



(11) **EP 2 999 408 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
22.04.2020 Bulletin 2020/17

(21) Application number: **14794148.8**

(22) Date of filing: **08.05.2014**

(51) Int Cl.:
A61K 49/00 (2006.01) A61B 5/00 (2006.01)

(86) International application number:
PCT/US2014/037339

(87) International publication number:
WO 2014/182927 (13.11.2014 Gazette 2014/46)

(54) **DDAO DERIVATIVES AND THEIR USE**

DDAO-DERIVATE UND DEREN VERWENDUNG

DÉRIVÉS DE DDAO ET LEUR UTILISATION

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

(30) Priority: **08.05.2013 US 201361821187 P**

(43) Date of publication of application:
30.03.2016 Bulletin 2016/13

(73) Proprietors:
• **Rutgers, the State University of New Jersey
New Brunswick, NJ 08909 (US)**
• **New Jersey Institute of Technology
Newark, NJ 07102 (US)**

(72) Inventors:
• **MUSTAEV, Arkady
New York, NY 10024 (US)**

• **PERLIN, David, S.
New York, NY 10022 (US)**
• **WIRPSZA, Laura
Ocean City, NJ 08226 (US)**

(74) Representative: **Barker Brettell LLP
100 Hagley Road
Edgbaston
Birmingham B16 8QQ (GB)**

(56) References cited:
**WO-A2-2008/013997 WO-A2-2012/064733
WO-A2-2013/169932 US-A- 4 892 858
US-A1- 2010 256 399 US-A1- 2011 003 343**

• **TUNG ET AL.: 'In Vivo Imaging of b-Galactosidase
Activity Using Far Red Fluorescent Switch'
CANCER RES. vol. 64, 2004, pages 1579 - 1583,
XP009129965**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/821,187, filed on May 8, 2013, and the benefits of International Application No. PCT/US2013/040182, filed on May 8, 2013, which in turn claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/644,283, filed on May 8, 2012.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] The invention described herein was supported in whole or in part by grants from the National Institutes of Health (NIH-GM-30717-21). The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The present invention relates to a novel near infrared label, compositions, and methods of using the label to identify targets *in vitro*, *in situ* and *in vivo*.

BACKGROUND OF THE INVENTION

[0004] Rapid detection of microbial pathogens in body tissues is a challenging task. Prompt diagnosis is crucial for successful therapeutic intervention in the case of infection by fast-growing pathogens or with immune-compromised individuals. The problem is aggravated due to increasing use of immunosuppressive agents in patients who receive transplants, implants, or more intensive anticancer therapies. Conventionally, detecting and monitoring infectious processes are achieved by use of various non-invasive imaging techniques, including conventional radiography, ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI). However, these techniques rely solely on morphological changes and, therefore, most abnormalities can only be detected at advanced stages of disease and diagnosis of early infections and differentiation between active and structural but indolent alterations following surgery or other interventions can be difficult. Also, morphological imaging methods cannot differentiate between sterile inflammation and infection and do not allow for timely monitoring to access the success of antimicrobial therapy at an early stage.

[0005] Fluorescence Molecular Tomography (FMT) is a powerful approach to image microbial infections in body tissues. It is based on using fluorescently labeled compounds (e.g. antibiotics) that recognize pathogen-specific targets, thereby rendering the pathogen cells fluorescent after binding to the targets. In FMT, imaging of fluorescent material (labeled microbial pathogens) in body is achieved through excitation by powerful light source and detection of the induced fluorescence. Moving the excitation source around a scanned object with simultaneous signal acquisition allows 3-D imaging of the area of interest. For these applications fluorescent group attached to the "address" molecule must have excitation and emission maxima in near-infrared (NIR) spectral range where body tissues are transparent.

[0006] The near infrared (NIR) range of the spectrum is a very promising area for fluorescence detection and imaging. Most fluorophores operate in the visible to ultraviolet range of the spectrum. In NIR much less background/noise is observed in both *in vitro* assays and tissue samples, thereby increasing detection sensitivity. Notably, body tissues have a transparency window in the region 650-800 nm with a maximal transparency at 680 nm, which enables non-invasive imaging of NIR dyes in the living body. Therefore, long wavelength emitting dyes are of a great demand for numerous biomedical applications such as imaging of pathogens inside the body. However, conventional NIR dyes tend to be large in size and possess groups with a stable electric charge (introduced to enhance a solubility of the compounds), and when conjugated to a targeting agent such as a drug, may affect the interaction of the modified drug with a target and cell permeability of the labeled drug derivatives. In particular, most current NIR fluorescent labels are bulky and contain a stable positive charge, which is likely to disable the "address" molecules by affecting their affinity to the targets. Therefore, new NIR labels, especially of small molecules, with desired properties are still in high demand.

[0007] WO2013/169932 discloses targeting agents and methods of using targeting agents to detect a fungal cell in a subject.

[0008] Tung et al; Imaging of β -Galactosidase Activity Using Far Red Fluorescent Switch", Cancer Research(2004), vol 64, pages 1579-1583, discloses that DDAOG, a conjugate of β -galactoside and 7-hydroxy-9H-(1, 3-dichloro-9,9-dimethylacridin-2-one)(DDAO) is not only a chromogenic β -gal substrate but that the cleavage product has far-red fluorescent properties detectable by imaging. Importantly, the cleavage substrate shows a 50-nm red shift, enabling its specific detection in a background of intact probe, a highly desirable feature for *in vivo* imaging. β -gal expressing 9L gliomas are shown to be readily detectable by red fluorescence imaging in comparison with native 9L gliomas. Herpes simplex virus amplicon mediated *LacZ* gene transfer into tumors is transiently and serially visualized over time. It is disclosed that the results indicate that *in vivo* real-time detection of β -gal is possible with fluorescence imaging technology.

SUMMARY OF THE INVENTION

[0009] In accordance with a first aspect of the invention there is provided a compound or pharmaceutically acceptable salt of solvate thereof in accordance with claim 1 of the appended claims.

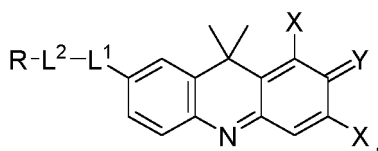
[0010] In accordance with a second aspect of the invention there is provided a targeting agent in accordance with claim 9 of the appended claims.

[0011] In accordance with a third aspect of the invention there is provided a method of detecting a cell or a target in a cell of a subject or detecting a microbe in a subject, the method having all of the steps set out in claim 12 of the appended claims.

[0012] In accordance with a fourth aspect of the invention there is provided a method detecting a cell or a target in a cell of a biological sample the method having all of the steps set out in claim 13 of the appended claims.

[0013] The present invention provides much needed small-size near-infrared fluorophores suitable for attachment to biomolecules of interest. Due to the small size, these compounds preserve targeting properties of the "address" molecules when attached to them. The small-size near-infrared fluorophores have improved light-emission properties over the existing NIR labels, and their reactive derivatives are convenient for bioconjugation. These have been demonstrated by successful imaging of fungal pathogens with cultured cells and in body tissues using Fluorescent Molecular Tomography (FMT). Specifically, the invention relates to new derivatives of NIR fluorophore DDAO or analogs with improved spectral properties. The near-infrared labeling compounds include, but are not limited to, signaling molecular beacons-like non-nucleic acid-based diagnostic compounds, which are quenched, but become fluorescent upon binding to their targets.

[0014] There are disclosed compounds of formula (I):



(I)

or a pharmaceutically acceptable salt or solvate thereof, wherein:

X at each occurrence is independently a halogen;

Y is O, S, or NR^Y, wherein R^Y is hydrogen or C₁-C₄ alkyl;

L¹ is -NH- or -O-;

L² is a bond, a linear or branched alkylene, or a linear or branched heteroalkylene; and

R is hydrogen, a moiety of a biologically active molecule, or a functional group that can react with a biologically active molecule to form a covalent bond. Preferably, X, Y, L¹, L², and R are selected so that the compound is near-infrared fluorescent.

[0015] In one embodiment, the present invention provides a bioconjugate comprising a targeting agent, a moiety of a DDAO or 7-aminoDDAO derivative, and a linking group, wherein the targeting agent is an agent capable of detecting a target of interest in a biological sample or a subject.

[0016] The small size of the suggested NIR reporter group in combination with its fair brightness provides highly beneficial properties, which will make the corresponding detection probes useful in a variety of biomedical and research applications that rely on imaging of specific targets in body tissues. Competitive approaches (e.g. positron emission tomography, or PET) are based on using of radionuclides as labels for design of diagnostic probes. Short life time of radionuclides (from minutes to hours) and costs associated with their production, fast transportation and handling are major issues associated with their use. Also frequent use of radionuclides raises health concerns. Therefore, fluorescent labels in diagnostic microbe-targeting probes represent good alternative to radionuclides in this fast developing field.

[0017] In contrast, conventional near-infrared fluorophores are rarely suitable for the detection approach described above since they are bulky and in many cases possess a stable positive charge, which reduces the affinity of the probes to their targets and cellular uptake.

[0018] In another aspect the present invention provides a composition comprising a NIR label compound and bioconjugate as claimed, and a pharmaceutically acceptable carrier.

[0019] In another aspect the present invention provides methods of detecting a cell or a target in a cell of a subject or a biological sample of a subject, or detecting microbes in a subject or a biological sample of a subject, using a NIR label compound or bioconjugate as herein claimed, or a composition thereof.

[0020] In one embodiment, the present invention provides DDAO or 7-aminoDDAO compounds as NIR labels, in

particular, derivatives of small-size NIR fluorophore DDAOs, such as 7-amino-DDAO derivatives, their compositions, methods of their preparation and synthesis, and methods of using them for detecting microbial targets *in vitro*, *in situ*, and *in vivo*.

[0021] These DDAO compounds include, but are not limited to, thiol- and amine-reactive NIR fluorescent labels as well as other click-reactive (such as azido- or alkynyl) derivatives.

