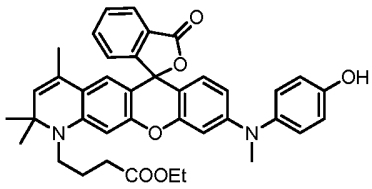

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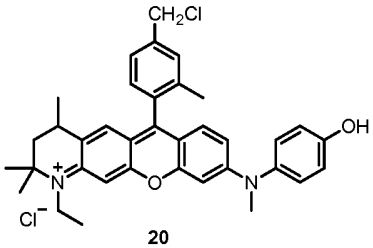

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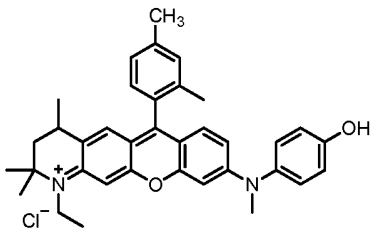
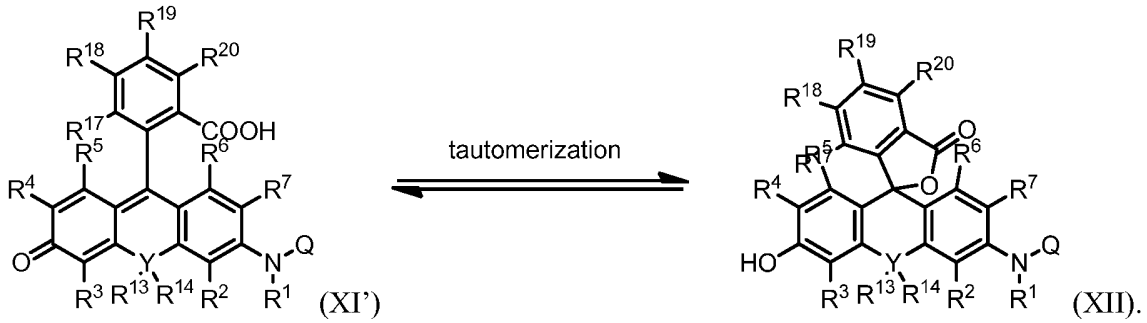


21

26. The compound of claim 1, wherein the compound has a structure of formula (II), and wherein B is O, Z is $YR^{13}R^{14}$ wherein Y is Si, Ge, or Sn, and R^8 is formula (III).

27. The compound of claim 26, wherein R^{13} and R^{14} are independently CH_3, or phenyl.

28. The compound of claim 26, wherein R^{21} is COOH, and the compound has a structure of (XI') or its tautomer (XII)

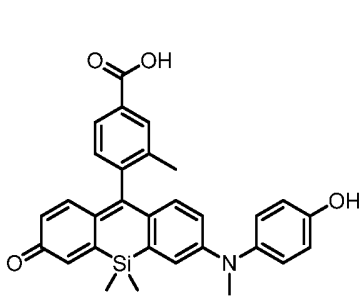


(XI') (XII).

29. The compound of claim 28, wherein the COOH group is esterified with a methyl, ethyl, or acetoxymethyl (AM) group.

30. The compound of claim 28, wherein the phenolic group is acylated with acetyl, propionyl, or butyryl groups, or is protected with acetoxymethyl (AM) groups.

31. The compound of claim 26, wherein the compound has one of formulae 22-24



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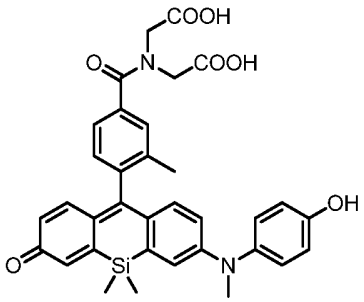
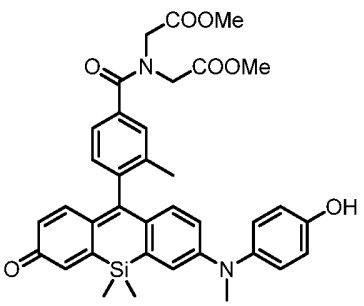


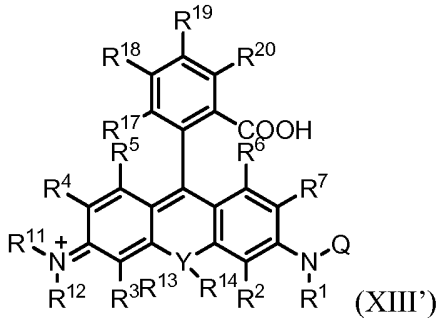
23



24

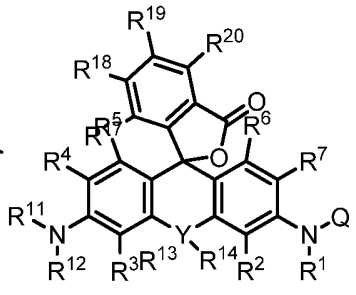
32. The compound of claim 1, wherein the compound has a structure of formula (II), and wherein B is $N^{+}R^{11}R^{12}$, Z is $YR^{13}R^{14}$ wherein Y is Si, Ge, or Sn, and R^{8} is formula (III).

33. The compound of claim 32, wherein R^{21} is COOH, and the compound has a structure of (XIII') or its tautomer (XIV)



(XIII')

tautomerization



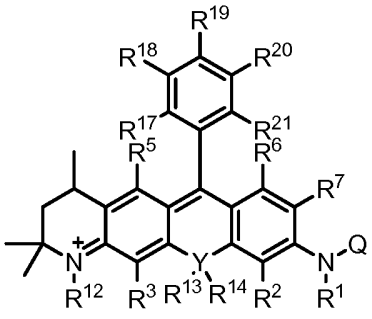
(XIV).

34. The compound of claim 32, wherein R^{11} in combination with R^{4}, or R^{12} in combination with R^{3}, or both, form a 5- or 6-membered ring that is saturated or unsaturated, or can further be fused with an aryl or heteroaryl ring, and can optionally be substituted by one or more alkyls, carboxylic acids, sulfonic acids ($-SO_3H$), or their salts, ester or amide derivatives.

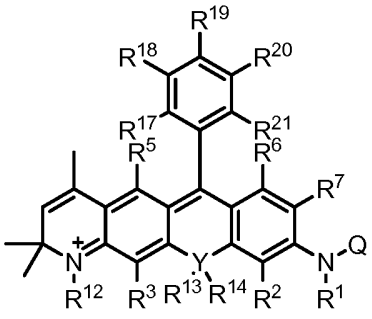
35. The compound of claim 32, wherein the COOH group is esterified with a methyl, ethyl, or acetoxymethyl (AM) group.

36. The compound of claim 32, wherein the phenolic group is acylated with acetyl, propionyl, or butyryl groups, or is protected with acetoxymethyl (AM) groups.

37. The compound of claim 32, wherein the compound has a formula of (XV) or (XVI)

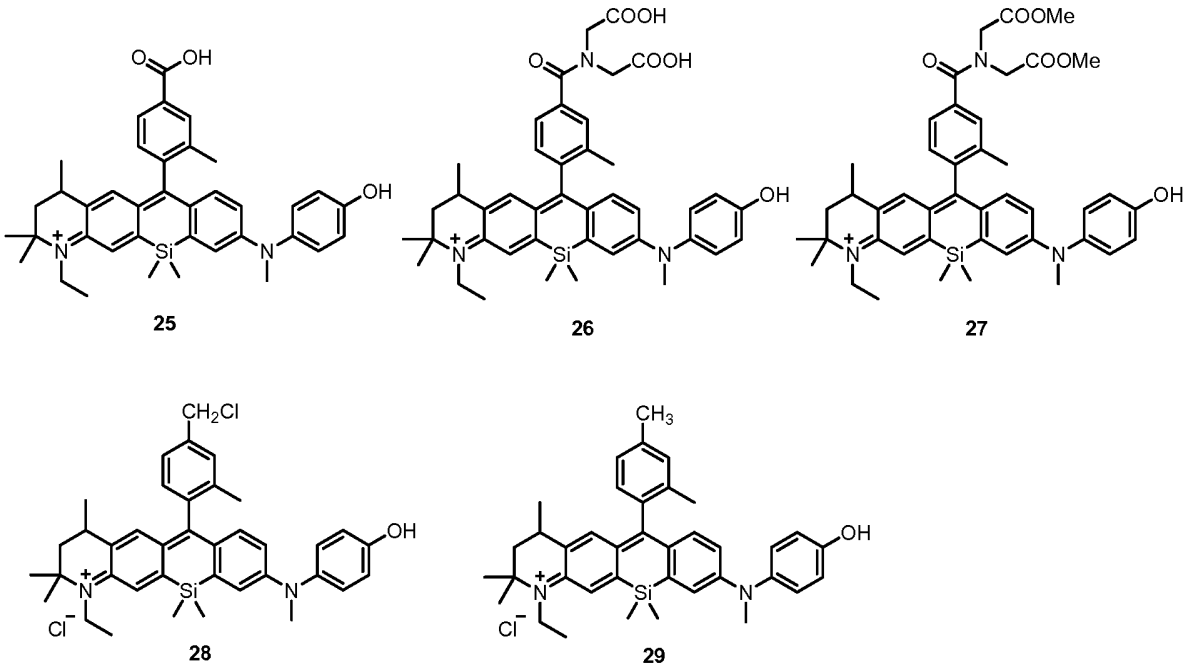


(XV)



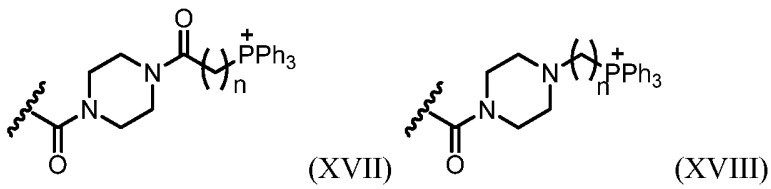
(XVI).

