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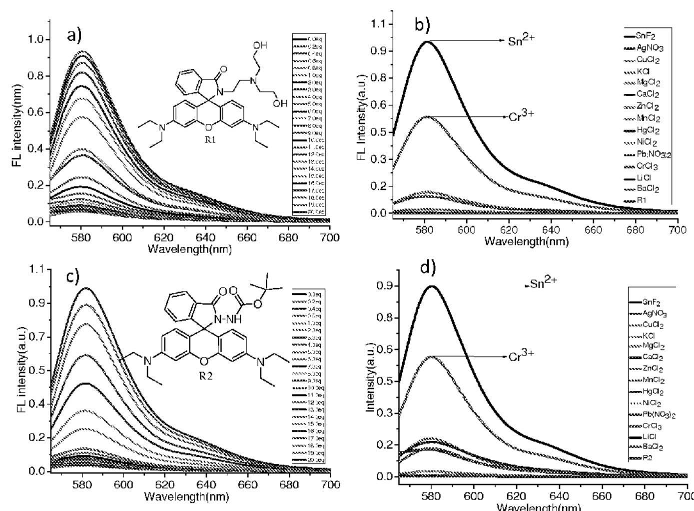

FIGURE 1

(57) Abstract: Rhodamine B derivative selectively chelates Sn^{2+} to act as a fluorescent probe.

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STANNOUS FLUORESCENT PROBE

FIELD OF THE INVENTION

Fluorescent probes selectively chelating Sn^{2+}.

BACKGROUND OF THE INVENTION

Stannous (Sn^{2+}) is added to toothpaste to prevent dental plaque and oral disease. Sn^{2+} is found to effectively inhibit certain bacteria that can lead to tooth decay in human interproximal dental plaque. More recently, there has been increasing interest in the biological roles of Sn^{2+} because tin is an essential trace mineral for humans and is found in the greatest amount in the adrenal gland, liver, brain, spleen and thyroid gland. There is some evidence that tin is involved in growth factors and cancer prevention. Deficiency of tin may result in poor growth and hearing loss, but excess tin accumulation can negatively affect respiratory and digestive systems. However, studies of the physiological role and bacteriostatic mechanism of tin ion are restricted by the lack of versatile Sn^{2+} detection methods applicable to living cells - either eukaryotic or prokaryotic.

There is a need for chemical probe that is highly selective for Sn^{2+} in the presence of various metal ions and will exhibit high fluorescence upon Sn^{2+} chelation. There is a further need for such probe for use in living cells. There is yet a further need for a probe that minimizes background noise while providing high fluoresce intensity in living cells consistent where Sn^{2+} is found in the cell.

It is an advantage is to have a probe that works well in pH conditions in an organelle that plays a role in Sn accumulation in the cell.

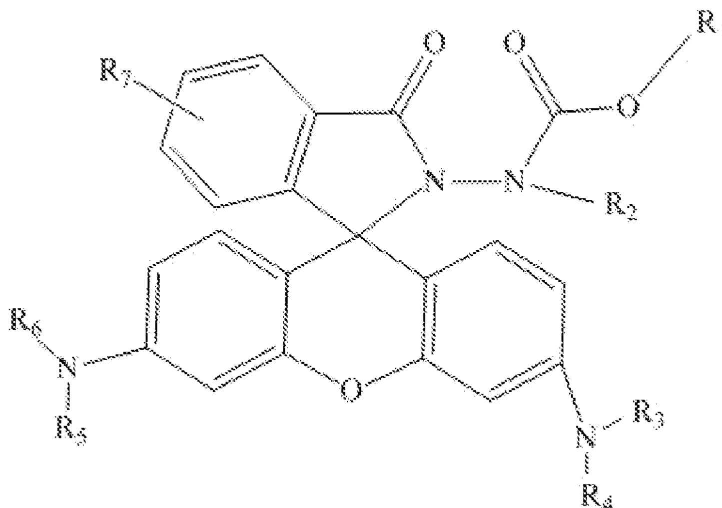
It is a further advantage to have a probe that is relatively easy and simple to synthesize.

SUMMARY OF THE INVENTION

The present invention address this need by the surprising discovery of a Sn^{2+} fluorescent probe containing rhodamine B derivative moiety as fluorophore, linked via amide moiety to a carbazate group. The use of this class of probe compounds is demonstrated as an imaging probe for monitoring Sn^{2+} in living cells to study the physiological function of Sn^{2+} in biological systems. This class of compounds is particularly useful given the additional surprising discovery that lysosomes appear to be an organelle where Sn concentrations are found. And given the rather acidic microenvironment of this organelle, the probes of the present invention exhibit high

fluorescent intensity and yet minimizes background noise, compared to other probes that are otherwise subject to low pH induced fluoresce. In other words, a comparative probe, given the acidity of the lysosome, leads to undesirably induce fluorescence emission (thereby generating background noise).

5 A first aspect of the invention provides for a compound having the following Formula (I):



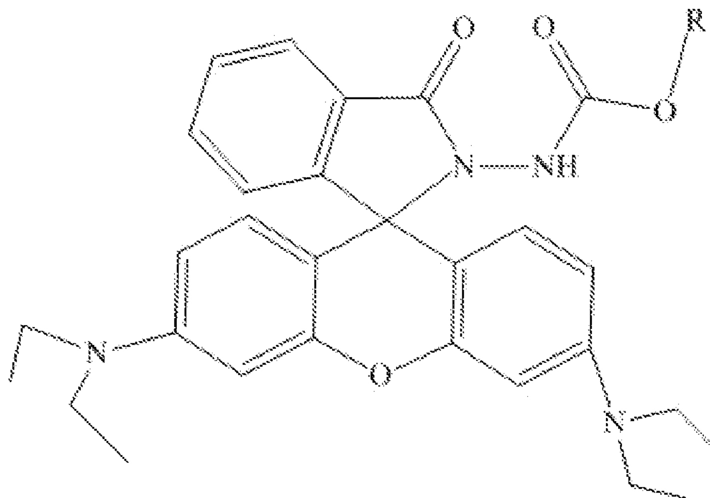
Formula (I)

wherein R_1 is unsubstituted, branched or unbranched, C_1-C_{12} alkyl, alkenyl, or alkynyl; and
10 wherein R_2, R_3, R_4, R_5, R_6, and R_7 are each independently a hydrocarbyl; or an optical isomer, diastereomer or enantiomer for Formula (I), or a salt thereof.

In one embodiment, R_1 is unsubstituted, branched or unbranched, C_1-C_{10} alkyl, preferably C_1-C_8 alkyl. In another embodiment, R_1 is selected from the group consisting of methyl, ethyl, propyl, butyl, isobutyl, pentanyl, and hexanyl, preferably isobutyl.

15 In one embodiment, R_2, R_3, R_4, R_5, R_6, and R_7 are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, aryl, heteroakyl, heteroalkenyl, heteroalkynyl, heterocycloalkyl, heterocycloalkenyl, heteroaryl, and wherein the aforementioned may be substituted or unsubstituted. In another embodiment, R_2, R_3, R_4, R_5, R_6, and R_7 are each independently selected from H, C_1-C_{10} alkyl, alkenyl, or alkynyl, and wherein
20 the aforementioned may be substituted or unsubstituted, preferably unsubstituted. In yet still another embodiment, R_2 and R_7 are hydrogen, and/or R_3, R_4, R_5, and R_6 are each independently selected from hydrogen, or C_1 to C_5 alkyl, branched or unbranched, preferably unsubstituted C_1 to C_5 alkyl. In yet still another embodiment, R_3, R_4, R_5, and R_6, are each independently selected from unsubstituted C_1 to C_3 alkyl.

Another aspect of the invention provides a compound of Formula (II):



Formula (II)

5

wherein R_1 is unsubstituted, branched or unbranched, C_1-C_{12} alkyl or alkenyl; or an optical isomer, diastereomer or enantiomer for Formula (I), or a salt thereof. In one embodiment, R_1 is an unsubstituted C_1-C_{10} alkyl, preferably R_1 is an unsubstituted, branched or unbranched, C_1-C_8 alkyl. In yet still another embodiment, R_1 is selected from the group consisting of methyl, ethyl,
10 propyl, butyl, isobutyl, pentanyl, and hexanyl, preferably isobutyl.