[0022] In another embodiment, the present invention provides diagnostic compounds containing pathogen-targeting molecules labeled with the DDAO-derived fluorophores.

[0023] In another embodiment, the present invention provides methods of detecting and monitoring infectious processes in a subject by using these new NIR labels.

[0024] In another embodiment, the present invention provides a method of detecting bacterial pathogens in a subject, such as in body tissues, by using the new NIR label, *in vitro* or *in vivo*.

[0025] In another embodiment, the present invention provides methods of *in vitro* fluorescent detection of the bacterial pathogens comprising use of the diagnostic probes. In another embodiment the present invention provides diagnostic probes comprising the DDAO NIR label and a pathogen-specific "address" molecule, and methods of using these diagnostic probes for detecting pathogens. These diagnostic probes and methods are useful for *in vivo* testing of the diagnostic probes with bacterial pathogens.

[0026] Also described are methods of synthesizing DDAO conjugates with antibacterial drugs as diagnostic probes.

[0027] The details of these and other aspects or of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028]

FIG. 1 depicts DDAO and the synthesis and light emission properties of 7-aminoDDAO and its cross-linkable derivatives.

FIG. 2 depicts antifungal drugs labeled with 7-aminoDDAO.

FIG. 3 illustrates the derivatization of poscanazole with 7-aminoDDAO.

FIG. 4 depicts the light absorption spectra of DDAO and 7-aminoDDAO derivatives in 50% MeOH.

FIG. 5 depicts fluorescence spectra for DDAO at pH 9.0 (A), 7-aminoDDAO (B), 7-aminoDDAO-Pos (C), and 7-aminoDDAO-Casp (D) in various solvents.

FIG. 6 depicts light transparency window for body tissues and the near infrared emission of 7-aminoDDAO.

FIG. 7 illustrates the light emission properties of Posconazole-7-aminoDDAO and Rifampicin-7-aminoDDAO derivatives.

FIG. 8 depicts the use of 7-aminoDDAO labeled casponfungin for imaging of infected mice kidneys.

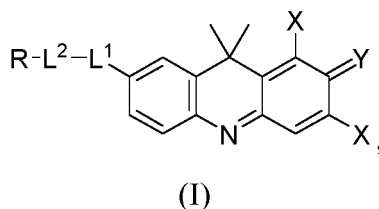
FIG. 9 depicts tomographic detection of *C. albicans* in live mice using fluorescent Casp-DDAO compound.

FIG. 10 illustrates synthesis and validation of light-emitting probes for diagnostic imaging of microbial infections.

DETAILED DESCRIPTION OF THE INVENTION

[0029] The present invention relates to derivatives and analogs of the fluorophore, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one), otherwise known as DDAO, methods of preparing and synthesizing DDAO derivatives, and the use of the DDAO derivatives as a label for detecting a target *in vitro*, *in situ* and *in vivo*.

[0030] There is disclosed a compound of formula (I):



or a pharmaceutically acceptable salt or solvate thereof, wherein:

X at each occurrence is independently a halogen;

Y is O, S, or NR^Y, wherein R^Y is hydrogen or C₁-C₄ alkyl;

L¹ is -NH- or -O-;

L^2 is a bond, a linear or branched alkylene, or a linear or branched heteroalkylene; and

R is hydrogen, a moiety of a biologically active molecule, or a functional group that can react with a biologically active molecule to form a covalent bond. Preferably, X, Y, L^1 , L^2 , and R are selected so that the compound is near-infrared fluorescent.

[0031] In one compound, the alkylene has from 1 to 20 carbon atoms, and the heteroalkylene has from 1 to 15 carbon atoms and from 1 to 5 heteroatoms independently selected from O, N and S.

[0032] In an embodiment of this aspect, L^2 is $-(CH_2)_m-$ or alternatively $-(CH_2CH_2O)_a-(CH_2)_b-$, wherein m is an integer selected from 1 to 10; a is 0 or an integer selected from 1 to 5, and b is an integer selected from 1 to 4.

[0033] In another embodiment of this aspect, R is $-NR^aR^b$, $-NCS$, $-NCO$, , amido, substituted or unsubstituted maleimido, or a click-reactive group (e.g., $-N_3$ or $-C\equiv CH$), or $-C(O)-R^1$;

wherein R^a and R^b are independently hydrogen or C_1-C_4 alkyl;

R^1 is $-N_3$ or $-OR^2$; and

R^2 is C_1-C_6 alkyl or succinimido.

[0034] In an embodiment of this aspect, R is a moiety of a biologically active small or large molecule.

[0035] In another embodiment of this aspect, the biologically active small molecule is an antibacterial or antifungal agent.

[0036] In another embodiment of this aspect, the biologically active large molecule is an antibody, an antigen, a carbohydrate, a peptide, a nucleic acid, a lipid, or a synthetic or natural polymer.

[0037] In another embodiment of this aspect, L^1 is $-NH-$.

[0038] In another embodiment, not claimed, L^1 is $-O-$.

[0039] In another embodiment of this aspect, L^1 is $-NH-$; and L^2 is $-(CH_2)_m-$, wherein m is 2 to 6.

[0040] In another embodiment of this aspect, L^1 is $-NH-$; and L^2 is $-CH_2CH_2CH_2CH_2-$.

[0041] In another embodiment of this aspect, Y is O.

[0042] In another embodiment, not claimed, Y is S.

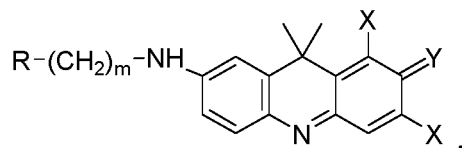
[0043] In another embodiment, not claimed, Y is NR^y , wherein R^y is H or C_1-C_4 alkyl.

[0044] In another embodiment of this aspect, both X are Cl

[0045] In another embodiment of this aspect, both X are Cl; and Y is O.

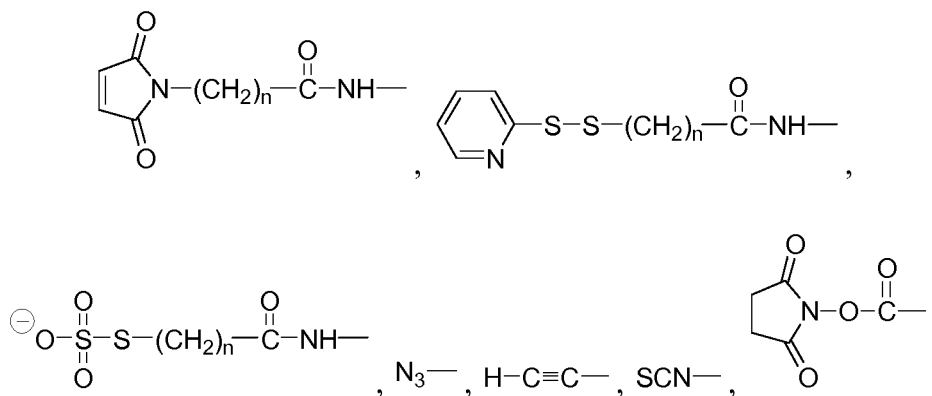
[0046] In another embodiment of this aspect, the compound is selected from the group consisting of 7-amino-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)(7-aminoDDAO), DDAO- $NH-(CH_2)_4-NH_2$, DDAO- $NH-(CH_2)_4-NCS$, DDAO- $NH-(CH_2)_4-NH-COCH_2X$ (wherein X is halogen), DDAO- $NH-(CH_2)_4-NH-3$ -maleimide, a conjugate between Caspofungin and DDAO, a conjugate between Posaconazole and DDAO, and a conjugate between Rifampicin and DDAO.

[0047] In another embodiment of this aspect, the compound has a formula (II):

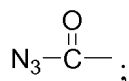


(II)

wherein R is selected from the group consisting of:



and



- 5 each X is independently a halogen;
Y is O,;
m is an integer selected from 1 to 10; and
n at each occurrence is independently an integer selected from 1 to 10.
- 10 **[0048]** In another embodiment of this aspect, each X is independently Cl,; m is 2 to 6; and n at each occurrence is 1 to 6.
[0049] In another embodiment of this aspect, L² is -(CH₂)_m-, wherein m is 2 to 6.
[0050] In another embodiment of this aspect, L² is -CH₂CH₂CH₂CH₂-.
[0051] In another embodiment of this aspect, Y is O.
[0052] In another embodiment, not claimed, Y is S.
- 15 **[0053]** In another embodiment, not claimed, Y is NR^y, wherein R^y is H or C₁-C₄ alkyl.
[0054] In another embodiment of this aspect, both X are Cl
[0055] In another embodiment of this aspect, both X are Cl; and Y is O.
[0056] In another embodiment of this aspect, both X are Cl; Y is O; m is 2 to 4; and n at each occurrence is 2 to 4.
[0057] In other embodiments, the present invention encompasses any other combinations of the structural features
- 20 described herein.
[0058] In another aspect, the present invention provides a composition comprising a compound according to any embodiments described herein, and a pharmaceutically acceptable carrier.
[0059] In another aspect the present invention provides a bioconjugate comprising a targeting agent, a moiety of a DDAO or 7-aminoDDAO derivative, and a linking group, wherein the targeting agent is an agent capable of detecting a
- 25 target of interest in a biological sample or a subject.
[0060] In one embodiment of this aspect, the targeting agent is an antibody, an antigen, a carbohydrate, a peptide, a nucleic acid, a lipid, a synthetic or natural polymer, a small molecule, or a combination thereof.
[0061] In another embodiment of this aspect, the target is a glycoprotein, lipopolysaccharide, lipopeptide, a component of a cell wall, a receptor, or a combination thereof.
- 30 **[0062]** In another embodiment of this aspect, the linking group is selected from peptide linkers, self-immolative linkers, acid sensitive linkers, multifunctional organic linking agents, bifunctional inorganic crosslinking agents, polymers, and combinations thereof.
[0063] In another embodiment of this aspect, the linking group is stable, biodegradable, and/or cleavable under physiological conditions.
- 35 **[0064]** In another embodiment of this aspect, the targeting agent is a small molecule and/or a drug.
[0065] In another embodiment of this aspect, the small molecule is an antibacterial, or antifungal agent.
[0066] In another aspect the present invention provides a composition comprising a bioconjugate according to any embodiments described herein, and a pharmaceutically acceptable carrier.
[0067] In another aspect the present invention provides a method of detecting a cell or a target in a cell of a subject,
- 40 comprising administering to the subject a compound and/or a bioconjugate according to any embodiment described herein, and detecting NIR fluorescence of the compound or the bioconjugate.
[0068] In another embodiment of this aspect, the compound is a conjugate between a DDAO derivative and a targeting agent.
[0069] In another embodiment of this aspect, said detecting comprises use of an imaging device.
- 45 **[0070]** In another aspect the present invention provides a cell or a target in a cell of a biological sample, comprising contacting the biological sample with a compound according to any embodiment of the present invention, and detecting NIR fluorescence of the compound.
[0071] In another embodiment of this aspect, the compound is a conjugate between a DDAO derivative and a targeting agent.
- 50 **[0072]** In another aspect the present invention provides a method of detecting a microbe in a subject, comprising administering to said subject a compound and/or a bioconjugate according to any embodiment described herein, and detecting NIR fluorescence of the compound or the bioconjugate.
[0073] In one embodiment of this aspect, the compound or bioconjugate comprises a DDAO or 7-aminoDDAO moiety.
[0074] In another embodiment of this aspect, the subject is administered a pretreatment antimicrobial drug prior to the
- 55 administration of said compound or bioconjugate.
[0075] In another embodiment of this aspect, said detecting comprises using an imaging device.
[0076] In another embodiment of this aspect, the imaging device is selected from the group consisting of an x-ray imaging device, an infrared imaging device, fluorescent imaging device, nuclear magnetic resonance imaging device,