38. The compound of claim 32, wherein the compound has one of the formulae 25-29:



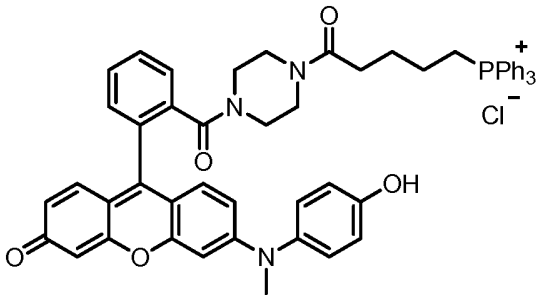
39. The compound of claim 1, wherein the compound comprises one or more free carboxyl groups, wherein at least one of the carboxyl group is conjugated with a positively charged triphenylphosphonium moiety through an amide bond linkage.

40. The compound of claim 39, wherein the linkage between the compound and the triphenylphosphonium moiety has the following formula (XVII) or (XVIII):



wherein n = 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

41. The compound of claim 39, wherein the compound has one of the formulae 30-33:



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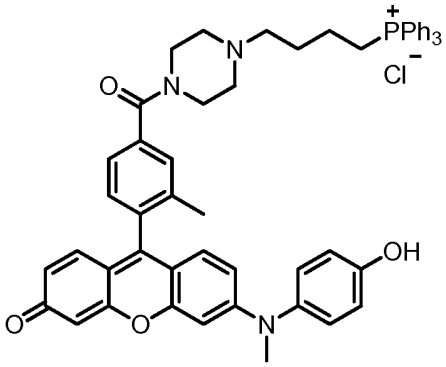
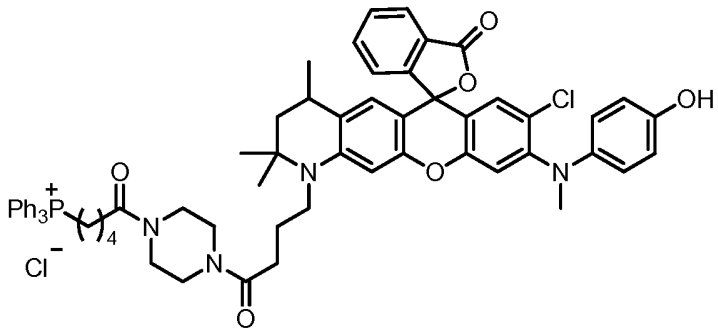
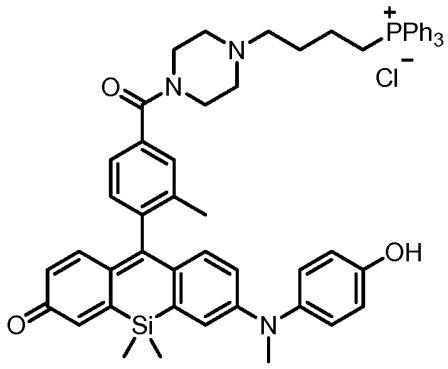


31



32



33

42. A fluorogenic probe composition comprising the compound of claim 1, and, optionally, a carrier.

43. The fluorogenic probe composition of claim 42, wherein the fluorogenic probe composition further comprises a solvent, an acid, a base, a buffer solution, or a combination thereof.

44. A method for detecting the presence of, and/or or determining the level of peroxynitrite in a sample, comprising:

a) contacting a compound of claim 1 with the sample to form a fluorescent compound; and

b) determining fluorescence property of the fluorescent compound.

45. The method of claim 44, wherein the sample is a chemical sample or biological sample.

46. The method of claim 45, wherein the sample is a biological sample comprising a microorganism, or a cell or tissue.

47. A method for detecting the presence of, or determining the level of peroxynitrite *in vivo* in an organism, comprising:

a) administering a compound of claim 1 to the organism to form a fluorescent compound; and

b) determining fluorescence property of the fluorescent compound.

48. A high-throughput screening method for detecting the presence of, or determining the level of, peroxynitrite in samples, wherein the high-throughput method comprises the steps of:

a) contacting a compound of claim 1 with the samples to form one or more fluorescent compounds; and

b) determining fluorescence properties of the fluorescent compounds to determine the presence and/or amount of peroxynitrite in the samples.

49. A high-throughput method for screening one or more target compounds that increase or decrease the level of peroxynitrite, wherein the high-throughput method comprises the steps of:

a) contacting a compound of claim 1 with target compounds to form one or more fluorescent compounds; and

b) measuring fluorescence properties of the florescent compounds to determine the presence and/or amount of the target compounds.

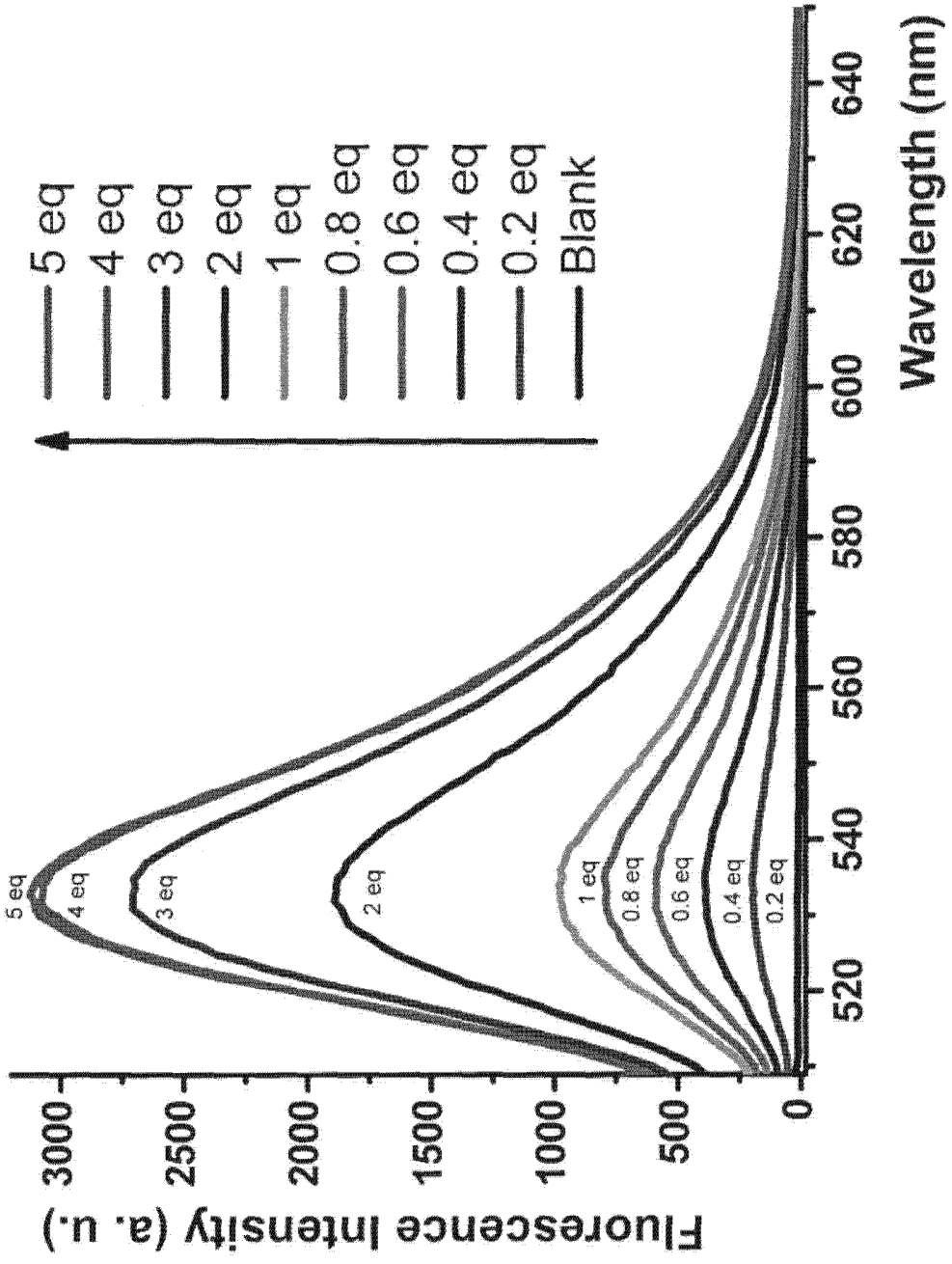

Fluorescence Intensity (a. u.)
3000
2500
2000
1500
1000
500
0
520
540
560
580
600
620
640
Wavelength (nm)
5 eq
4 eq
3 eq
2 eq
1 eq
0.8 eq
0.6 eq
0.4 eq
0.2 eq
Blank