In another aspect of the invention, a compound according to Formula (I) or (II) is provided, wherein the compound is selected from the group consisting of:

(a) Tert-butoxy-carboxamide, N-[3',6'-bis(diethylamino)-3-oxospiro [1H-isoindole-1,9'-[9H]xanthen]-H)-yl]- ;

15 (b) Tert-butoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-2(3H)-yl]-;

(c) Methoxy-carboxamide,N-[3',6'-bis(diethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-2(3H)-yl]-;

(d) Ethoxy-carboxamide,N-[3',6'-bis(diethylamino)-3-oxospiro[1H-isoindole-1,9'-
20 [9H]xanthen]-2(3H)-yl]-; and

(e) Methoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-2(3H)-yl]-; and

(f) Ethoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-2(3H)-yl]-.

In yet another embodiment, the compound is selected from: N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)propionamide; N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)butyramide; and N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)pentanamide.

5 Yet still another aspect of the invention provides for a method of detecting fluorescence in a biological cell comprising the steps: (a) incubating the biological cell with a compound described above (e.g., a compound of Formula (I) or Formula (II), or preferred or alternative compound embodiments within said Formulas (I) or (II)); (b) shining excitation light to the incubated cell , preferably wherein the shined light has wavelength of at least from 520 to 580 10 nm, alternatively at 560 nm; and (c) detecting light emission from the compound from 560 to 660 nm. In one embodiment, the method further comprises subjecting the biological cell to Sn^{2+}, alternatively wherein the biological cell is selected from an oral epithelial cell or S*treptococcus* genus of bacterium, alternatively wherein the biological cell is any eukaryotic cell. In another embodiment, the light emission detection is at a lysosome organelle of eukaryotic cell. In yet 15 still another embodiment, the method is conducted with at least one specific compound previously described above or herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 – 5 are provided. Figure 1 (a) and (c) is fluorescence spectra of comparative 20 Compound R1 and inventive Compound R2 upon addition of Sn^{2+}; (b) and (d) is fluorescent spectra of Compounds R1 and R2 upon various metal ions with an excitation of 560 nm, respectively.

Figure 2 shows dependence of fluorescence at 580 nm of comparative Compound R1 and inventive Compound R2 (10 μM) at different pH range. Excitation is at 560 nm.

25 Figure 3 are CLSM images of KB cells. (a1-c1) and (a2-c2) Cells are separately incubated with 10μM comparative Compound R1 and inventive Compound R2 for 30 min, (d1-f1) and (d2-f2) followed incubated with 50μM SnF_2 for 30 min. Emission is collected in red channel at 560–660 nm (b1, e1, b2, e2); a1, d1, a2, d2 are bright field images andc1, f1, c2, f2 are overlay images, respectively (λex = 543 nm).

30 Figure 4 are CLSM images of KB cells. (a1- d1) Cells are incubated with 10 μM R1 and 1μM LysoTracker Green for 30 min; (a2-d2) successively incubated with 10 μM R2, 50μM SnF_2, 1μM LysoTracker Green ,each for 20 min. Emission is collected in red channel (b1, b2) at 560–

660 nm (λex = 543 nm) or in green channel at 500-540 nm (λex = 488 nm); a1, a2, are bright field images and d1, d2 are overlay images, respectively.

Figure 5 is CLSM images of KB cells. (a1- d1) Cells are successively incubated with 50μM SnF_2, 10 μM of comparative Compound R1 and 1μM LysoTracker Green each for 20 min; (a2-d2) successively incubated with 50μM SnF_2, 10μM of inventive Compound R2 and 1μM LysoTracker Green, each for 20 min. Emission is collected in red channel (b1, b2) at 560–660 nm (λex = 543 nm) or in green channel at 500-540 nm (λex = 488 nm); a1, a2, are bright field images and d1, d2 are overlay images, respectively.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

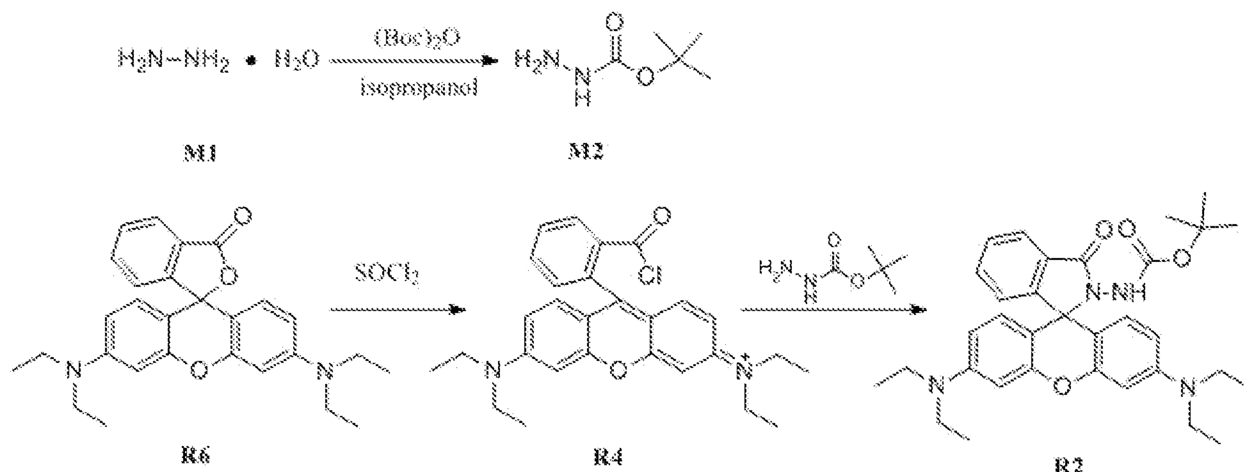
For purposes of the present invention the term "hydrocarbyl" is defined herein as any organic unit or moiety which is comprised of carbon atoms and hydrogen atoms. Included within the term hydrocarbyl are heterocycles. Non-limiting examples of various unsubstituted non-heterocyclic hydrocarbyl units include pentyl, 3-ethyloctanyl, 1,3-dimethylphenyl, cyclohexyl, cis-3-hexyl, 7, 7-dimethylbicyclo[2.2.1]-heptan-1-yl, and napth-2-yl. Included with the definition of "hydrocarbyl" are the aromatic (aryl) and non-aromatic carbocyclic rings. The term "heterocycle" includes both aromatic (heteroaryl) and non-aromatic heterocyclic rings.

The term "substituted" is used throughout the specification. The term "substituted" is defined herein as "encompassing moieties or units which can replace a hydrogen atom, two hydrogen atoms, or three hydrogen atoms of a hydrocarbyl moiety. Also substituted can include replacement of hydrogen atoms on two adjacent carbons to form a new moiety or unit." For example, a substituted unit that requires a single hydrogen atom replacement includes halogen, hydroxyl, and the like. A two hydrogen atom replacement includes carbonyl, oximino, and the like. A two hydrogen atom replacement from adjacent carbon atoms includes epoxy, and the like. Three hydrogen replacement includes cyano, and the like. An epoxide unit is an example of a substituted unit which requires replacement of a hydrogen atom on adjacent carbons. The term "substituted" is used through the present specification to indicate that a hydrocarbyl moiety, *inter alia*, aromatic ring, alkyl chain, can have one or more of the hydrogen atoms replaced by a substituent. When a moiety is described a "substituted" any number of the hydrogen atoms may be replaced. For example, 4-hydroxyphenyl is a "substituted aromatic carbocyclic ring," (N, N-dimethyl-5-amino)octanyl is a "substituted C_8 alkyl unit, 3-guanidinopropyl is a "substituted C_3 alkyl unit," and 2-carboxypyridinyl is a "substituted heteroaryl unit".

In one embodiment, wherein R_2, R_3, R_4, R_5, R_6, and R_7 are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, aryl, heteroakyl, heteroalkenyl, heteroalkynyl, heterocycloalkyl, heterocycloalkenyl, heteroaryl, substituted, and wherein the aforementioned may be substituted or unsubstituted. These terms are well known in
5 the art. For a detailed definition, see U.S. Pat. No. 6,919,346 B2 at column 2, line 61 to column 9, line 53, incorporated herein by reference.

Synthesis path of Tert-butoxy-carboxamide,N-[3',6'-bis(diethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-H)-yl]- (herein after "Compound R2" or simply "R2")

10 Compound R2, an exemplary compound of the present invention, is rhodamine B derivative moiety linked via amide moiety to tert-butyl carbazate group. The synthesis path of this compound is provided.