magnetic resonance spectroscopy device, and a positron emission tomography device.

[0077] In another aspect the present invention provides a kit for detecting a microbe in a biological sample or a subject comprising a compound or bioconjugate according to any one of embodiments described herein, and instructions for use.

5 DDAO Derivatives

[0078] In one embodiment the present invention provides DDAO derivatives that contain an amine or amino group at position 7 instead of a hydroxyl group. In a preferred embodiment the hydroxyl group is replaced with a moiety having the following formula $\text{NH}-(\text{CH}_2)_m\text{-A}$, wherein $m=1\text{-}10$ and $\text{A} = -\text{NH}_2$, $-\text{NCS}$, haloacetamido, acylmaleimido, or click-reactive (e.g., azido or alkynyl group). $(\text{CH}_2)_m$ may also be replaced with another spacer or polymer such as polyethylene glycol or other polymers that have the same properties and length. A DDAO fluorophore that contains an amino group at position 7 is herein referred to as 7-aminoDDAO. 7-aminoDDAO derivative (compound II of FIG. 1) has many superior properties compared to other NIR fluorophores: (1) it is small in size ($\text{FW} = 294$); (2) has long-wavelength emission (640-700 nm); (3) exhibits fair brightness; (4) is pH-independent; (5) has emission maximum 680 nm, at which the body tissues are the most transparent; and (6) can be easily attached to a molecule of interest either directly, or through its simple conversion to amine-, thiol-, or click-reactive forms. In contrast, DDAO itself is pH-dependent due to the presence of a phenol hydroxyl group, and has emission maximum at 660 nm, a molar absorbance of 48,000 (vs. 53,000 for 7-aminoDDAO), and a brightness of 3,600 (vs. 6,000 for 7-aminoDDAO).

[0079] The synthetic protocol for compound II includes simple one-step conversion, followed by fast, non-chromatography purification. In a further embodiment, the present invention provides DDAO derivatives that are 1.4 - 2.3 fold brighter than original DDAO. DDAO derivatives may be used for covalent labeling of a biomolecule of interest, such as a targeting agent. The synthetic intermediate 7-(4-aminobutyl)aminoDDAO can be easily converted to other reactive forms (e.g. thiol-, or click-reactive), which are useful for bioconjugation, using methods known in the art. The structures of some of these reactive compounds (III-V) are shown in FIG. 1.

[0080] In a further embodiment, the present invention provides compositions comprising DDAO derivatives, which may be directly conjugated or covalently attached to a targeting agent, or attached to a targeting agent using a linker. The targeting agent conjugated to a DDAO derivative may be used to detect a target in a biological sample, such as a target in a cell or in a subject. The targeting agent may be any agent capable of detecting a target of interest in a biological sample, including for example an antibody, an antigen, a carbohydrate, a peptide, a nucleic acid, a synthetic or natural polymer, a small molecule or a combination thereof. The target may be a peptide, nucleic acid, antibody, antigen, carbohydrate, peptide, lipid, or a combination thereof, such as a glycoprotein, lipopolysaccharide, lipopeptide, or a component of a cell wall or a receptor.

[0081] As used herein, the phrase "near-infrared fluorescent" or the like refers to the property of a molecule that emits in the range of about 600 to 800 nm, with an emission maximum in the range of about 650 to 750 nm, preferably emitting in the range of 650-750 nm, with an emission maximum about 660 to 700 nm, and more preferably having an emission maximum around 680 nm.

[0082] The term "antibody" refers to an immunoglobulin or antigen-binding fragment thereof, and encompasses any such polypeptide comprising an antigen-binding fragment of an antibody. The term includes but is not limited to polyclonal, monoclonal, monospecific, polyspecific, humanized, human, single-chain, single-domain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies. The term "antibody" also includes antigen-binding fragments of an antibody. Examples of antigen-binding fragments include, but are not limited to, Fab fragments (consisting of the VL, VH, CL and CH1 domains); Fd fragments (consisting of the VH and CH1 domains); Fv fragments (referring to a dimer of one heavy and one light chain variable domain in tight, non-covalent association); dAb fragments (consisting of a VH domain); single domain fragments (VH domain, VL domain, VHH domain, or VNAR domain); isolated CDR regions; (Fab')₂ fragments, bivalent fragments (comprising two Fab fragments linked by a disulphide bridge at the hinge region), scFv (referring to a fusion of the VL and VH domains, linked together with a short linker), and other antibody fragments that retain antigen-binding function.

[0083] The terms "polypeptide", "peptide", "protein", and "protein fragment" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, "nucleic acid" or "oligonucleotide" or "polynucleotide" or "nucleotide sequence" means at least two nucleotides covalently linked together. Nucleic acids may be single stranded or double stranded, or may contain portions of both double stranded and single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribonucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine. Nucleic acids may be obtained by chemical synthesis methods or by recombinant methods.

[0084] As used herein, the term "linker" or "linking group" refers to a chemical moiety that connects a molecule to

another molecule, covalently links separate parts of a molecule or separate molecules. The linker provides spacing between the two molecules or moieties such that they are able to function in their intended manner. Examples of linking groups include peptide linkers, enzyme sensitive peptide linkers/linkers, self-immolative linkers, acid sensitive linkers, multifunctional organic linking agents, bifunctional inorganic crosslinking agents, polymers and other linkers known in the art. The linker may be stable or degradable/cleavable. Various linkers are known in the art.

[0085] As used here, the term "biologically active compound" or the like refers to a compound having a physiological or biological effect on animals or humans, including but not limited to naturally occurring, semi-synthetic, or synthetic small or large molecule drugs, as well as cellular metabolites and signaling molecules (e.g. hormones, pheromones, etc.). Biologically active compounds capable of coupling to a DDAO or 7-aminoDDAO derivatives or analogs possess at least one functional group (e.g., hydroxyl, amine, or carboxyl, etc.) that can react with a DDAO or 7-aminoDDAO derivative or analog to form a conjugate. The method of forming such conjugate should be apparent to a person skilled in the art based on the present disclosure. Preferred biologically active compounds for the present invention include those that can serve as targeting agents for certain desired purposes, for example, binding to a specific target.

[0086] A biologically active compound can be a small molecule therapeutic agent, such as an antibacterial or antifungal agent. Examples of antibacterial agents include, but are not limited to, aminoglycosides, amikacin, gentamicin, kanamycin, neomycin, netilmicin, tobramycin, paromomycin, spectinomycin, ansamycins, geldanamycin, herbimycin, rifaximin, streptomycin, carbacephem, loracarbef, carbapenems, ertapenem, doripenem, imipenem, cilastatin, meropenem, cephalosporins, cefadroxil, cefazolin, cefalotin, cefalothin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefixime, cefdinir, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftaroline fosamil, ceftobiprole, glycopeptides, teicoplanin, vancomycin, telavancin, lincosamides, clindamycin, lincomycin, lipopeptide, daptomycin, macrolides, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spiramycin, monobactams, aztreonam, nitrofurans, furazolidone, nitrofurantoin, oxazolidinones, linezolid, posizolid, radezolid, torezolid, penicillins, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, piperacillin, temocillin, ticarcillin, bacitracin, colistin, polymyxin b, quinolones, fluoroquinolone, ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, sulfonamides, mafenide, sulfacetamide, sulfadiazine, silver sulfadiazine, sulfadimethoxine, sulfamethizole, sulfamethoxazole, sulfasalazine, sulfisoxazole, trimethoprim-sulfamethoxazole (co-trimoxazole) (tmp-smx), tetracyclines, demeclocycline, doxycycline, minocycline, oxytetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, pyrazinamide, rifampicin, rifabutin, rifapentine, streptomycin, arspenamine, chloramphenicol, fosfomycin, fusidic acid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, thiamphenicol, tigecycline, tinidazole, and trimethoprim.

[0087] Examples of antifungal agents include, but are not limited to, natamycin, rimocidin, filipin, nystatin, amphotericin B, candicin, miconazole, ketoconazole, clotrimazole, econazole, bifonazole, butoconazole, fenticonazole, isoconazole, oxiconazole, sertaconazole, sulconazole, tioconazole, fluconazole, itraconazole, isavuconazole, ravuconazole, posaconazole, voriconazole, terconazole, abafungin, terbinafine, amorolfine, naftifine, butenafine, anidulafungin, caspofungin, and micafungin.