FIG. 1A

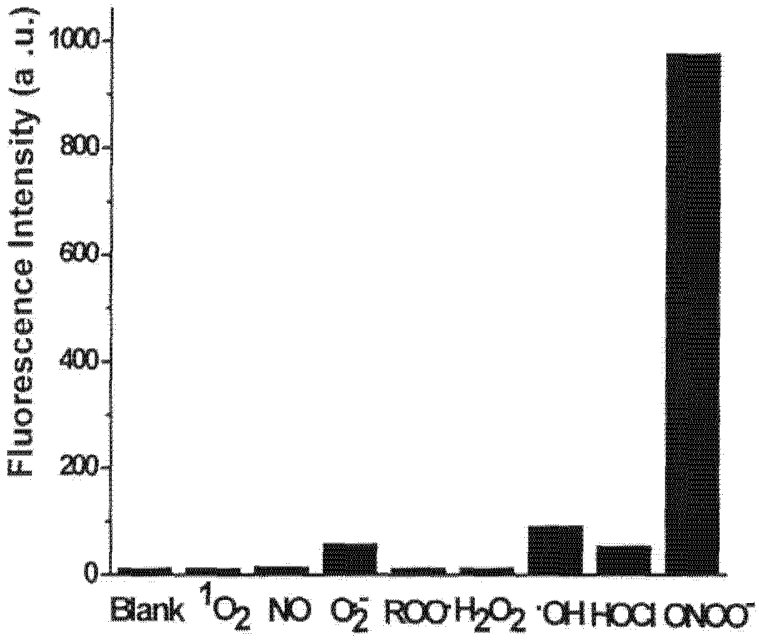

Fluorescence Intensity (a .u.)
1000
800
600
400
200
0
Blank
$^1O_2$
NO
O_2^-
ROO·
H_2O_2
·OH
HOCl
ONOO⁻

FIG. 1B

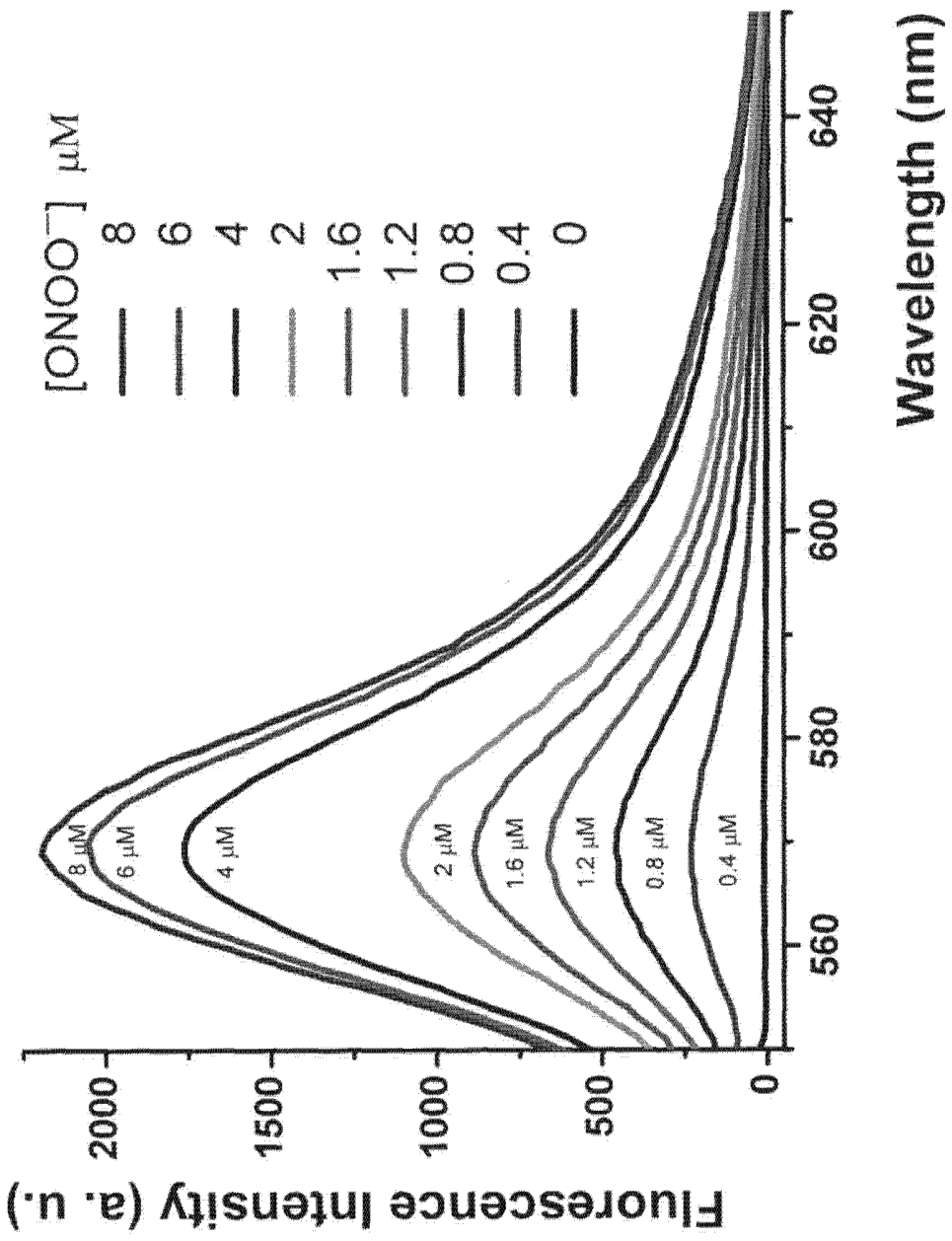

Fluorescence Intensity (a. u.)
2000
1500
1000
500
0
560
580
600
620
640
Wavelength (nm)
[ONOO⁻] μM
8
6
4
2
1.6
1.2
0.8
0.4
0
8 μM
6 μM
4 μM
2 μM
1.6 μM
1.2 μM
0.8 μM
0.4 μM

FIG. 2A

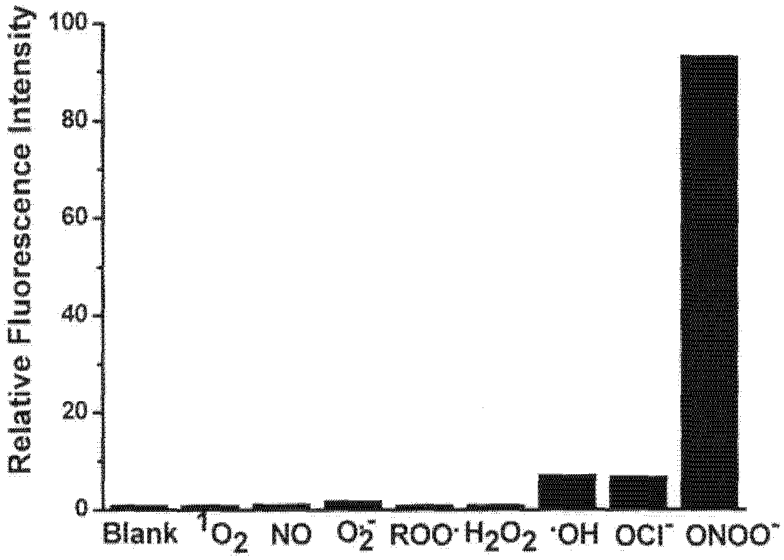

Relative Fluorescence Intensity
100
80
60
40
20
0
Blank
$^1O_2$
NO
O_2^-
ROO·
H_2O_2
·OH
OCl⁻
ONOO⁻