15 The synthesis of Compound R2 is described. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Rhodamine (95%) is obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). Other chemicals are provided from Shanghai No. 1 chemical reagent. Flash chromatography is carried out on silica gel (200-300 mesh). The ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra is recorded on a
20 Bruker DRX-500 spectrometer. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS). HRMS is recorded on LTQ-Orbitrap mass spectrometer (ThermoFIsher, San Jose, CA). Melting points are determined on a hot-plate melting point apparatus XT4-100A and uncorrected. UV-Vis spectra are recorded on a Shimadzu UV-2250 spectrophotometer. Fluorescence spectra are recorded on an Edinburgh

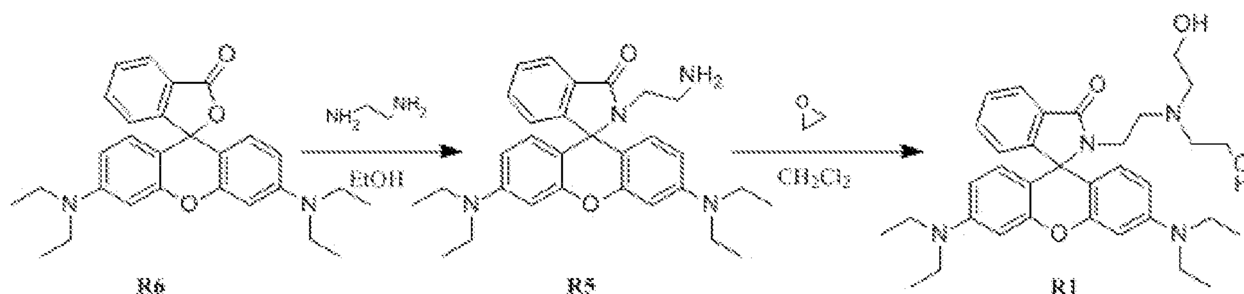
FLS-920 spectrophotometer. All pH measurements are made with a model Mettler -Toledo meter.

The synthesis of chemical intermediate M2 (of the schematic above) is described. Hydrazine monohydrate (5.2 g, 100.0 mmol) is stirred in 20 mL of isopropanol at 0 °C for 15 min, and treated dropwise with a solution of Boc_2O (10.0 g, 45.8 mmol) in 10 mL of isopropanol. The reaction turns cloudy upon addition and stirring is continued at room temperature for 20 min. The solvent is removed by rotary evaporation and the residue is dissolved in DCM and dried over $MgSO_4$. The DCM is removed by rotary evaporation and the remaining liquid is distilled under reduced pressure to obtain *t*-butyl carbazate (M2) as a white solid, ^1H NMR (400 MHz, $CDCl_3$) δ 6.42 (s, 1H), 3.60 (s, 2H), 1.37 (s, 9H).

Synthesis of chemical intermediate R4 of the above scheme is described. A solution of rhodamine B (442 mg, 1 mmol) in Cl_2SO (10 mL) is kept at room temperature overnight. The reaction mixture is evaporated under vacuum and co-evaporated with anhydrous CH_2Cl_2 (3 × 15 mL) to give rhodamine B acid chloride (R4). The crude acid chloride is dissolved in anhydrous CH_2Cl_2 (10 mL) and added dropwise to a solution of Boc-NH-NH_2 (132 mg, 1 mmol) and Et_3N (200 mL, 2 mmol) in anhydrous CH_2Cl_2 (15 mL). The reaction mixture is kept at room temperature for 10 min. Evaporation of the solvent yielded a crude that is purified by column chromatography using petroleum ether/ethyl acetate (3/1, v/v) to give R2 as a white solid (0.26 g, 50% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 7.96 (d, J = 7.6 Hz, 1H), 7.57 – 7.44 (m, 2H), 7.16 (d, J = 7.5 Hz, 1H), 6.52 (s, 2H), 6.38 (d, J = 1.8 Hz, 2H), 6.28 (d, J = 8.4 Hz, 2H), 3.34 (q, J = 7.0 Hz, 8H), 1.61 (s, 4H), 1.26 (s, 9H), 1.16 (d, J = 14.1 Hz, 12H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.92, 153.56, 152.87, 148.92, 132.58, 131.45, 129.05, 128.32, 123.80, 123.09, 108.24, 105.41, 97.68, 59.49, 57.65, 54.48, 44.13, 39.49, 12.31. HRMS calc. for $C_{33}H_{41}N_4O_4^+$ (M+H$^+$): 557.3122, found: 557.3111.

Comparative Example - Compound R1 or "R1"

Comparative compound R1, outside the scope of the present invention, is compared to inventive Compound R2. Compound R1 is: Spiro[1*H*-isoindole-1,9'-[9*H*]xanthen]-3(2*H*)-one, 2-[2-[bis(2-hydroxyethyl)amino]amino]ethyl]-3',6'-bis(diethylamino), and has the CAS Registry Number of 1217892-36-8 (C_{34}, H_{44}, N_4, O_4). The structure of Compound R1 and its synthesis is provided herein:



Synthesis of Compound R1

The synthesis of comparative Compound R1 is described. Synthesis of intermediate R5 is described: Rhodamine B hydrochloride (5.0 g, 10.4 mmol) and ethylenediamine (12.5 g, 208.8 mmol) is dissolved in EtOH (50 mL) and refluxed for 12 h. Most of solvent is removed by evaporation, and the residue is dispersed in water with magnetic stirring. Then the pink precipitate appeared and is recovered by filtration, washed thoroughly with water. At last pink precipitate is washed with petroleum ether, and then dried in vacuum, yielding Compound R5 as a pink powder (3.6 g, 72% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 2.3 Hz, 1H), 7.45 (d, *J* = 2.5 Hz, 2H), 7.14 – 7.05 (m, 1H), 6.44 (s, 1H), 6.42 (s, 1H), 6.37 (d, *J* = 2.3 Hz, 2H), 6.28 (d, *J* = 2.3 Hz, 1H), 6.26 (d, *J* = 2.4 Hz, 1H), 3.33 (q, *J* = 7.0 Hz, 9H), 3.19 (t, *J* = 6.6 Hz, 2H), 2.40 (t, *J* = 6.6 Hz, 2H), 1.62 (s, 5H), 1.16 (t, *J* = 7.0 Hz, 13H). *See* Shiraishi, Y.; Miyamoto, R.; Zhang, X.; Hirai, T. *Org. Lett.* 2007, *9*, 3921-3924.

Synthesis of R1 from R5 is described. Oxirane (0.44 g, 10.0 mmol) is added to a cooled (-5°C) solution of R5 (0.48 g, 0.1 mmol) in dichloromethane (10 mL). The solution is stirred for 4 h at -5°C and then overnight at room temperature before being concentrated under reduced pressure. The resulted mixture is purified by column chromatography using dichloromethane/ methanol (10/1, v/v) to give Compound R1 as a white solid (0.2 g, 40% yield): ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 7.91 (d, *J* = 2.9 Hz, 1H), 7.46 (dd, *J* = 5.5, 3.1 Hz, 2H), 7.13 – 7.06 (m, 1H), 6.46 (s, 1H), 6.44 (s, 1H), 6.39 (d, *J* = 2.4 Hz, 2H), 6.30 (d, *J* = 2.5 Hz, 1H), 6.28 (d, *J* = 2.5 Hz, 1H), 3.54 – 3.45 (m, 4H), 3.34 (q, *J* = 7.0 Hz, 9H), 3.22 (t, *J* = 5.7 Hz, 2H), 2.55 (t, *J* = 7.5 Hz, 4H), 2.23 (t, *J* = 5.0 Hz, 2H), 1.89 (s, 2H), 1.17 (t, J = 7.0 Hz, 13H). ^{13}C NMR (125 MHz, $CDCl_3$) *δ* 153.96, 150.99, 148.86, 133.12, 129.67, 129.32, 128.31, 124.24, 123.41, 107.85, 104.52, 97.76, 44.10, 27.81, 12.59. HRMS calc. for C34H45N4O4 ($M+H^+$): 573.3435, found: 573.3463.