[0088] Biologically active compounds also include large molecules, including but not limited to antibodies, antigens, carbohydrates, proteins, polypeptides, nucleic acids, lipids, or synthetic or natural polymers.

[0089] As used herein, the term "targeting agent" or the like refers to a moiety that recognizes, binds or adheres to a target molecule located, for example, in a cell, tissue, organism, or subset thereof. A targeting agent and its target molecule represent a binding pair of molecules, which interact with each other through any of a variety of molecular forces such as ionic, covalent, metal ions-mediated coordination, hydrophobic, van der Waals, and hydrogen bonding, so that the pair have the property of binding specifically to each other as known in the art.

[0090] The terms "group," "functional group," "moiety," "molecular moiety," or the like are somewhat synonymous in the chemical arts and are used to refer to distinct, definable portions or units of a molecule, and to units that perform some function or activity and are reactive with other molecules or portions of molecules. As used herein, a "moiety" or "molecular moiety" of a biologically active compound refers to a major portion of the biologically active compound that is expected to retain the function of the parent molecule when it forms a conjugate with a DDAO or 7-aminoDDAO derivative or analog. Examples of reactive functional groups that are suitable for the present invention include, but are not limited to, hydroxyl, amino, thiol, disulfide, carboxyl, carboxylate ester, sulfonic acid, sulfonate ester, amido, alkynyl, alkenyl, azido, cyanate, isocyanate, thiocyanate, isothiocyanate, nitrile, isonitrile, or the like.

[0091] As used herein, the term "alkylene" refers to a straight or branched aliphatic divalent hydrocarbon group, in one embodiment having from 1 to about 20 carbon atoms. In a further embodiment alkylene includes lower alkylene (typically C₁-C₆, and in some embodiments, preferably C₁-C₄).

[0092] As used herein, the term "heteroalkylene" refers an alkylene group wherein one or more carbon atoms in its backbone are replaced by heteroatoms, such as O, N, and S, which includes, e.g., an oligomeric ethylene glycol moiety. Both alkylene and heteroalkylene groups can be substituted by one or more substituents, such as hydroxyl, halogen,

amino, nitro, lower alkyl, C₁-C₄alkoxy, or the like.

[0093] As used herein, the term "halo" or "halogen" refers to F, Cl, Br, or I.

[0094] As used herein, the term "click-reactive group" or the like refers to a functional group that can participate in certain highly specific reactions (called bioorthogonal, since the reactants link only to each other, but not to any other biomolecules, or media components) to link two molecular moieties together, which has been called "click chemistry." Examples of "click-reactive groups include, but are not limited to, azido and terminal alkynyl group, which can be linked to each other in reactions such as: i) copper-free (with strained alkyne), or copper-catalyzed Huisgen cycloaddition or the 1,3- dipolar cycloaddition between an azide and a terminal alkyne to form a 1,2,4-triazole, ii) a Diels-Alder reaction, which is a cycloaddition reaction between a diene and a dienophile, and/or iii) nucleophilic substitution reactions in which one of the reactive species is an epoxy or aziridine compound with significant ring strain. See, e.g., US 20050222427 (Sharpless, et al.). Any clickable compound capable of linking a DDAO derivative with a biologically active compound is regarded as being within the scope of the present invention.

[0095] In certain embodiments the targeting agent may be a small molecule and/or a drug. Examples of small molecules include antifungal agents such as echinofungins and triazoles.

[0096] The DDAO derivatives of the invention may be formulated as a pharmaceutical composition, and may be administered to a mammalian host, such as a human patient, in a variety of forms adapted to the chosen route of administration, i.e., orally or parenterally, by intravenous, intramuscular, topical, subcutaneous, or other routes. Thus, the pharmaceutical composition of the invention may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent. They may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the compositions of the invention may be used in the form of elixirs, syrups, and the like.

[0097] Any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. To administer the pharmaceutical composition to a patient, it is preferable to formulate the molecules in a composition comprising one or more pharmaceutically acceptable carriers. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce allergic, or other adverse reactions when administered using routes well-known in the art. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like.

[0098] The pharmaceutical composition of the present invention can be administered to a subject by any of a number of means known in the art. A "subject" refers to a human and a non-human animal. Examples of a non-human animal include all vertebrates, e.g., mammals, such as non-human primates (particularly higher primates), dog, rodent (e.g., mouse or rat), guinea pig, cat, and non-mammals, such as birds, amphibians, reptiles, etc. In a preferred embodiment, the subject is a human. In another embodiment, the subject is an experimental animal or animal suitable as a disease model. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[0099] The pharmaceutical composition of the invention may also be administered intravenously or intra-peritoneally by infusion or injection, among many other routes. Solutions may be prepared, for example, in water. However, other solvents may also be employed. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms, and other formulation ingredients as is known in the art.

[0100] The pharmaceutical dosage forms suitable for injection or infusion should be preferably sterile, fluid and stable under the conditions of manufacture and storage. The prevention of the action of microorganisms may be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Others are also suitable. In many cases, it may be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride.

[0101] Sterile injectable solutions may be prepared by incorporating the pharmaceutical composition of the invention in the required amount into an appropriate solvent or medium with various other ingredients, e.g., those enumerated above, as needed, which may be followed by sterilization. The above-described pharmaceutical composition containing the nanoparticles can be used to treat cancer.

[0102] In a further embodiment, the present invention provides a method to detect a target in a cell of interest comprising contacting a cell with a pharmaceutical composition comprising a DDAO derivative attached to a targeting agent.

[0103] In a further embodiment, the present invention provides a method to detect a cell comprising contacting a cell in a biological sample with a pharmaceutical composition comprising a DDAO derivative attached to a targeting agent. One with ordinary skill in the art will adapt the proper modality to detect pharmaceutical compositions as described. Such modalities include Fourier transform infrared spectroscopy.

[0104] In a further embodiment, the present invention provides a method to detect a target in a subject comprising administering to a subject a pharmaceutical composition comprising a DDAO derivative attached to a targeting agent. One with ordinary skill in the art will adapt the proper modality to detect pharmaceutical compositions as described. Such modalities include Fourier transform infrared spectroscopy.

[0105] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly

understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described.

[0106] The following non-limiting examples set forth herein below illustrate certain aspects of the invention.

EXAMPLES

Synthesis of DDAO-Based Fluorescent Derivatives

Example 1. DDAO-NH-(CH₂)₄-NH₂ (compound II of FIG. 1)

[0107] 10 mg DDAO (7-hydroxy-9H (1,3-dichloro-9,9-dimethyl acridin-2-one)) (33 μmol) was dissolved in 100 μl 1M diaminobutane diacetate in 80 % aqueous DMSO.

[0108] After 10 h incubation at 95 °C TLC analysis in acetonitrile-water (14:1) developing system detected intense-blue colored product migrating lower (R_f = 0.45) than the original product (R_f = 0.9). The reaction mixture was supplemented with 2 ml of water and extracted with ethylacetate (3 x 5 ml). The pH of the water layer was adjusted to 11-11.5 by 10 M KOH followed by extraction with ethylacetate (2 x 5 ml). The organic layer was collected and evaporated to dryness under reduced pressure affording 4 mg of compound I. UV λ_{max} = 673 nm (ε = 53 000 M⁻¹cm⁻¹). MS: DDAO-NH-(CH₂)₄-NH₂ (+1) 378.0887 (found); 378.288 (calculated).

Example 2. DDAO-NH-(CH₂)₄-NCS (compound III of FIG. 1)

[0109] 15 mM Solution of compound I in 150 μl of DMSO was supplemented with 2 molar equivalent of 1,1-thiocarbonylimidazole dissolved in 0.2 ml of chloroform. After 30 min incubation at room temperature the mixture was supplemented with 1 ml of trifluoroacetic acid and incubation continued at 40 °C for another 45 min. TLC analysis in hexane - acetone (2:1) developing system showed complete conversion of the original compound (R_f = 0) to reaction product (R_f = 0.5). The reaction mixture was diluted by water and the product extracted in chloroform. The solvent was removed by evaporation in vacuo, and the residue dried by co-evaporation with acetonitrile. The product was purified by preparative TLC in hexane-acetone (2:1) developing system. Yield: 4 μmol. UV: λ_{max} = 673 nm (ε = 53 000 M⁻¹cm⁻¹). MS: DDAO-NH-(CH₂)₄-NCS (+1) 420.0453 (found); 420.352 (calculated).

Example 3. DDAO-NH-(CH₂)₄-NH-AcBr (compound IV of FIG. 1)

[0110] Equivalent amounts of *p*-nitrophenyl bromoacetate and compound I in DMF (0.46 μmol) were mixed and left for 5 minutes at room temperature. The sky blue product with R_f = 0.58 was purified by TLC in ethyl acetate developing system. The product was eluted by 100% MeOH and evaporated to final concentration 2.16 mM. Yield: 0.22 μmol (~48%). UV: λ_{max} = 673 nm (ε = 53,000 M⁻¹cm⁻¹). MS: DDAO-NH-(CH₂)₄-NH-AcBr 499.045(found) 499.213 (calculated). DDAO-NH-(CH₂)₄-NH-AcBr (+H) 500.044 (found); 500.221 (calculated).

Example 4. DDAO-NH-CH₂)₄-NH-3MI (compound V of FIG. 1)

[0111] Equivalent amounts of compound I (0.46 μmol) and N-succinimidyl-3-maleimidopropionate (Mlpr-OSuc) were mixed in 122 μL DMF and kept at 0°C for 15 minutes. TLC analysis in a 100% ethyl acetate developing system revealed two products, blue (R_f=0.5) and one purple. The Blue product was purified by preparative TLC in ethyl acetate developing system and eluted with MeOH. The eluate was concentrated by evaporation under reduced pressure affording 0.1 ml of 1.37 mM solution. Yield: 0.14 μmol (~30%). UV: λ_{max} = 673 nm (ε = 53,000 M⁻¹cm⁻¹). MS: DDAO-NH-(CH₂)₄-NH-3MI 529.154 (found); 529.40 (calculated). DDAO-NH-(CH₂)₄-NH-3MI (+H) 530.167 (found); 530.40 (calculated).