FIG. 2B

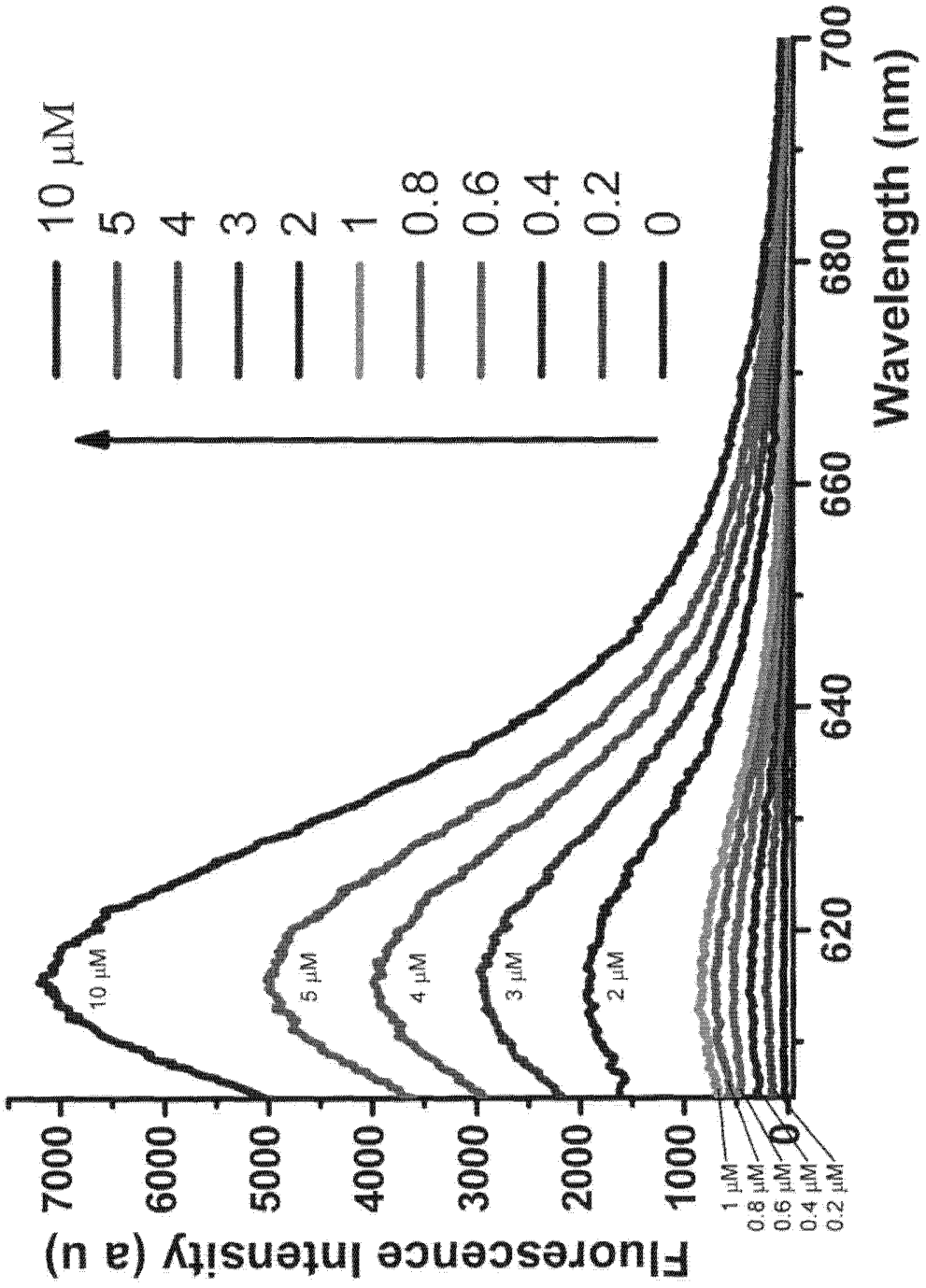

Fluorescence Intensity (a u)
7000
6000
5000
4000
3000
2000
1000
620
640
660
680
700
Wavelength (nm)
10 μM
5
4
3
2
1
0.8
0.6
0.4
0.2
0
10 μM
5 μM
4 μM
3 μM
2 μM
1 μM
0.8 μM
0.6 μM
0.4 μM
0.2 μM
0

FIG. 3A

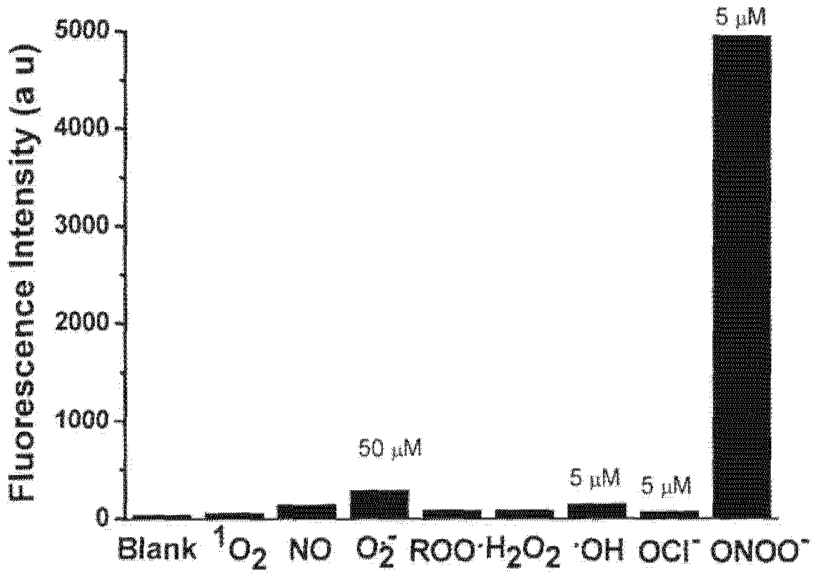

Fluorescence Intensity (a u)
5000
4000
3000
2000
1000
0
5 μM
50 μM
5 μM
5 μM
Blank
$^1O_2$
NO
O_2^-
ROO·
H_2O_2
·OH
OCl^-
$ONOO^-$

FIG. 3B

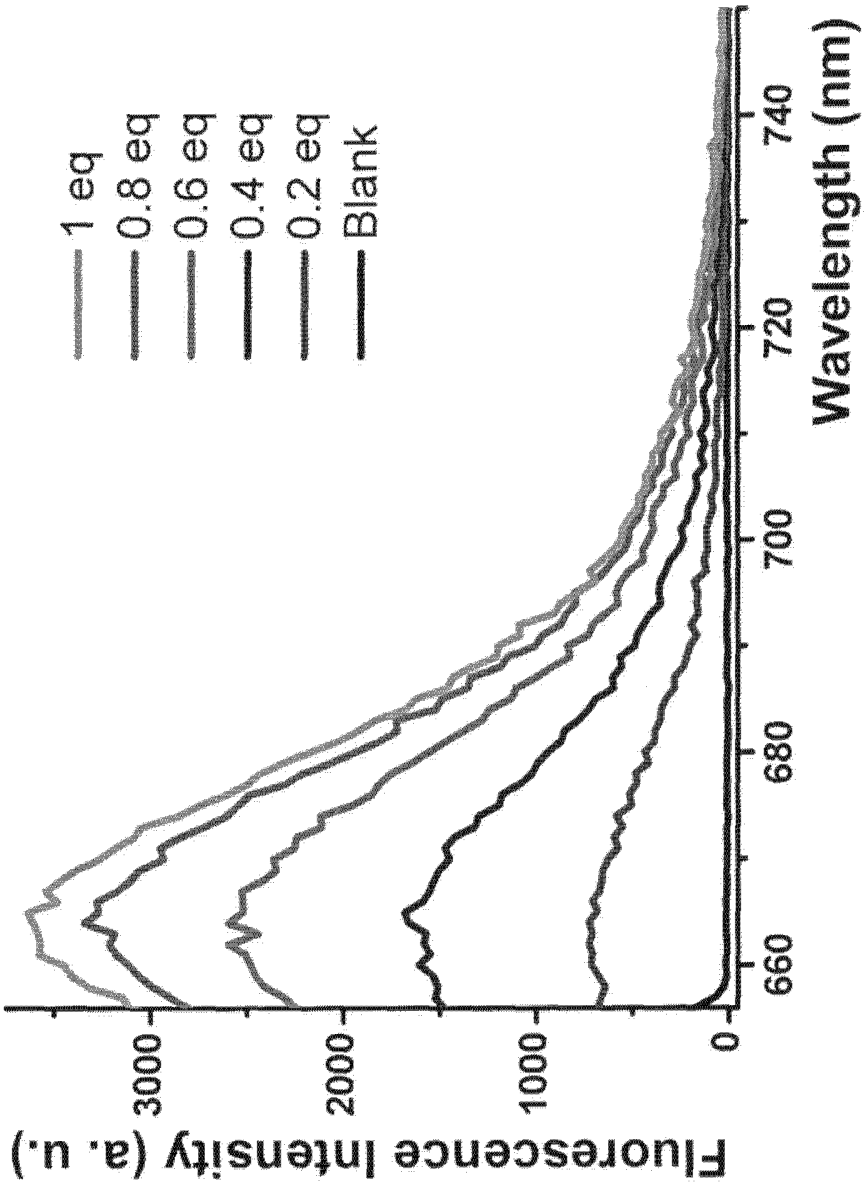

1 eq
0.8 eq
0.6 eq
0.4 eq
0.2 eq
Blank
Fluorescence Intensity (a. u.)
0
1000
2000
3000
660
680
700
720
740
Wavelength (nm)

FIG. 4A

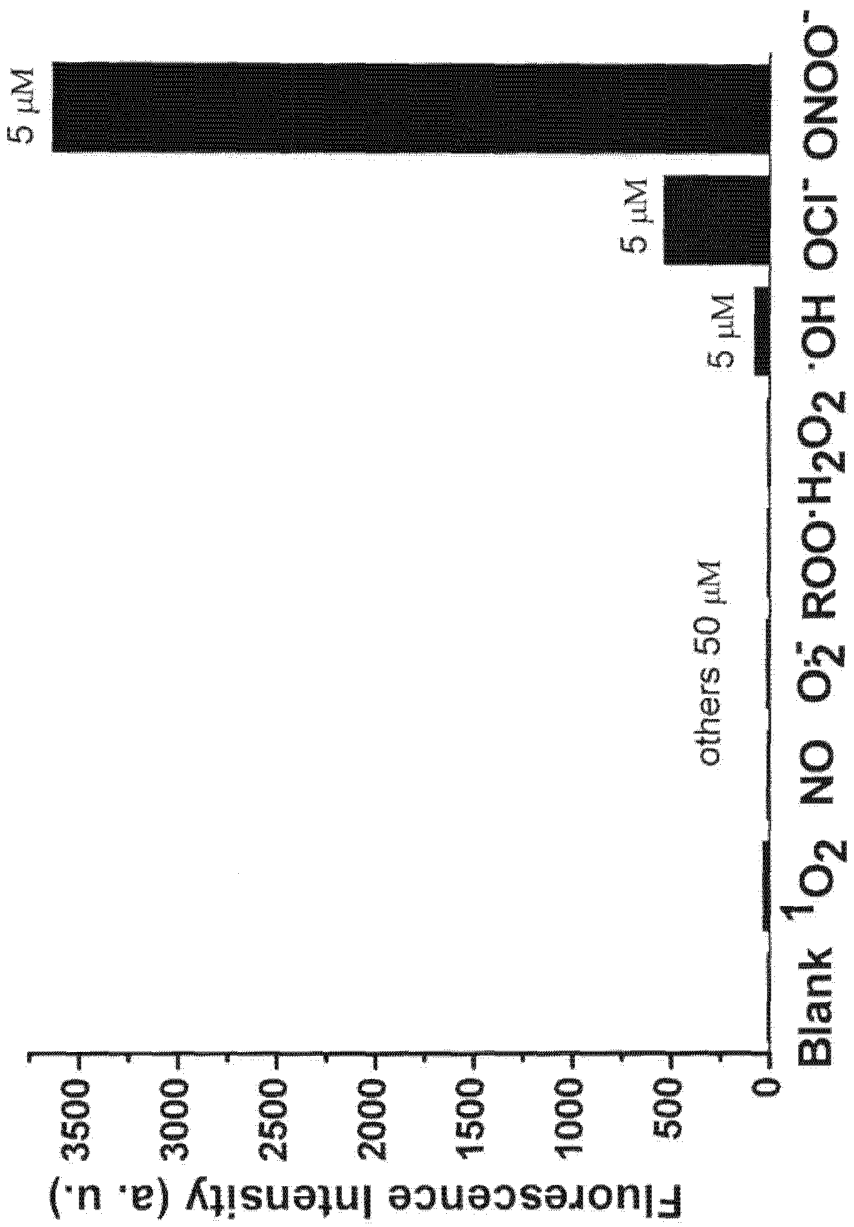

Fluorescence Intensity (a. u.)
3500
3000
2500
2000
1500
1000
500
0
Blank
$^1O_2$
NO
O_2^-
ROO·
H_2O_2
·OH
OCl^-
$ONOO^-$
others 50 μM
5 μM
5 μM
5 μM