Derivations of Compound R2

Many further derivations from this basic molecule of Compound R2 can be made by those skilled in the art consistent with Formulas (I) and (II) by using starting materials and

intermediates that are known or commercially available or by further modifying these molecules by known methods. Non-limiting examples of these compounds within the scope of Formula (I) and/or Formula (II) including the following:

(1) tert-butyl (3',6'-diamino-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C25H24N4O4), (Molecular Weight: 444.48);

(2) tert-butyl (3',6'-bis(dimethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C29H32N4O4), (Molecular Weight: 500.59);

(3) tert-butyl (3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C33H40N4O4), (Molecular Weight: 556.70);

(4) tert-butyl (3',6'-bis(ethylamino)-2',7'-dimethyl-3-oxospiro [isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C31H36N4O4), (Molecular Weight: 528.64);

(5) tert-butyl (3',6'-diamino-2',7'-dimethyl-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C27H28N4O4), (Molecular Weight: 472.54);

(6) tert-butyl (3-oxo-3',6'-di(pyrrolidin-1-yl)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C33H36N4O4), (Molecular Weight: 552.66);

(7) tert-butyl (3-oxo-3',6'-bis(phenylamino)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C37H32N4O4), (Molecular Weight: 596.67);

(8) tert-butyl (3-oxo-3',6'-di(piperidin-1-yl)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C35H40N4O4), (Molecular Weight: 580.72);

(9) tert-butyl (3',6'-dimorpholino-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C33H36N4O6), (Molecular Weight: 584.66);

(10) tert-butyl(2',7'-dibutyl-3',6'-bis(diethylamino) -3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C41H56N4O4), (Molecular Weight: 668.91);

(11) tert-butyl (2',7'-dimethyl-3-oxo-3',6'-di(piperidin-1-yl)spiro [isoindoline-1,9'-xanthen]-2-yl)carbamate (Chemical Formula: C37H44N4O4), (Molecular Weight: 608.77);

(12) tert-butyl (3-oxo-1',2',3',4',10',11',12',13'-octahydrospiro[isoindoline-1,7'-pyrano [2,3-f:6,5-f']diquinolin]-2-yl)carbamate (Chemical Formula: C31H32N4O4), (Molecular Weight: 524.61);

(13) tert-butyl (3-oxo-1',2',3',4',8',9',10',11'-octahydrospiro[isoindoline-1,6'-pyrano

[3,2-g:5,6-g']diquinolin]-2-yl)carbamate (Chemical Formula: C31H32N4O4), (Molecular Weight: 524.61);

(14) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)propionamide (Chemical Formula: C31H36N4O3), (Molecular Weight: 512.64);

5 (15) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)butyramide (Chemical Formula: C32H38N4O3), (Molecular Weight: 526.67); and

(16) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)pentanamide; (Chemical Formula: C33H40N4O3), (Molecular Weight: 540.70).

10 Metal Ion Sensing:

The procedure for metal ion sensing is described. Solutions of the metal ions (10.0 mM) are prepared in deionized water. A stock solutions of Compounds R1 and R2 (0.2 mM) are each prepared in ethanol and then diluted to 20 μM with ethanol–water (1: 1, v/v, pH 7.04) for spectral measurement. For titration experiments, a 2.0 mL solution of Compounds R1 and R2 (20 μM)
15 are filled in a respective quartz optical cell of 1 cm optical path length. Sn^{2+} stock solution is added into the quartz optical cell gradually by micro-pipette. Spectral data is recorded at 5 min after addition. In selectivity experiments, the test samples are prepared by placing appropriate amounts of metal ion stock into 2.0 mL solution of R1, R2 (20 μM). For fluorescence measurements, excitation is provided at 560 nm, while emission is collected from 565 to 700 nm.

20 pH titration of Compounds R1 and R2 is described. Stock solutions of Compounds R1 and R2 are respectively added to sodium phosphate buffers of various pH to a final concentration of 10 μM. The fluorescence emission spectra is recorded as a function of pH using λ_{ex} at 560 nm. The titration curves are plotted by fluorescence emission intensities at 580 nm versus pH.

Cell culture preparation is described. The KB cell line was provided by of Biochemistry
25 and Cell Biology (China). Cells are grown in MEM (Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine serum) and 5% CO_2 at 37℃. Cell (5×10^{-8} L^{-1}) are plated on 18 nm glass coverslips and allowed to adhere for 24 hours. The *Streptococcus* mutans (ATCC® 700610™) is prepared by inoculating the single colony from the BHI agar plate into 5 mL BHI broth and incubating at 37°C for 48 h.

30 Fluorescence imaging is described. Confocal fluorescence imaging is performed with an OLYMPUS IX81 laser scanning microscope and a 60 × oilimmersion objective lens. The microscope is equipped with multiple visible laser lines (405, 488, 543 nm). Images are collected and processed with Olympus FV10-ASW software. For fluorescence imaging of

intracellular Sn^{2+}: 10 μM of Compound R1 or R2 in the culture media containing 0.2% (v/v) DMSO is added to the cells. The cells are incubated at 37 °C for 30 min, and washed with PBS three times to remove the excess probe and bathed in PBS (2 mL) before imaging. After washing with PBS (2 mL × 3) to remove the excess probe, the cells are treated with 50 μM SnF_2 for 30 min. Excitation of R1 or R2 loaded cells at 543 nm is carried out with a semiconductor laser, and emission is collected at 560–660 nm (single channel). 50 μM SnF_2 in the culture media is added to the cells. The cells are incubated at 37 °C for 30 min, and washed with PBS three times to remove the excess SnF_2. After washing with PBS (2 mL × 3) to remove the excess SnF_2, the cells are treated with 10 μM R1 or R2 separately for 30 min, and washed with PBS three times to remove the excess probe and bathed in PBS (2 mL) before imaging. Cell imaging is then carried out as the former.

Colocalization experiments are described. 10 μM of Compound R1 or R2 in the culture media containing 0.2% (v/v) DMSO is added to the cells. The cells are incubated at 37°C for 30 min, and washed with PBS three times to remove the excess probe and bathed in PBS (2 mL) before imaging. After washing with PBS (2 mL × 3) to remove the excess probe, the cells are treated with 50 μM SnF_2 at 37 °C for 30 min, and washed with PBS three times to remove the excess SnF_2 and bathed in PBS (2 mL) before imaging. After washing with PBS (2 mL × 3) to remove the excess SnF_2, the cells are treated with 1.0 μM LysoTracker® Green DND at 37 °C for 30 min. The cells are bathed with 2 mL of PBS for fluorescence imaging equipped with the appropriate excitation and emission filters for R1 or R2 (λ_{ex} = 543 nm, λ_{em} = 560−660 nm), Lysotracker Green (λ_{ex} = 488 nm, λ_{em} = 500−540 nm). 50 μM SnF_2 in the culture media is added to the cells. The cells are incubated at 37 °C for 30 min, and washed with PBS three times to remove the excess SnF_2. After washing with PBS (2 mL × 3) to remove the excess SnF_2, the cells are treated with 10 μM of Compound R1 or R2 separately for 30 min, and washed with PBS three times to remove the excess probe and bathed in PBS (2 mL) before imaging. Cell imaging is then carried out as the former. After washing with PBS (2 mL × 3) to remove the excess probes, the cells were treated with 1.0 μM LysoTracker® Green DND at 37 °C for 30 min. Cell imaging is then carried out as the former.

For fluorescence imaging of Sn^{2+} in bacteria is described. Freshly diluted *Streptococcus* mutans (ATCC® 700610™) is sub cultured in the presence of the 10 μM Compound R1 or R2, separately at 37ºC on a shaker bed at 400 rpm for 60 min. Then the bacteria are collected by centrifugation at 8,000 rpm for 2 min and rinsed with Saline (pH = 7.0). The process is repeated three times before imaging. After washing with Saline (2 mL × 3) to remove the excess probes,

the bacteria is cultured in the presence of the 50 μM SnF_2 at 37 °C on a shaker bed at 400 rpm for 60 min. Then the bacteria are collected by centrifugation at 8,000 rpm for 2 min and rinsed with Saline (pH = 7.0). The process was repeated three times before imaging. The light source is at = 543 nm provided excitation and emission are collected in the range = 560-660 nm.

5 Metal ion response is described. Fluorescent 'turn on' probe is conducive for detection target. The solution of Compound R1 or R2 (20 μM) in ethanol–water (1: 1, v/v, PH 7.04) is non-fluorescent. With addition of Sn^{2+} (0–20 eq) , fluorescence at 580 nm is turned on and grown drastically with an excitation of 560 nm (Fig 1a and 1c) due to the ring open reaction of rhodamine induced by Sn^{2+} chelating. High-level selectivity is of paramount importance for an 10 excellent chemosensor. Compounds R1 and R2 show selectivity on sensing Sn^{2+}. The solution of R1 and R2 (20 μM) in ethanol–water (1: 1, v/v, PH 7.04) are tuning on just in the presence of Sn^{2+} and Cr^{3+}, while other transition and heavy metal ions such as K^+, Ag^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, displayed minimal enhancement with an excitation of 560 nm (Fig 1b and 1d). Metal ion response of R1 and R2 are suited for detection of Sn^{2+} in living 15 *Streptococcus* mutans cells.