Example 5. Caspofungin - DDAO derivative (FIG. 2, top)

[0112] Caspofungin (2.6 mg, 2 μmol) was dissolved in the solution of 230 μl of 5 mM DDAO-NH-(CH₂)₄-NCS in DMF and 0.5 μl of triethylamine (TEA) was added followed by incubation at 60 °C for 90 min. TLC in acetonitrile-water (5:1) developing system detected a blue-colored reaction product with R_f = 0.65. R_fs for caspofungin and DDAO-NH-(CH₂)₄-NCS were 0.48 and 1.0 respectively. The product was purified by preparative TLC in acetonitrile - water (7:1) developing system, eluted by 50 % aqueous methanol and the solution evaporated under reduced pressure to final concentration 0.33 mM. Yield: 50%. UV: λ_{max} = 673 nm (ε = 53 000 M⁻¹cm⁻¹). DDAO-NH-(CH₂)₄-NCS-Caspofungin (+H) 1515.7242 (found); 1515.673 (calculated).

Synthesis of Posaconazole-DDAO Derivatives (FIG. 2, bottom).**Example 6. Compound III (FIG. 3).**

[0113] A solution of 38 mg of posaconazole in 0.3 ml of DMSO was supplemented with 70 mg of succinic anhydride and 60 ml of methylimidazole. After 5 h incubation at 45° C TLC analysis in chloroform - ethanol - 20 % ammonium hydroxide (100:10:1) revealed ca. 95 % conversion of posaconazole to acylation product. The mixture was poured dropwise into 3 ml of 1 M citric acid and left for 30 min at 4° C. The precipitate was collected by centrifugation, washed with water (4 x 3 ml) and dried in vacuo under gentle heating. Yield: 30 mg. High resolution MS (MALDI) *m/z*: anal. calc. for $C_{41}H_{46}N_8O_7F_2$ 800.87; found 799.3.

Example 7. Compound IV (FIG. 3).

[0114] To a solution of 30 mg of Compound I in 0.3 ml of THF were added 10 mg of 4-nitrophenol and 25 mg of DCC. After 30 min incubation at 20° C TLC analysis in a chloroform-ethanol (3:1) developing system revealed nearly quantitative conversion of compound III to reaction product. The precipitate was removed by centrifugation, and the reaction mixture to evaporated to dryness under reduced pressure. High resolution MS (MALDI) *m/z*: anal. calc. for $C_{47}H_{49}N_9O_9F_2$ 921.97; found 923.4.

Example 8. Posaconazole-DDAO derivative.

[0115] Two milligrams of compound IV of FIG. 2 were dissolved in 0.1 ml of 20 mM solution of compound I of FIG. 1. The mixture was supplemented with 2 μ l of triethylamine and left for 20 min at room temperature. TLC analysis in ethylacetate - ethanol (8:1) developing mixture revealed complete conversion of compound I to reaction product. The mixture was diluted by 2 ml of water, and the residue was collected by centrifugation, dissolved in DMF and subjected to preparative TLC in the same system. Yield: 0.5 μ mol.

Example 9. Derivatization of Core DDAO Compounds

[0116] To derivatize core DDAO compound Hamilton reaction previously discovered with simpler phenol-, or naphthol-derivatives was used (Malmberg, E., W., Hamilton, C., S., J. Am. Chem. Soc. 70, 2415, (1948); Willenz, J. J. Chem. Soc., 1955, 2049). The reaction included acid-catalyzed attack of amino-compounds on mesomeric keto-form of the aromatic hydroxy-derivatives. The reaction product with 1,4-diaminobutane (FIG. 1) was obtained with high yield and purified by extraction. The resulting DDAO amino-derivative was converted to corresponding isothiocyanate (ITC) (compound III) by treatment with thiocarbonyldiimidazole followed by incubation with trifluoroacetic acid (FIG. 1). Thiol-reactive DDAO derivatives (compounds IV and V) were obtained by treatment of compound II with 4-nitrophenylbromoacetate, or succinimide ester of 3-maleimidopropionic acid respectively. Obtained 7-aminoDDAO derivative III was used to label antifungal drugs posaconazole and caspofungin (FIG. 2). Caspofungin was derivatized by the ITC in single-step reaction as one of the drugs two aliphatic amino groups. To introduce DDAO fluorescent label in posaconazole molecule the drug was first acylated at hydroxyl group by succinic anhydride in DMSO in the presence of nucleophilic catalyst, N-methylimidazole (FIG. 3). The resulting product was converted to an activated ester by incubation with 4-nitrophenol and DCC. This synthetic intermediate was introduced in reaction with 1,4-diaminobutyl-DDAO compound to yield the final product, which was purified using preparative TLC.

Example 10. Light absorption and fluorescent spectra of 7-aminoDDAO, caspofungin-DDAO, and posaconazole-DDAO derivatives

[0117] Modification of DDAO (FIG. 4) resulted in detectable blue shift of the light absorption maximum (653 nm and 673 nm correspondingly). The molar extinction of 7-aminoDDAO (55 000 M⁻¹cm⁻¹) was determined by the attachment of reference chromophores with known molar absorptivity (Table 1). Light absorption spectra of the labeled caspofungin and posaconazole derivatives were close to superposition of those for the 7-(4-aminobutyl)amino-DDAO and the corresponding drugs, which is illustrated in FIG. 4 on the example of posaconazole derivative. The fluorescence spectra (FIG. 5B) of 7-(4-aminobutyl)amino-DDAO exhibited blue shift compared to ionized form of DDAO (FIG. 5A). Thus, excitation and emission maxima for DDAO were 653 nm and 660 nm correspondingly, while for 7-(4-aminobutyl)amino-DDAO they shifted to 671 nm and 679 nm correspondingly. This beneficial spectral shift brings the emission maximum in the region where the body tissues are the most transparent, see FIG. 6, which shows light absorption spectrum of body tissue and emission properties of reporter DDAO fluorophore. 7-Amino DDAO is chemically and photochemically stable, and thus reduces photo bleaching. Increasing content of the organic solvent (MeOH) resulted in enhancement

of the light emission and characteristic change in excitation spectrum. Thus substitution 50 % methanol for water did not affect the shape of the excitation spectrum for 7-aminoDDAO, but increased the light emission ca. 2.5 fold. Placing the compound in 100 % MeOH resulted in dramatic change of the excitation spectrum profile shifting the maximum from 670 nm to 620 nm, while only slightly shifting emission maximum from 680 to 670 nm. Notably, the light emission intensity dropped 1.7 fold. Remarkably, the shape of the excitation spectrum curve for ionized form of DDAO was the same in 50 % and 100 % methanol. Also, in contrast to 7-aminoDDAO 1.3 fold increase in the emission was observed in 100 % methanol compared to 50 % methanol.

[0118] Similar trends in fluorescence spectra were observed for the 7-aminoDDAO fluorophore attached to caspofungin. As seen from FIG. 5D for the labeled drug the emission in 50 % methanol increased 2 fold compared to the emission in water, however contrary to the free 7-aminoDDAO dye in 100 % methanol further 1.3 fold increase was observed for caspofungin derivative emission. However, posaconazole derivative behaved differently. Thus in water the emission was completely quenched (FIG. 5C). The presence of 50 % methanol in the medium greatly increased the emission, which was further (ca. 3 fold) enhanced in 100 % methanol. The suppression of the fluorophore light emission in posaconazole derivative is likely due to the presence of extended light-absorbing moiety that can stack to the fluorophore, causing contact quenching (FIG. 7). The same change in fluorescence is expected upon interaction of the labeled drug with its cellular target, whereby stacking interactions of the fluorophore with the drug moiety have to be destroyed (FIG. 7B). This beneficial "signaling" effect is expected to greatly increase the contrast of imaging by minimizing the background signal of non-bound compound, which remains "dark".

Table 1. Molar absorption of 7-(4-aminobutyl)aminoDDAO calculated by using various chromophores attached to the aliphatic amino group.

Attached compound	Molar absorption for attached compounds (ϵ / M ⁻¹ cm ⁻¹)	Calculated molar absorption for DDAO residue (ϵ / M ⁻¹ cm ⁻¹)
Posaconazole	34,000	59,000
DNP	17,600	32,000
BODIPY-FL	82,000	70,000
	Average	53,000

Table 2. Relative Brightness (RB) and quantum yield (QY) in 3 different solvents for DDAO and its derivatives at [1 μ M].

Compounds	(ϵ / M ⁻¹ cm ⁻¹)	RB (H ₂ O)	RB (50% MeOH)	RB (MeOH)	QY (H ₂ O)	QY (50% MeOH)	QY (MeOH)
DDAO (pH 9.0)	48,000	6,000	9,000	12,000	0.125	0.19	0.25
DDAO NH ₂	53,000	10,000	21,000	13,000	0.19	0.40	0.25
DDAO Pos	53,000	0.0	3,300	13,000	0.0	0.16	0.24
DDAO Casp	53,000	6,000	13,800	22,000	0.11	0.26	0.42
Cy 5.5 (Ref)	250,000	57,500	N/A	N/A	0.23	N/A	N/A

[0119] Table 2 illustrates light emission properties of DDAO-based diagnostic probes and reference compounds, and presents the data on quantum yield and brightness for 7-aminoDDAO compound at various conditions. These parameters were determined using a reference fluorophore Cy5.5, (which has the excitation and emission maxima nearly identical to those for 7-aminoDDAO) and published data for original DDAO fluorophore. It is seen that synthesized 7-aminoDDAO derivative possessed higher brightness ($\phi = 10\,000$) compared to ionized original DDAO compound ($\phi = 6\,000$). This was perhaps due to higher light absorptivity and higher quantum yield of the amino-derivative. The difference in brightness further increased in 50 % methanol (21 000 and 9000 correspondingly). However, in 100 % methanol the brightness became comparable (13 000 and 12 000 correspondingly).