FIG. 4B

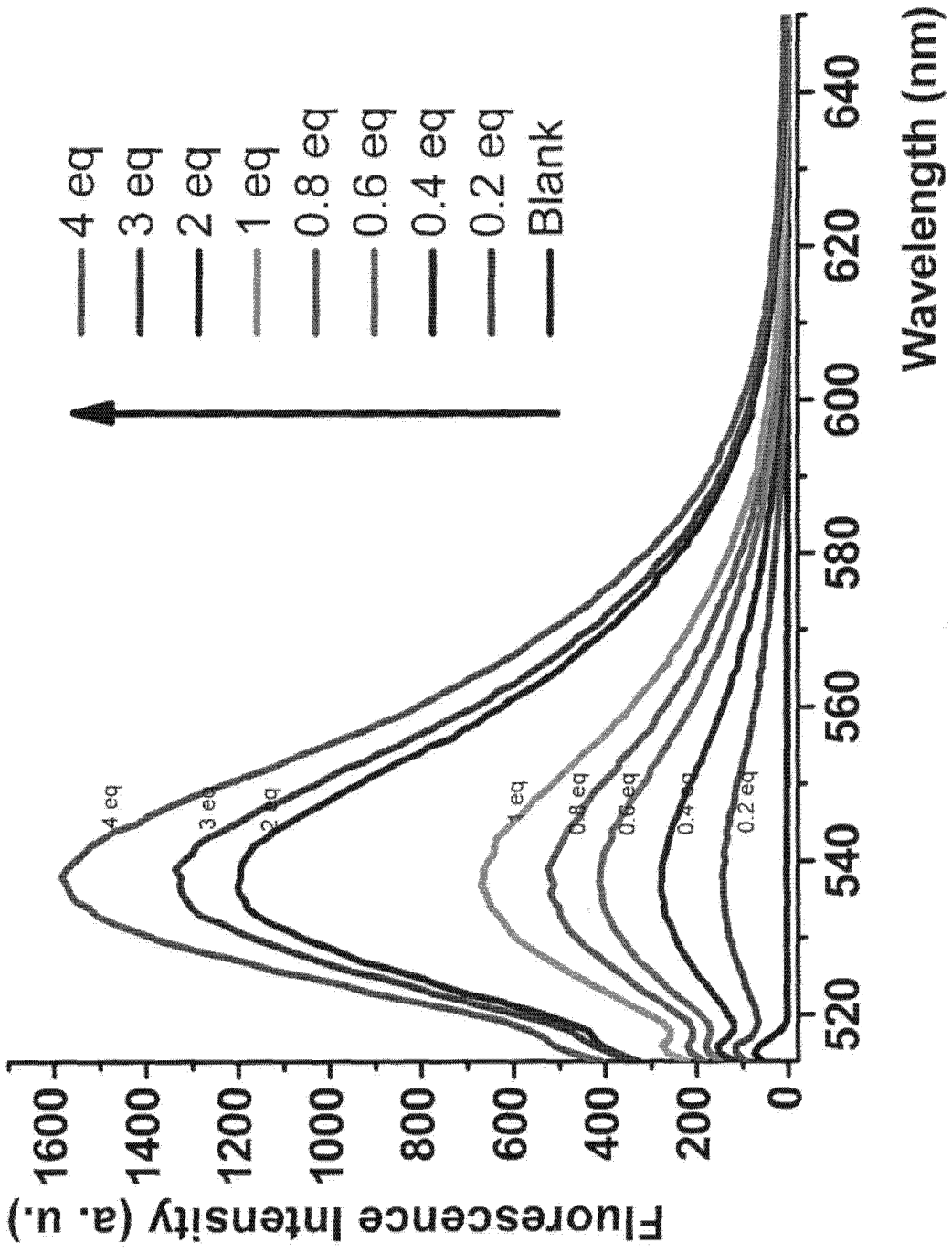

Fluorescence Intensity (a. u.)
Wavelength (nm)
4 eq
3 eq
2 eq
1 eq
0.8 eq
0.6 eq
0.4 eq
0.2 eq
Blank

FIG. 5A

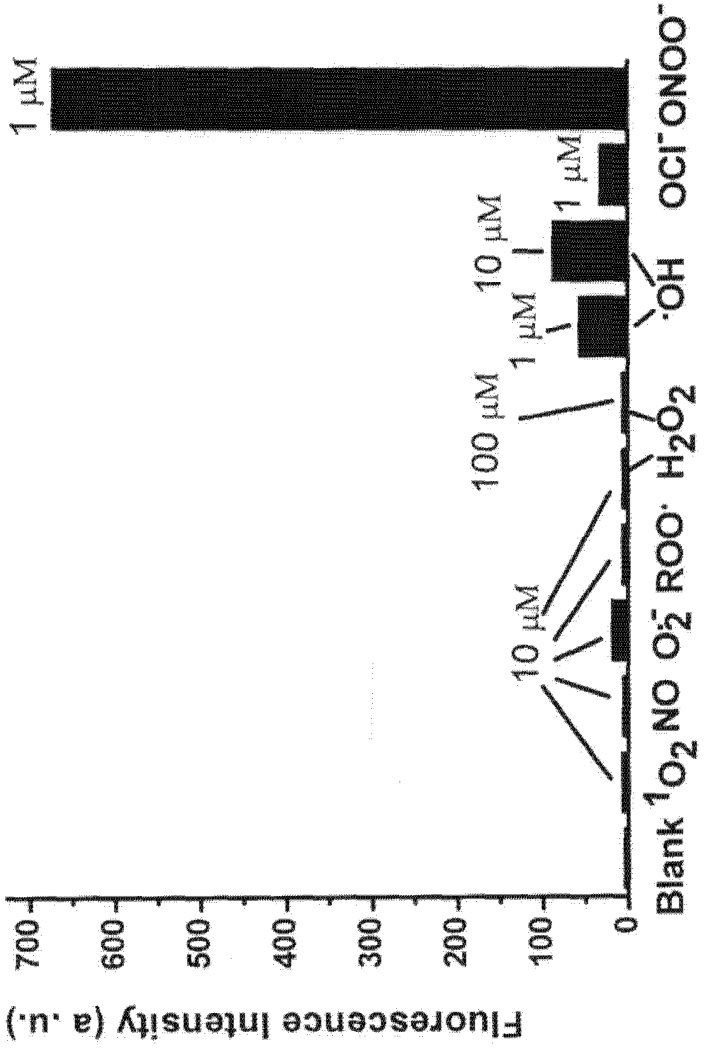

Fluorescence Intensity (a .u.)
0
100
200
300
400
500
600
700
Blank
$^1O_2$
NO
O_2^-
ROO·
H_2O_2
·OH
OCl^-
$ONOO^-$
10 μM
100 μM
1 μM
10 μM
1 μM
1 μM