Figure 1 (a) and (c) show the fluorescence spectra of comparative Compound R1 and inventive Compound R2 (20 μM) in ethanol–water (1: 1, v/v, pH 7.04) upon addition of 0–20 eq of Sn^{2+}. Figure 1 (b) and (d) show the fluorescent spectra of Compounds R1 and R2 (2.0×10^{-5} M) upon various metal ions (20.0×10^{-5} M) in ethanol–water (1: 1, v/v, pH 7.04) with an 20 excitation of 560 nm.

The impact of pH values on fluorescence is described. The pirolactam ring of the rhodamine derivatives will open in a certain pH range and indicates the fluorescence of rhodamine. It is therefore necessary to check the fluorescence properties of Compounds R1 and R2 in solutions with different pH values. Furthermore, in the cell, the acidity of different 25 organelles may vary greatly. For example, the normal pH of lysosomes is 4.5-5.5, which may induce ring opening of R1 or R2. Considering the application of Sn^{2+} probe R1 and R2 intracellular or extracellular may be disturbed by the pH, the acid–base titration experiments are carried out by adjusting the pH with an aqueous solution of NaOH and HCl in Phosphate-Buffered Saline (Fig.2 a and b). The titration revealed that the pH range for inducing 30 Compounds R1 or R2 fluorescence turning on is 2.5-6 or 2-4.5, respectively. It is predicted that R1 will be turned on by lysosomes in cell without Sn^{2+} present. Figure 2 shows the dependence of fluorescence at 580 nm of comparative Compound R1 and inventive Compound R2 (10 μM) at different pH in pbs solution. Excitation was at 560 nm.

Fluorescence imaging of intracellular Sn^{2+} is described. $Sn^{2+}$is usually added to toothpaste, so oral epithelial cells are most likely to come into contact with Sn^{2+}. The Kb cells are a good candidate for explored Sn^{2+} distribution in cell level by fluorescence imaging. Here the practical applicability of R1 and R2 as a Sn^{2+} probe in the fluorescence imaging of living Kb cells is
5 investigated. Firstly, the Kb cells are separately stained with 10 μM of Compound R1 or R2 at 37 °C for 30 min. As determined by laser scanning confocal microscopy, R1 gave fluorescence emission in a site of Kb cells without Sn^{2+} present (fig 3 .b1); R2 gave scarcely fluorescence (fig 3. b2). This result is consistent with the pH titration experiment that lysosomes acidity may induce R1 fluorescence emission. R2 gave scarcely fluorescence due its lower pH response.
10 This demonstrates the superiority of R2 over R1 given the greater diversity of pH environments that R2 may be used, and subsequently less background noise, particularly in more acidic environments like lysosomes.

Furthermore, when the cells are supplemented with Compound R1 or R2 in the PBS for 30 min at 37 °C and then incubated with 50 μM Sn^{2+} under the same conditions, inventive
15 Compound R2 gave a significant fluorescence increasing from the certain intracellular region (Fig 3 .c2, f2) whereas comparative Compound R1 showed slightly changing in fluorescence intensity (fig 3 .c1, f1). Accordingly, cell imaging experiment indicate that R2 is more suited for detection Sn^{2+} at a cell level. Furthermore, R1 and R2 may be specificity targeting for lysosomes, due to R1 and R2 bear the groups similar to 'dimethylethylamino' that is the targeting anchor for
20 lysosomes. Figure 3 shows CLSM images of KB cells (a1-c1) and (a2-c2). Cells separately incubated with 10μM of Compounds R1 and R2 for 30 min, (d1-f1) and (d2-f2) followed incubated with 50μM SnF_2 for 30 min. Emission was collected in red channel at 560–660 nm (b1, e1, b2, e2); a1, d1, a2, d2 are bright field images and c1, f1, c2, f2 are overlay images, respectively (λex = 543 nm).

25 Colocalization experiments are described. To probe the intracellular locations of Compounds R1 or R2, Kb cells were co-stained with R1 and LysoTracker® Green DND or R2, Sn^{2+} and LysoTracker® Green DND, which is a commercially available marker for lysosomes and has good separation in excitation and emission spectra with R1 and R2. As shown in Figure 4a1−d1, the fluorescence images between R1 and lysotracker overlapped very well. This result is
30 consistent with the pH titration experiment and fluorescence imaging of intracellular Sn^{2+}, that R1 accumulates in lysosomes and exhibits acidity induced fluorescence emission. Furthermore, Sn^{2+} can also locate in lysosomes and enhance the fluorescence of R1 (fig 3 .c1, f1). Fluorescence of R2 induced by Sn^{2+} and lysotracker overlapped very well, which indicates that

R2 can detection Sn^{2+} in lysosomes; at the same time fluorescence signal of R2 demonstrates distribution of Sn^{2+}.

Figure 4 shows CLSM images of KB cells (a1- d1). Cells are incubated with 10 μM of comparative Compound R1 and 1μM LysoTracker Green for 30 min; (a2-d2) successively incubated with 10 μM of inventive Compound R2, 50μM SnF_2, 1μM LysoTracker Green ,each for 20 min. Emission is collected in red channel (b1, b2) at 560–660 nm (λex = 543 nm) or in green channel at 500-540 nm (λex = 488 nm); a1, a2, are bright field images and d1, d2 are overlay images, respectively.

Without wishing to be bound by theory, Compounds R1 and R2 may be specificity targeting for lyosomes because these compounds have a groups similar to "dimethylethylamino" which is reported as a targeting anchor for lysosomes. Therefore, it's not clear whether Sn^{2+} is widely distributed in the cell or accumulated in lysosomes. To help answer this question, comparative colocalization experiment is carried out. Firstly, the Kb cells are stained with 50 μM Sn^{2+} at 37 °C for 30 min, and then separately incubated with 10 μM of Compound R1 and 1μM LysoTracker® Green DND or Compound R2 and 1 μM LysoTracker® Green DND under the same conditions. As determined by laser scanning confocal microscopy, R1 and R2 give fluorescence emission in a site of Kb cells (Fig.5 b1, b2), which overlap with LysoTracker® Green DND very well (Fig.5 d1, b2). There was no fluorescence in other site intracellular. This surprising result indicated that Sn^{2+} is internalized into cells and leading to accumulation of the ions in lysosomes. This may be a transmission path of Sn. Accordingly, inventive Compound R2 is superior over R1 in this application. Many pharmaceutical agents, including various large and small molecules, must be delivered specifically to particular cell organelles in order to efficiently exert their therapeutic action. Such delivery is still mainly an unresolved problem, but targeting detection is helpful attempt.

Figure 5 shows CLSM images of KB cells (a1- d1). Cells are successively incubated with 50μM SnF_2 , 10 μM of comparative Compound R1 and 1μM LysoTracker Green each for 20 min; (a2-d2) successively incubated with 50μM SnF_2, 10μM of inventive Compound R2 and 1μM LysoTracker Green ,each for 20 min. Emission is collected in red channel (b1, b2) at 560–660 nm (λex = 543 nm) or in green channel at 500-540 nm (λex = 488 nm); a1, a2, are bright field images and d1, d2 are overlay images, respectively.

Fluorescence imaging of Sn^{2+} in bacteria is described. It has been reported that Sn^{2+} can inhibit *Streptococcus* mutans. The practical applicability of Compounds R1 and R2 as a Sn^{2+} probe in the fluorescence imaging of living *Streptococcus* mutans (ATCC® 700610™) are

investigated. Firstly, the *Streptococcus* mutans (ATCC® 700610™) are separately stained with 10μM Compound R1 or R2 at 37℃ for 60 min. As determined by laser scanning confocal microscopy, Compounds R1 and R2 gave no fluorescence emission without Sn^{2+} present (Fig 6. b1, b2). When the *Streptococcus* mutans are supplemented with R1 or R2 in the PBS for 60 min
5 at 37℃ and then incubated with 50 μM Sn^{2+} under the same conditions, R1and R2 gave a significant fluorescence increasing (fig 6 .e1, e2). The overlay of fluorescence and Brightfield images revealed that the fluorescence signals are localized in the *Streptococcus* mutans (ATCC® 700610™) (Figure 6.f1,f2), indicating that the Sn^{2+} plays its physiological role within bacteria. This data provides evidence for antibacterial mechanism of Sn^{2+}.