Example 11. Use of caspofungin-DDAO derivative for imaging of fungal infections in mice

[0120] FIG. 8 shows the results on imaging of mice kidney infected with a fungal pathogen along with control images. Mice were infected via intravenous inoculation with 5×10^5 CFU of wild type *Candida albicans* and an infection that occurs most prominently in the kidneys. After 48 hours post infection, a fixed concentration of 0.12 μ g/mL of CSF-DDAO is

added via tail vein injection at 0, 2, 4 and 8 hours to assess the optimal time for visualization of the infection. At each time point, the mice were imaged in a non-invasive whole-body animal imaging system to detect fluorescence energy. Animals infected with *Candida albicans* show proliferation of the fungal infection in the kidneys after 48 hours. The addition of CSF probe resulted in progressive labeling of cells in the target organs over time, as determined by whole body imaging. Maximum labeling occurred at 8 hours. CSF-DDAO did not accumulate in the kidneys in the absence of infection. It is seen that treatment of the infected organ with caspofungin-7-aminoDDAO derivative resulted in bright fluorescent signal. At the same time no signal was detected in uninfected and/or untreated organs.

[0121] The tomographic detection of *C. albicans* in live mice using fluorescent CSF-DDAO compound is shown in FIG. 9. Mice were infected and treated with caspofungin-containing diagnostic probe essentially as described in the previous section, followed by FMT imaging after indicated time elapsed from the infection event. The animals imaging was performed after 2 or 10 hours following the injection of the diagnostic probe. Peak signal intensity is presented for each scan.

Example 12. Synthesis of Rifampicin-DDAO compound.

[0122] Rifamycin B (0.1 mmol) in 0.5 ml of anhydrous THF was supplemented with 0.5 mmol of DCC and kept at room temperature for 30 min, followed by addition of 0.5 mmol of 1,6-diaminohexane. After 10 min incubation aminoethyl derivative of Rif was purified by TLC in a chloroform/ethanol developing system (2:1). The product was eluted by methanol and evaporated to dryness in vacuo. Aminoethyl Rif derivative and NCS-DDAO compound III of FIG. 1 (1 mmol of each) were dissolved in 0.05 ml of DMF and mixed. After incubation at 60° C for 1 h the Rif-DDAO adduct was purified by TLC in chloroform-ethanol developing system (3:1), eluted by methanol and evaporated to dryness under reduced pressure. The residue was dissolved in DMSO (0.2 ml) and kept at -80° C. Yield: 40 %.

Example 13. Determination of minimal inhibitory concentration (MIC) for Rif-DDAO compound with *B. subtilis* and *S. aureus*.

[0123] This was performed by using serial dilution method in 96-well plates with starting concentration of bacteria 10⁵ CFU/ml. The bacterial growth was inspected visually after 12 h incubation at 37° C. The results are shown in FIG. 10.

Example 14. Imaging of cultured *B. subtilis* cells using Rif-DDAO derivative.

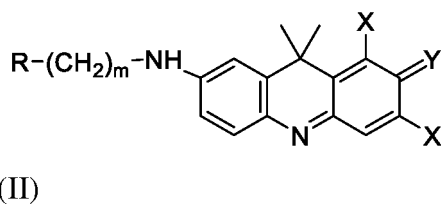
[0124] Bacteria were grown to the density ~0.2 a.u./ml followed by addition of Rif-DDAO compound to final concentration 0.5 μM. After incubation for 30 min the cells were collected by centrifugation and imaged using fluorescent microscopy. The results are presented in FIG. 10.

[0125] The synthesized DDAO derivatives, in particular 7-aminoDDAO derivatives, are excellent candidates for bio-imaging. This has been successfully demonstrated by visualization of a fungal pathogen in mice kidney. Beneficial spectral properties of the fluorophore combined with its small size make it useful in design of various diagnostic affinity probes in cellular studies as well as in biomedical applications.

[0126] The foregoing description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention as defined by the claims. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. The following claims define the scope of the invention.

Claims

1. A compound of formula II:



or a pharmaceutically acceptable salt or solvate thereof, wherein:

X at each occurrence is a chlorine;
Y is O;
m is an integer selected from 1 to 10;
and

R is a moiety of a biologically active molecule, or a functional group that can react with a biologically active molecule to form a covalent bond.

2. The compound of claim 1, wherein:

R is $-NR^aR^b$, $-NCS$, $-NCO$, C_1-C_6 alkyl, amido, substituted or unsubstituted maleimido, a click-reactive group, or $-C(O)-R^1$;
wherein R^a and R^b are independently hydrogen or C_1-C_4 alkyl;
 R^1 is $-N_3$ or $-OR^2$; and
 R^2 is C_1-C_6 alkyl or succinimido.

3. The compound of claim 2, wherein said click-reactive group is $-N_3$ or $-C\equiv CH$.

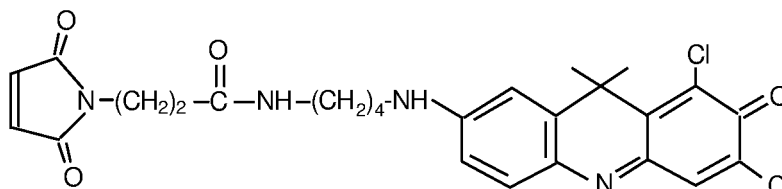
4. The compound of any one of claims 1 or 2, wherein R is a moiety of a biologically active molecule.

5. The compound of claim 4, wherein said biologically active molecule is

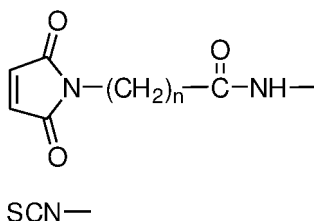
- (i) a small molecule and/or a drug, or an antifungal agent or
- (ii) a large molecule selected from an antibody, an antigen, a carbohydrate, a peptide, a nucleic acid, a lipid, or a synthetic or natural polymer.

6. The compound of any one of claims 1 to 5, wherein $m=4$.

7. The compound of claim 1, selected from the group consisting of 7-amino-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)(7-aminoDDAO), DDAO-NH-(CH_2)₄-NH₂, DDAO-NH-(CH_2)₄-NCS, DDAO-NH-(CH_2)₄-NH-COCH₂X (wherein X is halogen), DDAO-NH-(CH_2)₄-NH-3-maleimide, and



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



NH_2 , NCS , halideacetamido, acylmaleimido, a click-reactive azido, and an alkyn group, wherein n at each occurrence is independently an integer selected from 1 to 10.

9. A bioconjugate comprising a targeting agent, a moiety of a 7-aminoDDAO, and a linking group through which said 7-aminoDDAO is coupled to said targeting agent, wherein the targeting agent is an agent capable of detecting a target of interest in a biological sample or a subject.

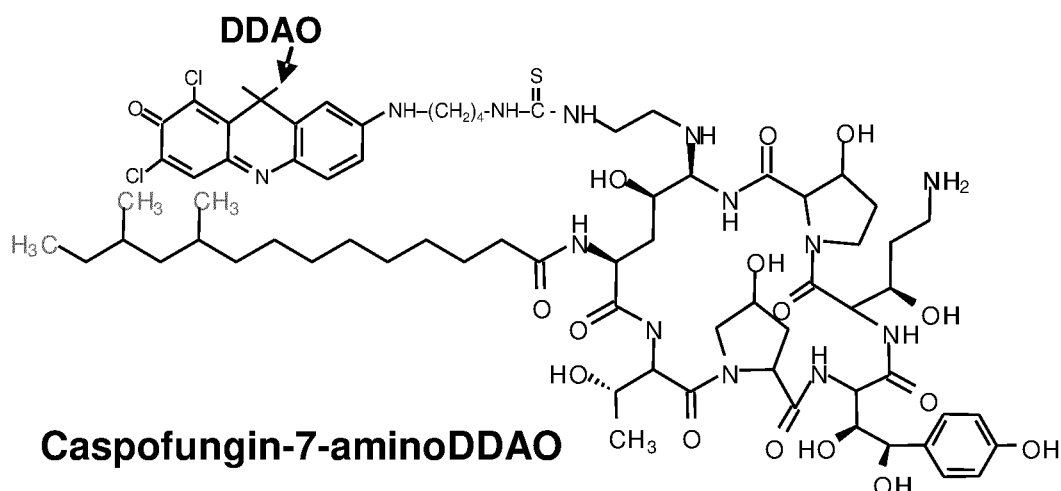
10. The bioconjugate of claim 9, having one or more of the following features:

- (i) wherein the targeting agent is an antibody, an antigen, a carbohydrate, a peptide, a nucleic acid, a lipid, a synthetic or natural polymer, a small molecule, or a combination thereof,
- (ii) wherein the target of said targeting agent is a glycoprotein, lipopolysaccharide, lipopeptide, a component of a cell wall, a receptor, or a combination thereof,
- (iii) wherein the linking group is selected from peptide linkers, self-immolative linkers, acid sensitive linkers, multifunctional organic linking agents, bifunctional inorganic crosslinking agents, polymers, and combinations thereof,
- (iv) wherein the linking group is stable, or biodegradable, or cleavable under physiological conditions, or combinations thereof; and
- (v) wherein the targeting agent is a pharmaceutically active agent, and
- (vi) wherein said small molecule is an antifungal agent.