FIG. 5B

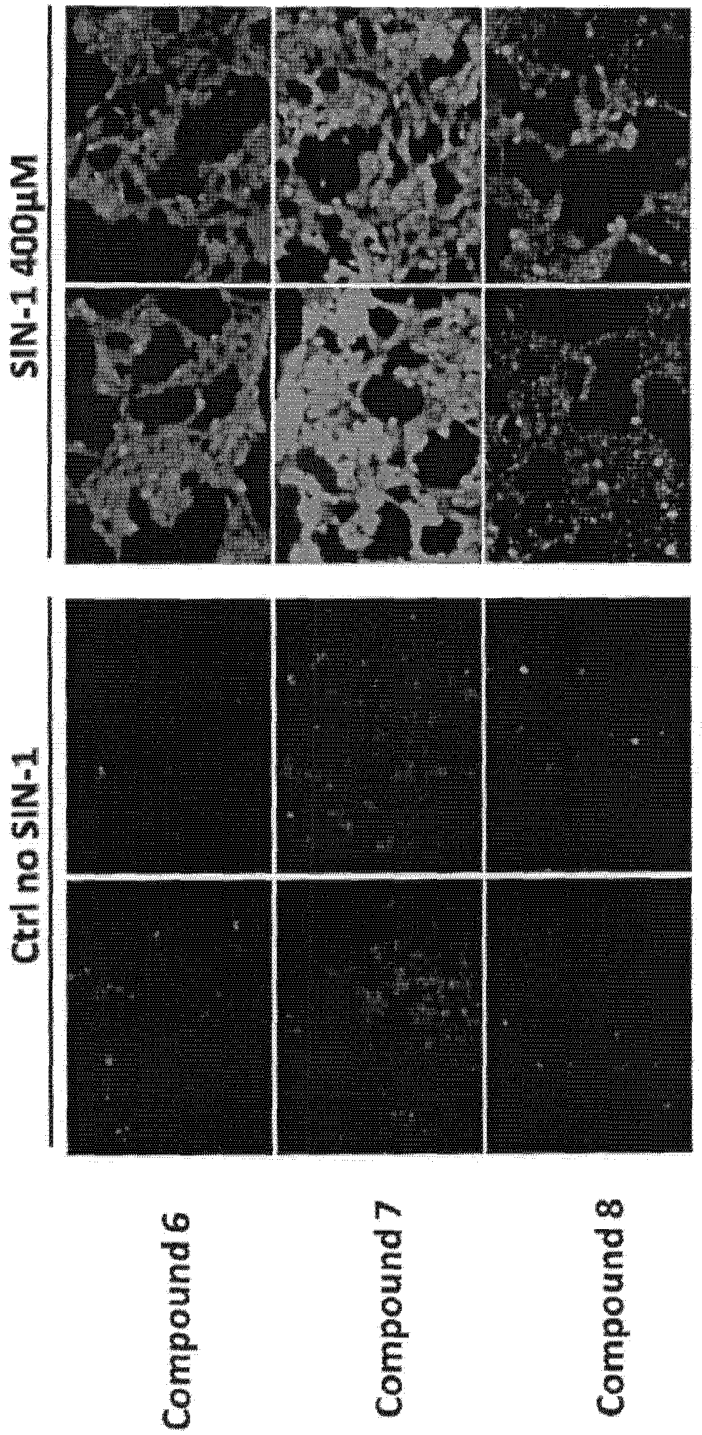


FIG. 6

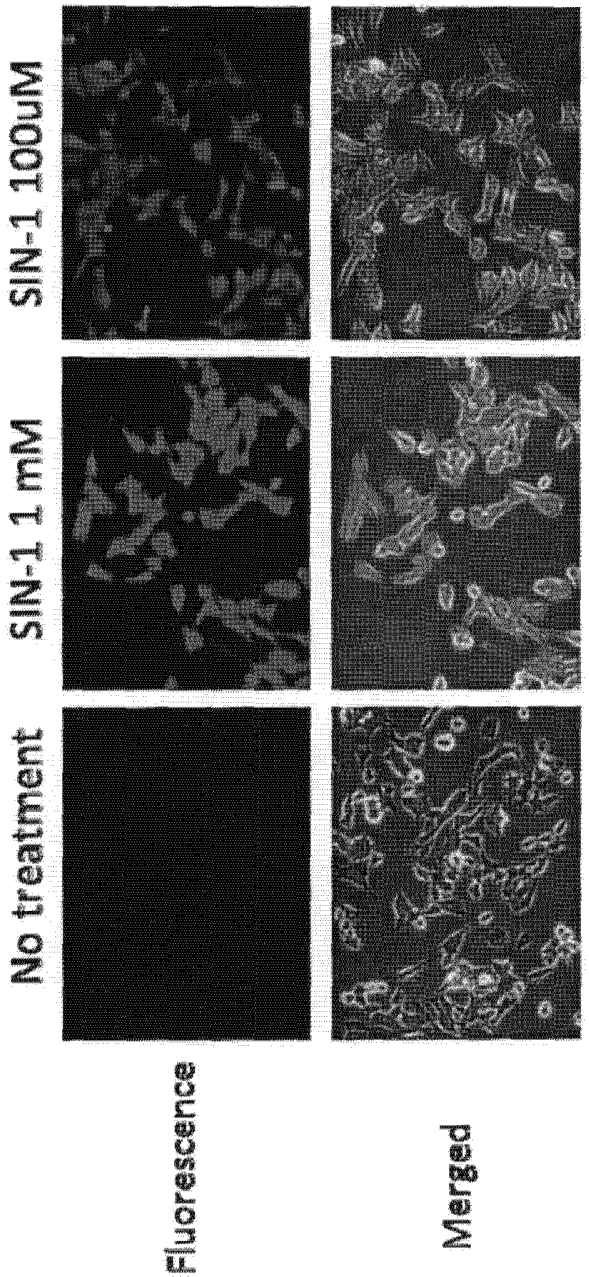


FIG. 7

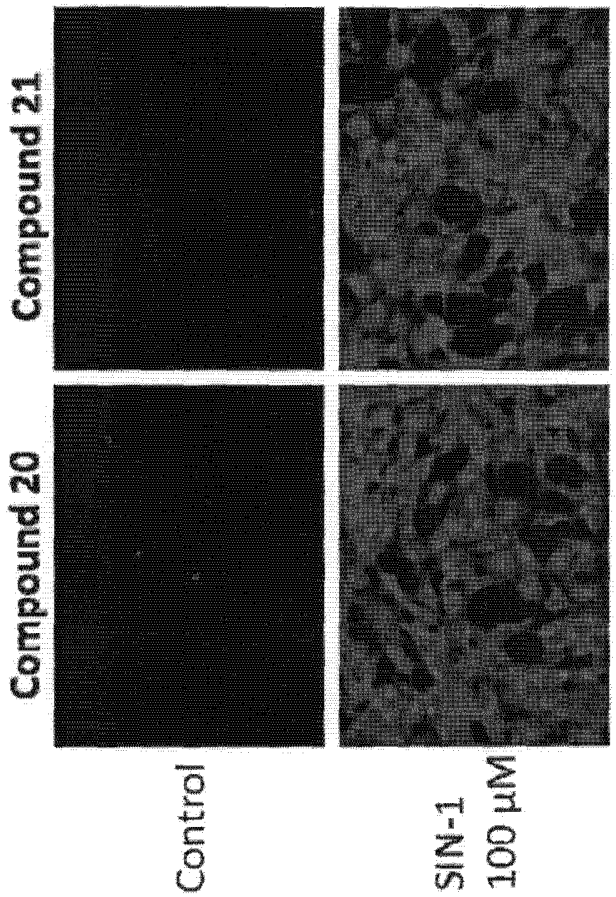


FIG. 8

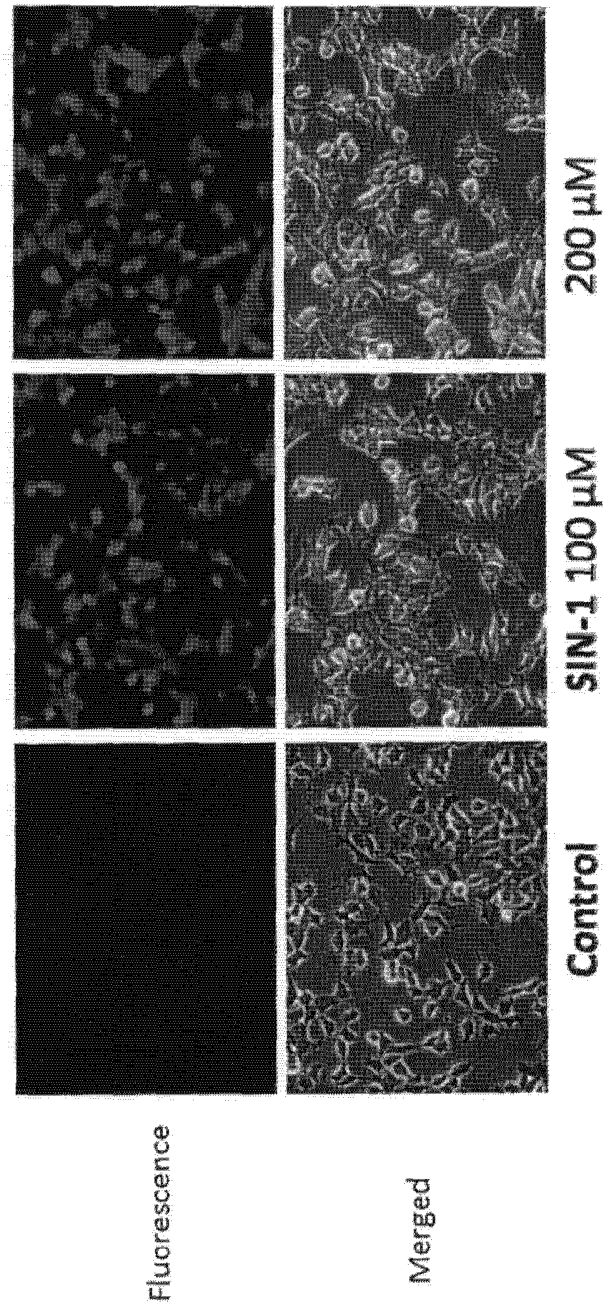


FIG. 9

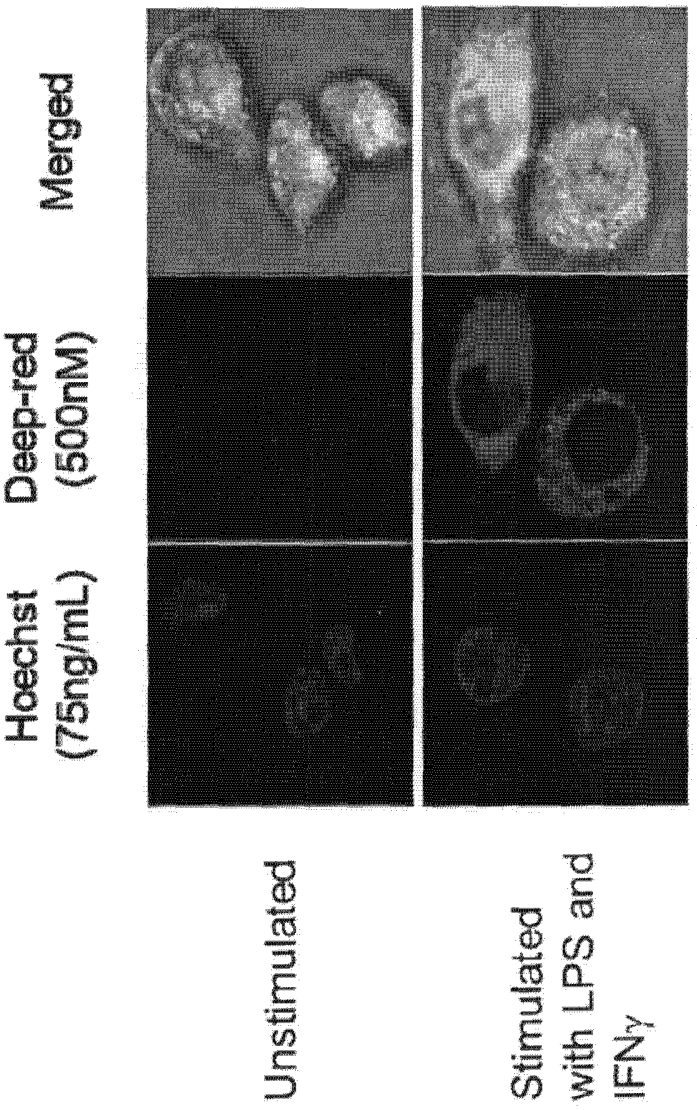

Hoechst (75ng/mL)
Deep-red (500nM)
Merged
Unstimulated
Stimulated with LPS and IFNγ