10 Figure 6 shows CLSM images of streptococcus mutans (ATCC® 700610™). (a1-c1) and (a2-c2) Cells are separately incubated with 10μM comparative Compound R1 and inventive Compound R2 for 30 min, (d1-f1) and (d2-f2) followed incubated with 50μM SnF_2 for 30 min. Emission is collected in red channel at 560–660 nm (b1, e1, b2, e2); a1, d1, a2, d2 are bright field images andc1, f1, c2, f2 are overlay images, respectively (λex = 543 nm).

15 In summary, the biological application of inventive Compound R2 is demonstrated by the imaging of Sn^{2+} in Kb cells and *Streptococcus* mutans (ATCC® 700610™). Furthermore compound R2 as lysosomes tracker is demonstrated by the distribution of Sn^{2+} in cells and bacteria, which is helpful to research of pharmaceutical agents delivery and antibacterial mechanism of Sn^{2+}.

20 The dimensions and values disclosed herein are not to be understood as being strictly limited to the exact numerical values recited. Instead, unless otherwise specified, each such dimension is intended to mean both the recited value and a functionally equivalent range surrounding that value. For example, a dimension disclosed as "40 mm" is intended to mean "about 40 mm."

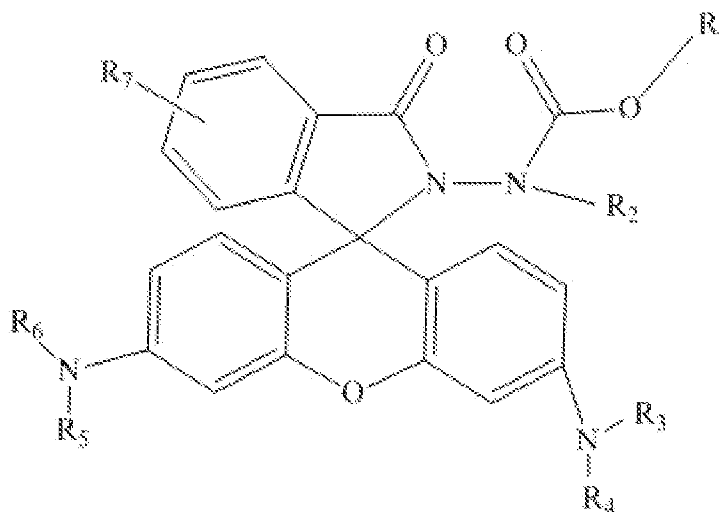
25 Every document cited herein, including any cross referenced or related patent or application and any patent application or patent to which this application claims priority or benefit thereof, is hereby incorporated herein by reference in its entirety unless expressly excluded or otherwise limited. The citation of any document is not an admission that it is prior art with respect to any invention disclosed or claimed herein or that it alone, or in any combination with any other
30 reference or references, teaches, suggests or discloses any such invention. Further, to the extent that any meaning or definition of a term in this document conflicts with any meaning or definition of the same term in a document incorporated by reference, the meaning or definition assigned to that term in this document shall govern.

While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this
5 invention.

CLAIMS

What is claimed is:

1. A compound of the following Formula (I):



Formula (I)

wherein R_1 is unsubstituted, branched or unbranched C_1-C_{12} alkyl, alkenyl, or alkynyl; and wherein R_2, R_3, R_4, R_5, R_6, and R_7 are each independently a hydrocarbyl;
or an optical isomer, diastereomer or enantiomer for Formula (I), or a salt thereof.

2. The compound of claim 1, wherein R_2, R_3, R_4, R_5, R_6, and R_7 are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, aryl, heteroakyl, heteroalkenyl, heteroalkynyl, heterocycloalkyl, heterocycloalkenyl, heteroaryl, and wherein the aforementioned may be substituted or unsubstituted.

3. The compound of claim 2, wherein: R_2, R_3, R_4, R_5, R_6, and R_7 are each independently selected from H, C_1-C_{10} alkyl or alkenyl, and wherein the aforementioned may be substituted or unsubstituted.

4. The compound of claim 3, wherein:

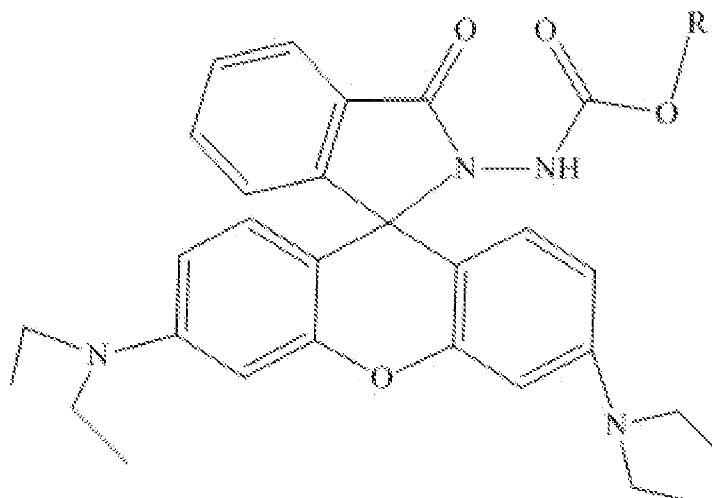
R_2 is hydrogen;

R_3, R_4, R_5, and R_6 are each independently selected from hydrogen, or unsubstituted C_1-C_5 alkyl, branched or unbranched; and

R_7 is hydrogen.

5. The compound of claim 4, wherein R_1 is unsubstituted, branched or unbranched C_1-C_{10} alkyl.

6. A compound of the following Formula (II):



Formula (II)

wherein R_1 is unsubstituted, branched or unbranched C_1-C_{12} alkyl or alkenyl;
or an optical isomer, diastereomer or enantiomer for Formula (I), or a salt thereof.

7. The compound of claim 6, wherein R_1 is a C_1-C_{10} alkyl, thereof.

8. The compound of claim 7, wherein R_1 is C_1-C_8 alkyl.

9. The compound of claim 10, wherein the compound is selected from the group consisting of:
(a) tert-butyl (3',6'-diamino-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(b) tert-butyl (3',6'-bis(dimethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(c) tert-butyl (3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(d) tert-butyl (3',6'-bis(ethylamino)-2',7'-dimethyl-3-oxospiro
[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(e) tert-butyl (3',6'-diamino-2',7'-dimethyl-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(f) tert-butyl (3-oxo-3',6'-di(pyrrolidin-1-yl)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(g) tert-butyl (3-oxo-3',6'-bis(phenylamino)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;
(h) tert-butyl (3-oxo-3',6'-di(piperidin-1-yl)spiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;

(i) tert-butyl (3',6'-dimorpholino-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;

(j) tert-butyl(2',7'-dibutyl-3',6'-bis(diethylamino)

-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)carbamate;

(k) tert-butyl (2',7'-dimethyl-3-oxo-3',6'-di(piperidin-1-yl)spiro

[isoindoline-1,9'-xanthen]-2-yl)carbamate;

(l) tert-butyl (3-oxo-1',2',3',4',10',11',12',13'-octahydrospiro[isoindoline-1,7'-pyrano

[2,3-f:6,5-f']diquinolin]-2-yl)carbamate;

(m) tert-butyl (3-oxo-1',2',3',4',8',9',10',11'-octahydrospiro[isoindoline-1,6'-pyrano

[3,2-g:5,6-g']diquinolin]-2-yl)carbamate;

(n) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)propionamide;

(p) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)butyramide; and

(q) N-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)pentanamide.

10. The compound of claim 1, wherein the compound is selected from the group consisting of:

(a) Tert-butoxy-carboxamide, N-[3',6'-bis(diethylamino)-3-oxospiro

[1H-isoindole-1,9'-[9H]xanthen]-H)-yl]- ;

(b)Tert-butoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-

[9H]xanthen]-2(3H)-yl]-;

(c)Methoxy-carboxamide,N-[3',6'-bis(diethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-

2(3H)-yl]-;

(d)Ethoxy-carboxamide,N-[3',6'-bis(diethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-

2(3H)-yl]-;

(e)Methoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-

[9H]xanthen]-2(3H)-yl]-; and

(f)Ethoxy-carboxamide,N-[3',6'-bis(dimethylamino)-3-oxospiro[1H-isoindole-1,9'-[9H]xanthen]-

2(3H)-yl]-.