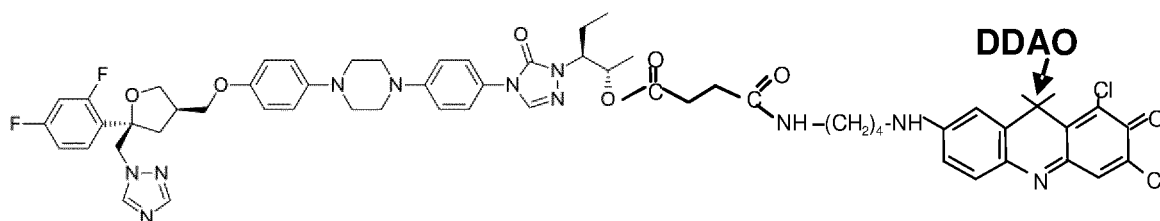
11. A method of detecting a cell or a target in a cell of a subject, or detecting a microbe in a subject, comprising administering to the subject a bioconjugate compound of any one of claims 1, 2 to 5 and 7, or a bioconjugate of any one of claims 9 or 10 exciting said compound with photons comprising NIR wavelength photons and detecting NIR fluorescence of the compound or the bioconjugate.

12. A method of detecting a cell or a target in a cell of a biological sample, comprising contacting the biological sample with a bioconjugate compound of any one of claims 1, 2 to 5 and 7 exciting said compound with photons comprising NIR wavelength photons and detecting NIR fluorescence of the compound.

13. The compound of claim 1, selected from the group consisting of



and

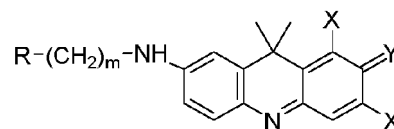


Posaconazole-7-aminoDDAO

14. The compound of claim 8, wherein R is BrAc-NH-.

Patentansprüche

1. Verbindung der Formel II:



(II)

oder ein pharmazeutisch unbedenkliches Salz oder Solvat davon, wobei:

X bei jedem Auftreten für Chlor steht;

Y für O steht;

m für eine ganze Zahl von 1 bis 10 steht

und

R für eine Gruppierung eines biologisch aktiven Moleküls oder eine funktionelle Gruppe, die mit einem biologisch aktiven Molekül unter Bildung einer kovalenten Bindung reagieren kann, steht.

2. Verbindung nach Anspruch 1, wobei:

R für -NR^aR^b, -NCS, -NCO, C₁-C₆-Alkyl, Amido, substituiertes oder unsubstituiertes Maleinimido, eine Click-reaktive Gruppe oder -C(O)-R¹ steht;

wobei R^a und R^b unabhängig für Wasserstoff oder C₁-C₄-Alkyl stehen;

R¹ für -N₃ oder -OR² steht und

R² für C₁-C₆-Alkyl oder Succinimido steht.

3. Verbindung nach Anspruch 2, wobei es sich bei der Click-reaktiven Gruppe um -N₃ oder -C≡CH handelt.

4. Verbindung nach Anspruch 1 oder 2, wobei R für eine Gruppierung eines biologisch aktiven Moleküls steht.

5. Verbindung nach Anspruch 4, wobei es sich bei dem biologisch aktiven Molekül um

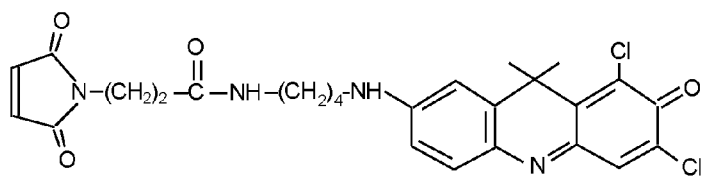
(i) ein kleines Molekül und/oder einen Arzneistoff oder ein Antimykotikum oder

(ii) ein großes Molekül, das aus einem Antikörper, einem Antigen, einem Kohlenhydrat, einem Peptid, einer Nukleinsäure, einem Lipid oder einem synthetischen oder natürlichen Polymer ausgewählt ist,

handelt.

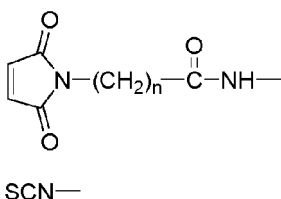
6. Verbindung nach einem der Ansprüche 1 bis 5, wobei m = 4.

7. Verbindung nach Anspruch 1, ausgewählt aus der Gruppe bestehend aus 7-Amino-9H-(1,3-dichlor-9,9-dimethylacridin-2-on) (7-Amino-DDAO), DDAO-NH-(CH₂)₄-NH₂, DDAO-NH-(CH₂)₄-NCS, DDAO-NH-(CH₂)₄-NH-COCH₂X (wobei X für Halogen steht), DDAO-NH-(CH₂)₄-NH-3-Maleinimid und



8. Verbindung nach Anspruch 1,

wobei R aus der Gruppe bestehend aus



NH₂, NCS, Halogenidacetamido, Acylmaleinimido, einem Click-reaktiven Azido und einer Alkingruppe ausgewählt ist, wobei
n bei jedem Auftreten unabhängig für eine ganze Zahl von 1 bis 10 steht.

9. Biokonjugat, umfassend ein Targeting-Mittel, eine Gruppierung eines 7-Amino-DDAO und eine Verknüpfungsgruppe, über die das 7-Amino-DDAO an das Targeting-Mittel gekoppelt ist, wobei es sich bei dem Targeting-Mittel um ein Mittel handelt, das ein Ziel von Interesse in einer biologischen Probe oder einem Individuum detektieren kann.

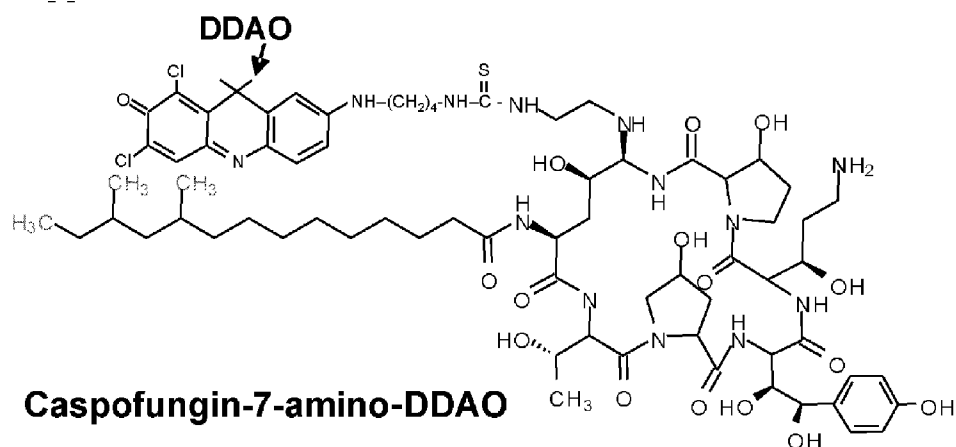
10. Biokonjugat nach Anspruch 9 mit einem oder mehreren der folgenden Merkmale:

- (i) wobei es sich bei dem Targeting-Mittel um einen Antikörper, ein Antigen, ein Kohlenhydrat, ein Peptid, eine Nukleinsäure, ein Lipid, ein synthetisches oder natürliches Polymer, ein kleines Molekül oder eine Kombination davon handelt,
- (ii) wobei es sich bei dem Ziel des Targeting-Mittels um ein Glykoprotein, ein Lipopolysaccharid, ein Lipopeptid, eine Komponente einer Zellwand, einen Rezeptor oder eine Kombination davon handelt,
- (iii) wobei die Verknüpfungsgruppe aus Peptid-Linkern, selbststimulativen Linkern, säureempfindlichen Linkern, multifunktionellen organischen Verknüpfungsmitteln, bifunktionellen anorganischen Verknüpfungsmitteln, Polymeren und Kombinationen davon ausgewählt ist,
- (iv) wobei die Verknüpfungsgruppe stabil oder biologisch abbaubar oder unter physiologischen Bedingungen spaltbar oder Kombinationen davon ist und
- (v) wobei es sich bei dem Targeting-Mittel um einen pharmazeutischen Wirkstoff handelt und
- (vi) wobei es sich bei dem kleinen Molekül um ein Antimykotikum handelt.

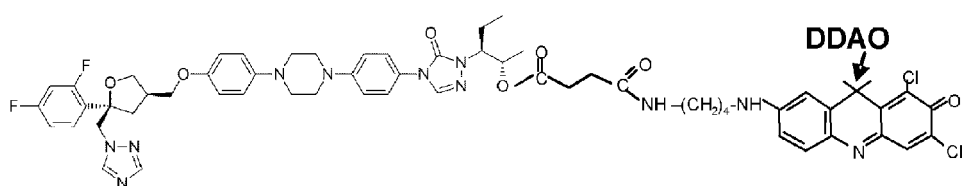
11. Verfahren zum Detektieren einer Zelle oder eines Ziels in einer Zelle eines Individuums oder zum Detektieren einer Mikrobe in einem Individuum, bei dem man dem Individuum eine Biokonjugat-Verbindung nach einem der Ansprüche 1, 2 bis 5 und 7 oder ein Bio konjugat nach Anspruch 9 oder 10 verabreicht, die Verbindung mit Photonen, die Photonen mit NIR-Wellenlänge umfassen, anregt und NIR-Fluoreszenz der Verbindung bzw. des Biokonjugats detektiert.

12. Verfahren zum Detektieren einer Zelle oder eines Ziels in einer Zelle einer biologischen Probe, bei dem man die biologische Probe mit einer Biokonjugat-Verbindung nach einem der Ansprüche 1, 2 bis 5 und 7 in Kontakt bringt, die Verbindung mit Photonen, die Photonen mit NIR-Wellenlänge umfassen, anregt und NIR-Fluoreszenz der Verbindung detektiert.