FIG. 10

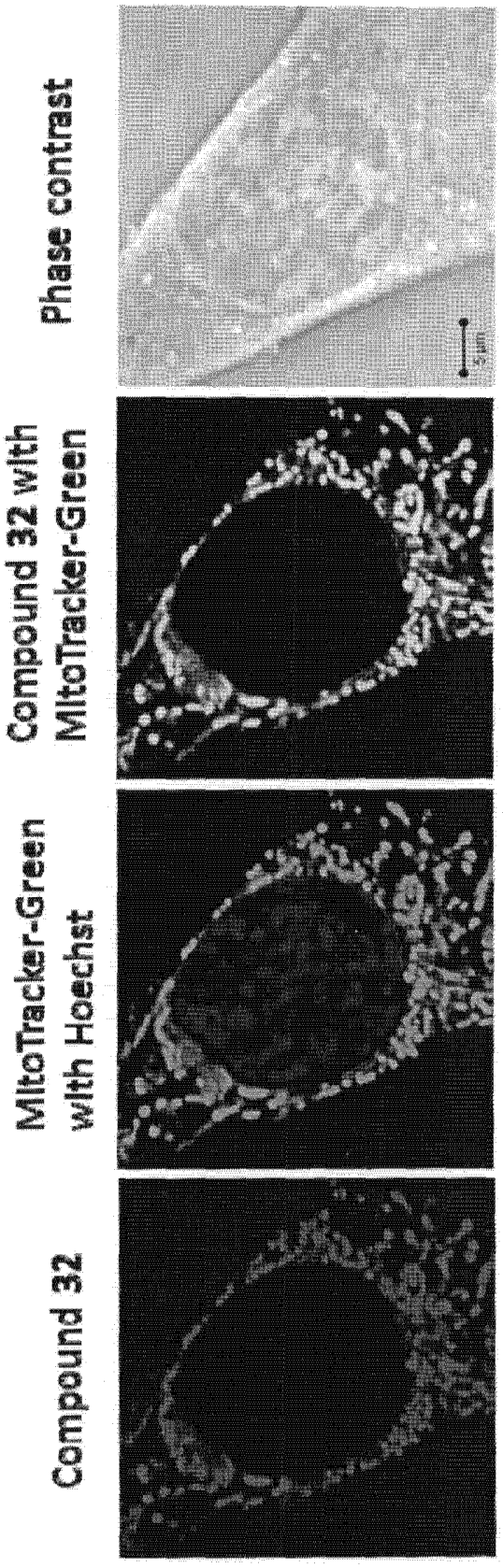

Phase contrast
Compound 32 with MitoTracker-Green
MitoTracker-Green with Hoechst
Compound 32

FIG. 11

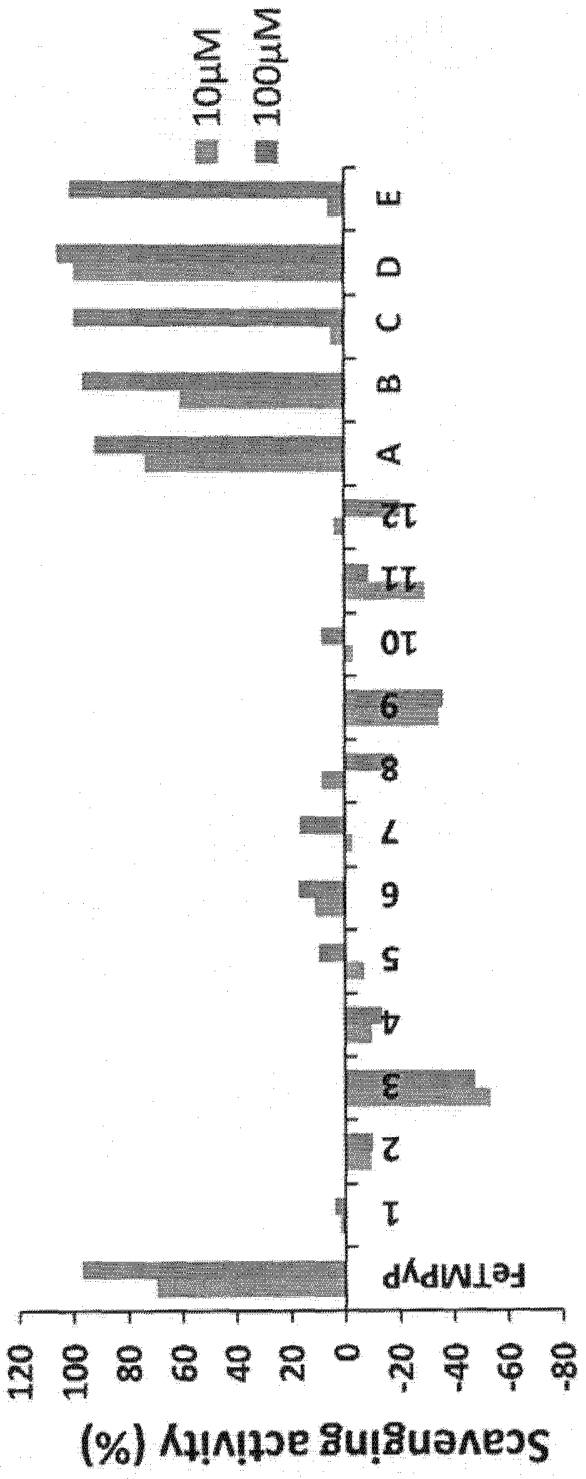

10μM
100μM
Scavenging activity (%)
120
100
80
60
40
20
0
-20
-40
-60
-80
FeTMPyP
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E