11. A method of detecting fluorescence in a biological cell comprising the steps:

(a) incubating the cell with a compound of claim 1;

(b) shining excitation light to the incubated cell;

(c) detecting light emission from the compound from 560 nm to 660 nm.

12. The method of claim 11, subjecting the biological cell to Sn^{2+}.

13. The method of claim 11, wherein the biological cell is selected from an oral epithelial cell or a S*treptococcus* genus of bacterium

14. The method of claim 12, wherein the biological cell is a eukaryotic cell, and wherein said emission detection is a lysosome.

15. The method of claim 14, wherein the compound is a compound of claim 9 or 10.

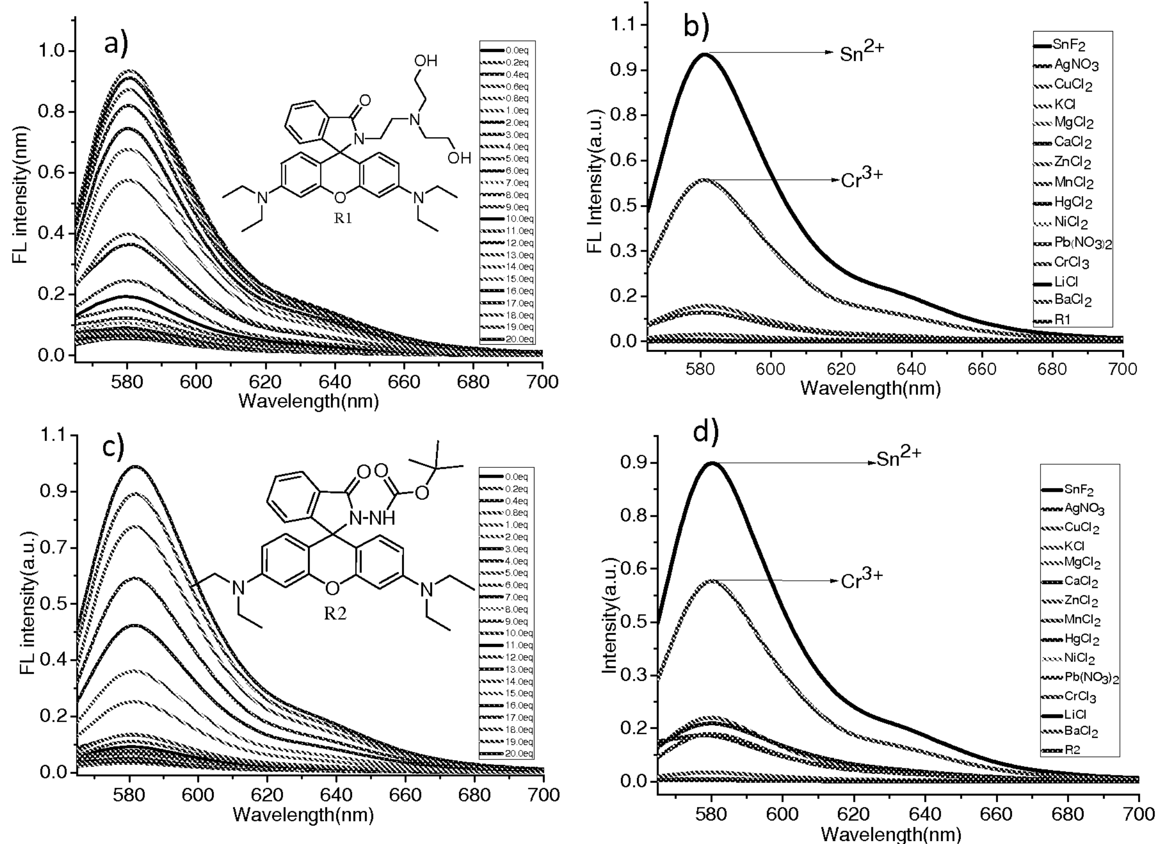


FIGURE 1

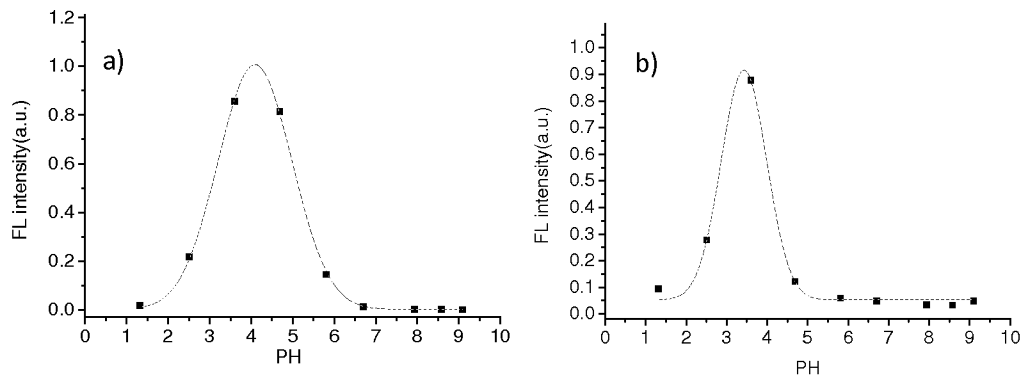


FIGURE 2

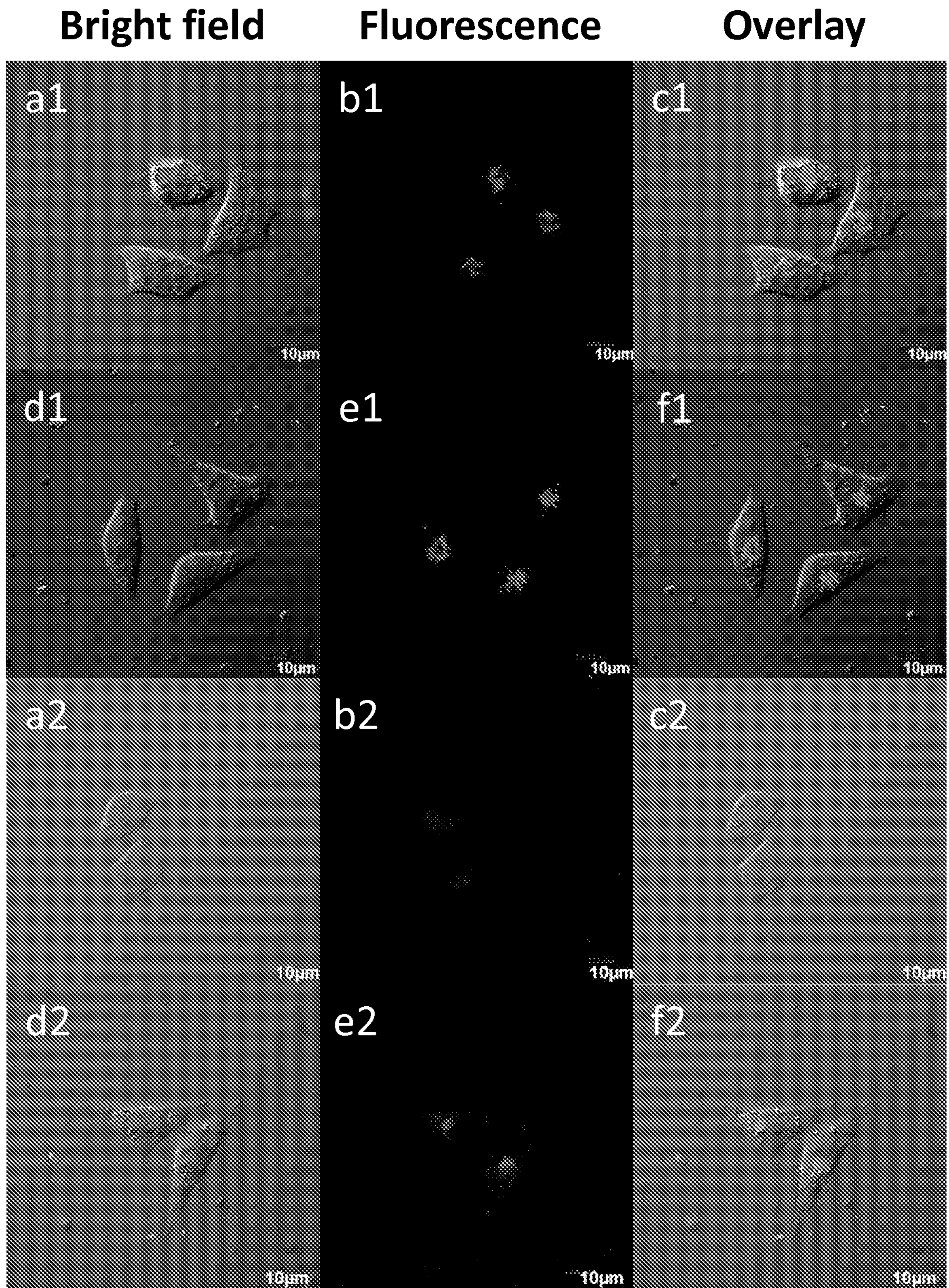


FIGURE 3

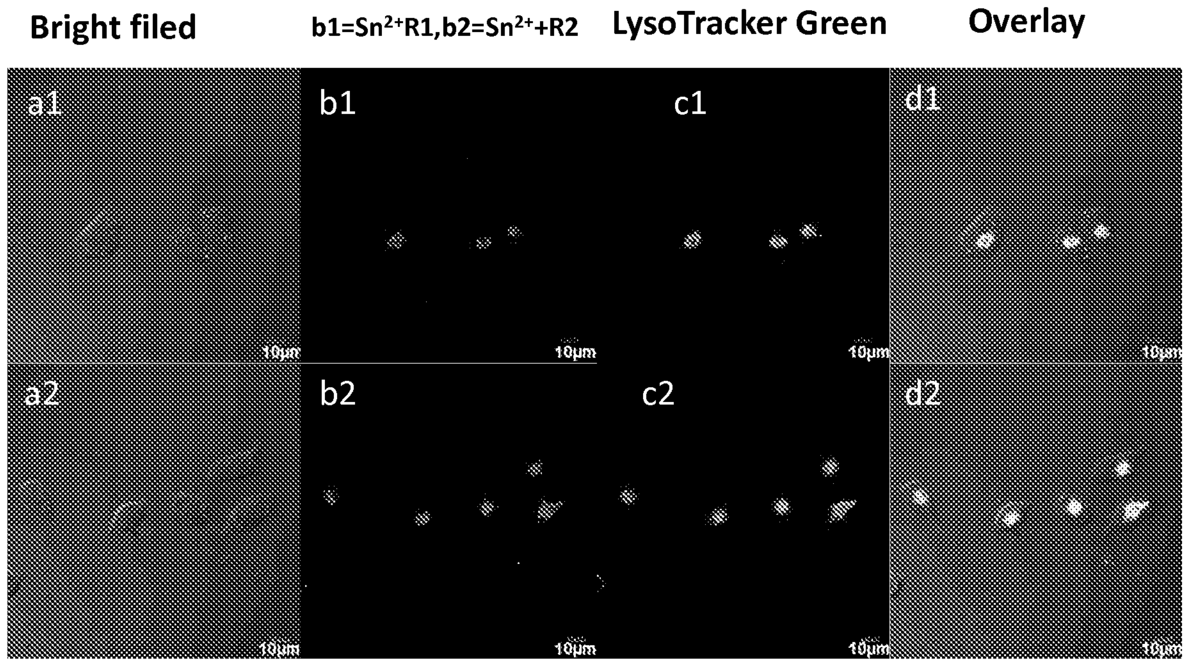


FIGURE 4

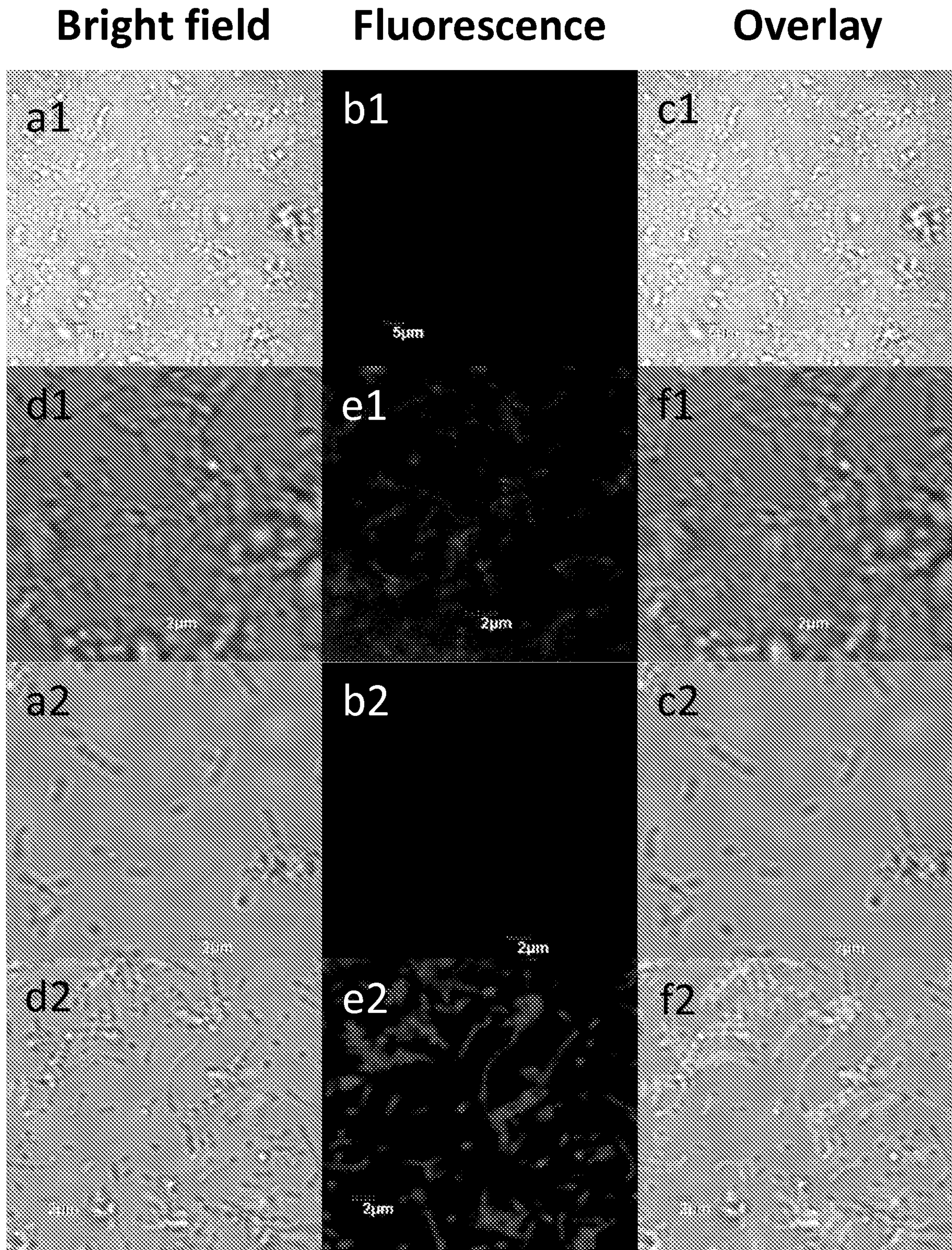


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/073769

A. CLASSIFICATION OF SUBJECT MATTER

C07D 491/107(2006.01)i; G01N 21/64(2006.01)i

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)

C07D; G01N

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)

CNKI, CNABS, WPI, EPODOC, REGISTRY, CAPLUS: tin, stannous, fluorescent, probe, substructure search according to formual (I)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADAMCZYK， MACIEJ. "Efficient fluorescein spirolactam and bis-spirolactam synthesis" *Synthetic Communications,* Vol. No.17, No. vol.31, 31 December 2001 (2001-12-31), pages 2681-2690	1-15
A	CN 102516254A (UNIV CAPITAL NORMAL ET AL.) 27 June 2012 (2012-06-27) the whole document	1-15

☐ 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
07 May 2014	**23 May 2014**

Name and mailing address of the ISA/

STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA(ISA/CN)
6,Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China

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Authorized officer

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Telephone No. **(86-10)62086353**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/073769

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.: **11-15**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] **Claims 11-15 are directed to a method of treatment of the human/animal body(Rule 39.1(iv) PCT). Nonetheless, the search report has been made and based on the alleged effects of the compounds/composition.**

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).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/CN2014/073769

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
CN 102516254A	27 June 2012	None	None