FIG. 12

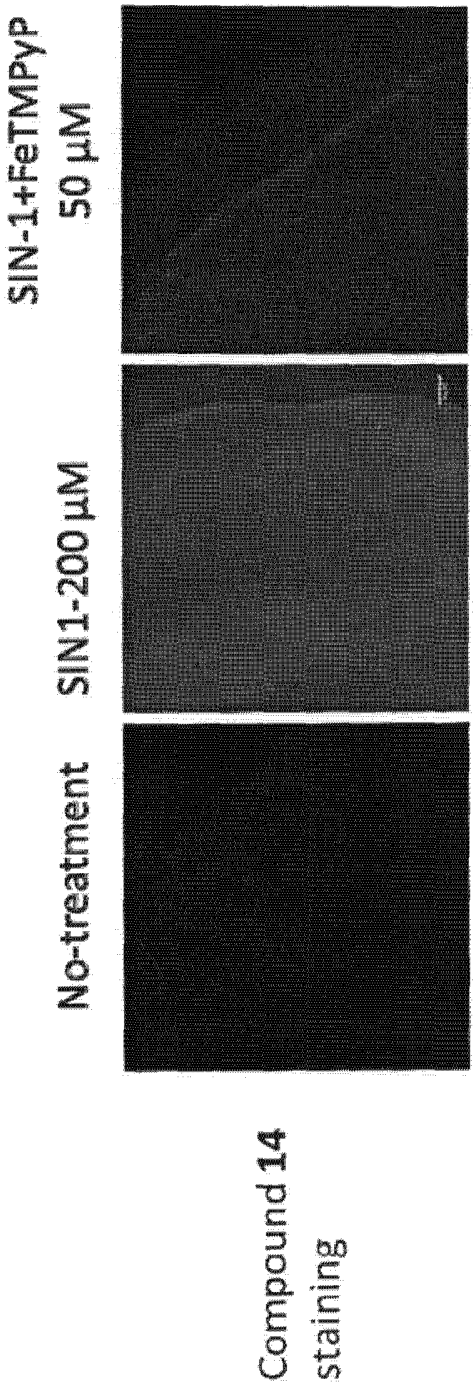

SIN-1+FeTMPyP
50 μM
SIN1-200 μM
No-treatment
Compound 14
staining

FIG. 13

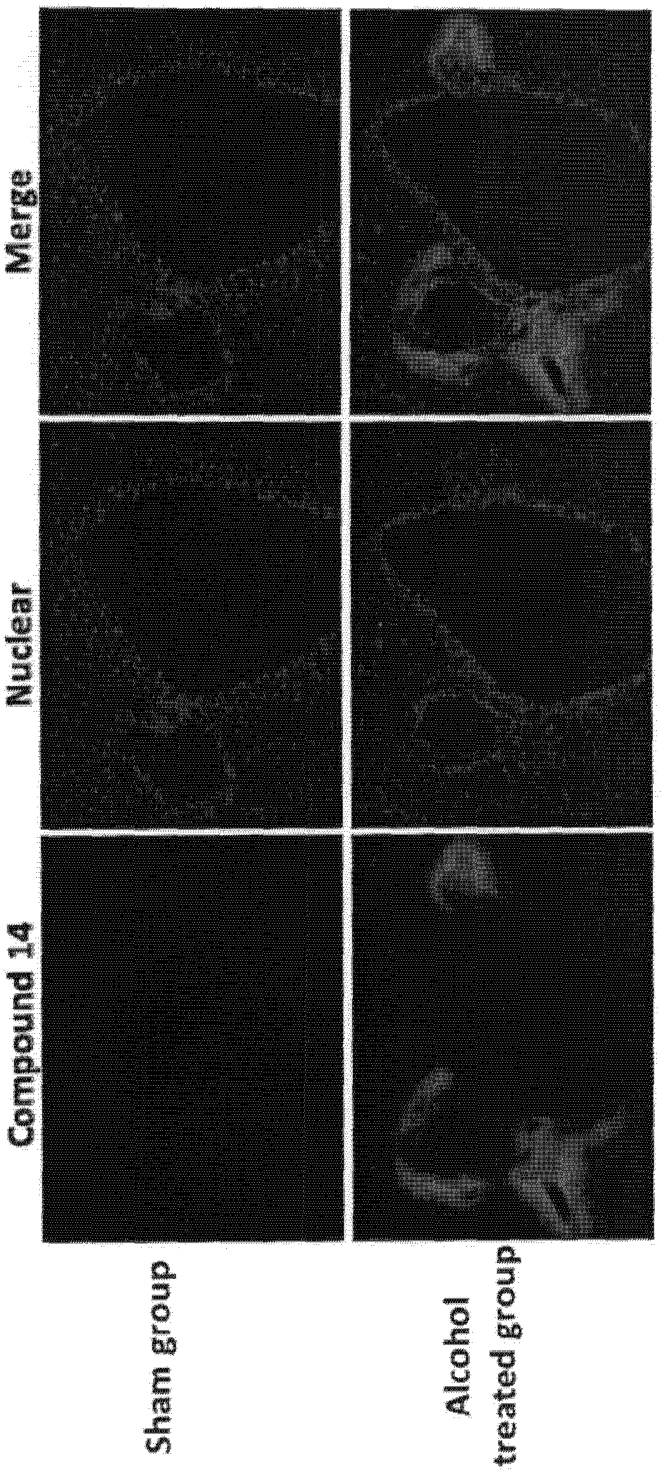

Compound 14
Nuclear
Merge
Sham group
Alcohol treated group

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2013/071155

A. CLASSIFICATION OF SUBJECT MATTER

See the Extra Sheet

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: C07D407/-; C09K3/- ; B41M5/-

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 practicable, search terms used)

WPI;EPODOC;CNKI;IEE;CNPAT;STN (registry, caplus); fluor+, probe, diarylamine, peroxynitrite, spiro, isobenzofuran, xanthen,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US20100099556A1 (ZINK IMAGING INC.) 22 Apr. 2010 (22.04.2010) see formula I in description and paragraphs 0091-0099 in description	1-2, 8, 12
X	PENG Tao et al., HKGreen-3: A Rhodol-Based Fluorescent Probe for Peroxynitrite, Organic Letters, Volume 12, No. 21, pages 4932-4935, see compounds 1, HKGreen-3 and HKGreen-3A, schemes 2-3 in pages 4933-34	1-8, 10, 12-13
X	PENG Tao et al., Construction of a Library of Rhodol Fluorophores for Developing New Fluorescent Probes, Organic Letters, Volume 12, No. 3, pages 496-499, see page 497 compounds 3l, 3m, 3r, 4l, 4m and 4r	1-2, 6-8, 10, 12-13

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 Apr. 2013 (12.04.2013)	**16 May 2013 (16.05.2013)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer WANG, Bo Telephone No. (86-10)62086314

Form PCT/ISA /210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2013/071155

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN101983202A (MORNINGSIDE VENTURES LTD et al.) 02 Mar. 2011 (02.03.2011) see compounds1a-1d, 10, 12, 12a, 14, 22 in claim 30, compounds 18-20, 25, 26, 29 in examples 4-6 of the description	1-8, 10, 12-13, 42-43
A	CN101983202A (MORNINGSIDE VENTURES LTD et al.) 02 Mar. 2011 (02.03.2011) see the whole document	9, 11, 14-41
X	US2009082495A1 (GOODYEAR TIRE&RUBBER CO et al.) 26 Mar. 2009 (26.03.2009) see the last compound in claim 3	1-2, 8, 12
X	JP 2009047814A (KONICA MINOLTA BUSINESS TECHNOLOGIES KK et al.) 05 Mar. 2009 (05.03.2009) see compound (1-a) in paragraph 20 of the description	1-2, 8, 12
X	CN101228147A (ZINK IMAGING INC.) 23 Jul. 2008 (23.07.2008) see formula I and compounds II-VI in table I of the description	1-2, 8, 12
X	JP7076587A (NIPPON SODA CO) 20 Mar. 1995 (20.03.1995) see compounds in table I of the description	1-3, 8, 12
X	US5421870A (CIBA GEIGY AG) 06 Jun. 1995 (06.06.1995) see the compound in example 15 of the description	1-2, 8, 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2013/071155

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 44-49
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 44-49 are directed to a diagnostic method practiced on the human/animal body.Thus, the subject-matter of claims 44-49 is not required to be searched by this Authority (Rule 39.1(iv) PCT).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See the Extra Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

claims 1-43, relating to the compounds of formula (I) and formula (II), wherein Z is selected from O, S, $SiR^{13}R^{14}$, $GeR^{13}R^{14}$, $SnR^{13}R^{14}$, the preparation methods, compositions and uses thereof, that is, the parts relating to claims Nos. 1-7(part), 8-41, 42-43(part).

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2013/071155

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
US2010099556A1	22.04.2010	US8372782B2	12.02.2013
		EP2485900A1	15.08.2012
		WO2011044049A1	14.04.2011
CN101983202A	02.03.2011	EP2250177A1	17.11.2010
		US2009253118A1	08.10.2009
		JP2011516432A	26.05.2011
		WO2009121247A1	08.10.2009
		US8114904B2	14.02.2012
		HK1149546A0	07.10.2011
US2009082495A1	26.03.2009	DE102008039101A1	09.04.2009
		BR200803566A2	19.05.2009
JP 2009047814A	05.03.2009	None	
CN101228147A	23.07.2008	EP1879876A2	23.01.2008
		WO2006124602A3	06.12.2007
		WO2006124602A2	23.11.2006
		JP2008540774T	20.11.2008
		US2006293523A1	28.12.2006
JP7076587A	20.03.1995	JP3374382B2	04.02.2003
US5421870A	06.06.1995	DE59307983D1	19.02.1998
		FI934242A	31.03.1994
		ES2112409T3	01.04.1998
		EP0591106A1	06.04.1994
		JP6200182A	19.07.1994
		EP0591106B1	14.01.1998
		BR9303948A	05.04.1994

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2013/071155

Continue:

A. CLASSIFICATION OF SUBJECT MATTER

C07D407/02（2006.01）i
C09K3/00（2006.01）i
B41M5/327（2006.01）i
B41M5/20（2006.01）i

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet):

This International Searching Authority found 8 inventions in this international application, as follows:

The first invention: claims 1-43, relating to the compounds of formula (I), wherein Z is selected from O and S, the preparation methods, pharmaceutical compositions and uses thereof;

The second invention: claims 1-43, relating to the compounds of formula (I), wherein Z is NR^{13}, the preparation methods, pharmaceutical compositions and uses thereof;

The third invention: claims 1-43, relating to the compounds of formula (I), wherein Z is $CR^{13}R^{14}$, the preparation methods, pharmaceutical compositions and uses thereof;

The forth invention: claims 1-43, relating to the compounds of formula (I), wherein Z is $SiR^{13}R^{14}$, $GeR^{13}R^{14}$, $SnR^{13}R^{14}$, the preparation methods, pharmaceutical compositions and uses thereof;

The fifth invention: claims 1-43, relating to the compounds of formula (II), wherein Z is selected from O and S, the preparation methods, pharmaceutical compositions and uses thereof;

The sixth invention: claims 1-43, relating to the compounds of formula (II), wherein Z is NR^{13}, the preparation methods, pharmaceutical compositions and uses thereof;

The seventh invention: claims 1-43, relating to the compounds of formula (II), wherein Z is $CR^{13}R^{14}$, the preparation methods, pharmaceutical compositions and uses thereof;

The eighth invention: claims 1-43, relating to the compounds of formula (II), wherein Z is selected from $SiR^{13}R^{14}$, $GeR^{13}R^{14}$ and $SnR^{13}R^{14}$, the preparation methods, pharmaceutical compositions and uses thereof.

The common technical feature of the eight inventions cannot be considered as a special technical feature within the meaning of PCT Rule 13.2. These inventions or groups of inventions are not so linked as to form a single inventive concept under PCT Rule 13.1.