13. Verbindung nach Anspruch 1, ausgewählt aus der Gruppe bestehend aus



und

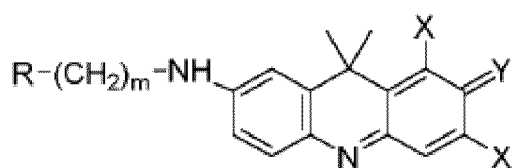


Posaconazol-7-amino-DDAO

14. Verbindung nach Anspruch 8, wobei R für BrAc-NHsteht.

Revendications

1. Composé de formule II :



(II)

ou sel ou solvate pharmaceutiquement acceptable correspondant :

X, en chaque occurrence, étant un chlore ;

Y étant O ;

m étant un entier choisi parmi 1 à 10 ;

et

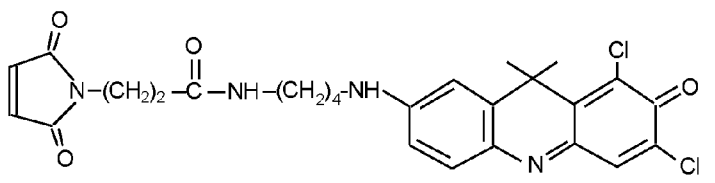
R étant un fragment d'une molécule biologiquement active, ou un groupe fonctionnel qui peut réagir avec une molécule biologiquement active pour former une liaison covalente.

2. Composé selon la revendication 1 :

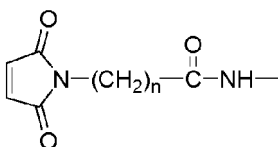
R étant -NR^aR^b, -NCS, -NCO, C₁₋₆-alkyle, amido, maléimido substitué ou non substitué, un groupe réactif en

chimie click, ou $-C(O)-R^1$;
 R^a et R^b étant indépendamment hydrogène ou C_{1-4} -alkyle ;
 R^1 étant $-N_3$ ou $-OR^2$; et
 R^2 étant C_{1-6} -alkyle ou succinimido.

3. Composé selon la revendication 2, ledit groupe réactif en chimie click étant $-N_3$ ou $-C\equiv CH$.
4. Composé selon l'une quelconque des revendications 1 et 2, R étant un fragment d'une molécule biologiquement active.
5. Composé selon la revendication 4, ladite molécule biologiquement active étant
 - (i) une petite molécule et/ou un médicament, ou un agent antifongique ou
 - (ii) une grande molécule choisie parmi un anticorps, un antigène, un glucide, un peptide, un acide nucléique, un lipide, ou un polymère synthétique ou naturel.
6. Composé selon l'une quelconque des revendications 1 à 5, où $m = 4$.
7. Composé selon la revendication 1, choisi dans le groupe constitué par 7-amino-9H-(1,3-dichloro-9,9-diméthylacridin-2-one)(7-aminoDDAO), DDAO-NH-(CH₂)₄-NH₂, DDAO-NH-(CH₂)₄-NCS, DDAO-NH-(CH₂)₄-NH-COCH₂X (X étant halogène), DDAO-NH-(CH₂)₄-NH-3-maléimide, et



8. Composé selon la revendication 1, R étant choisi dans le groupe constitué par :

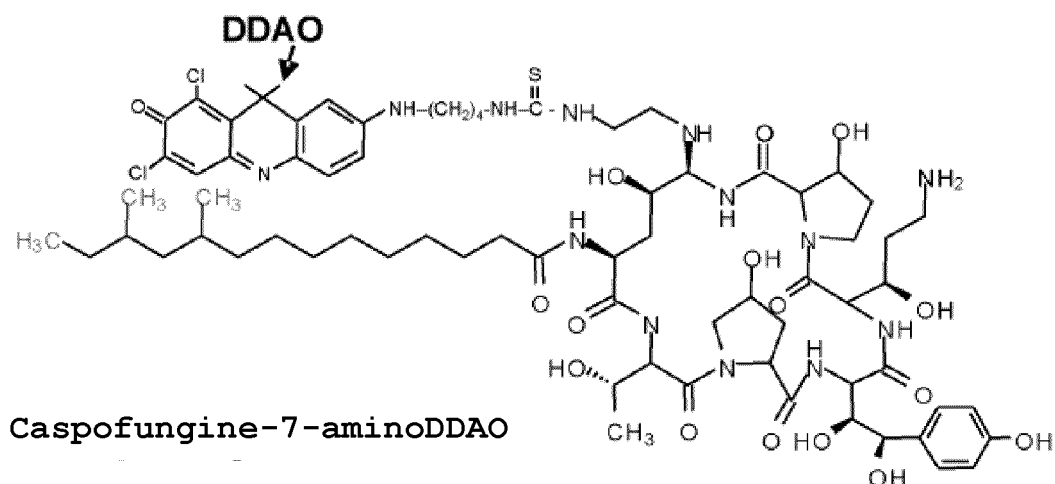


SCN-

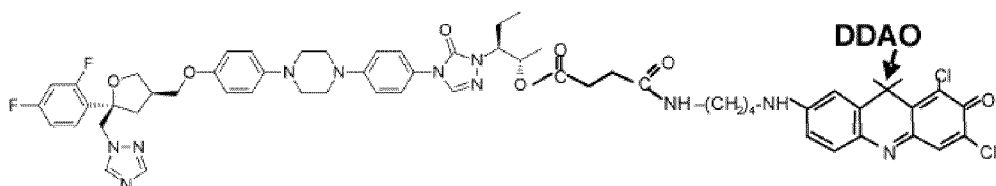
- NH₂, NCS, halogénoacétamido, acylmaléimido, un azoture réactif en chimie click, et un groupe alcyne, n, en chaque occurrence, étant indépendamment un entier choisi parmi 1 à 10.
9. Bioconjugué comprenant un agent de ciblage, un fragment d'un 7-aminoDDAO, et un groupe lieur par l'intermédiaire duquel ledit 7-aminoDDAO est couplé audit agent de ciblage, l'agent de ciblage étant un agent capable de détecter une cible d'intérêt dans un échantillon biologique ou un sujet.
10. Bioconjugué selon la revendication 9, possédant une ou plusieurs des caractéristiques suivantes :
 - (i) l'agent de ciblage étant un anticorps, un antigène, un glucide, un peptide, un acide nucléique, un lipide, un polymère synthétique ou naturel, une petite molécule, ou une combinaison correspondante,
 - (ii) la cible dudit agent de ciblage étant une glycoprotéine, un lipopolysaccharide, un lipopeptide, un composant d'une paroi cellulaire, un récepteur, ou une combinaison correspondante,
 - (iii) le groupe lieur étant choisi parmi des lieurs de type peptide, des lieurs auto-immolables, des lieurs sensibles à l'acide, des agents lieurs organiques multifonctionnels, des agents réticulants inorganiques bifonctionnels, des polymères, et des combinaisons correspondantes,
 - (iv) le groupe lieur étant stable, ou biodégradable, ou clivable dans des conditions physiologiques, ou des combinaisons correspondantes ; et

(v) l'agent de ciblage étant un agent pharmaceutiquement actif, et
 (vi) ladite petite molécule étant d'un agent antifongique.

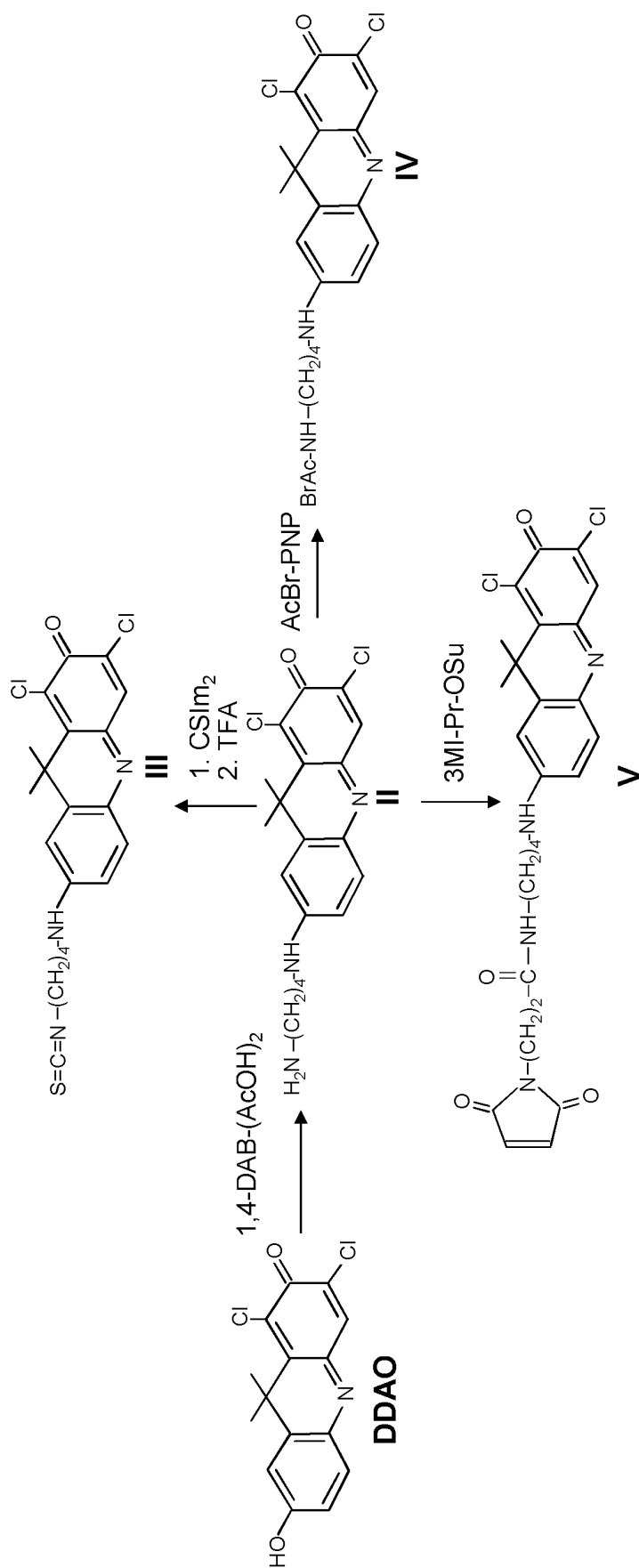
11. Procédé de détection d'une cellule ou d'une cible dans une cellule d'un sujet, ou de détection d'un microbe dans un sujet, comprenant l'administration au sujet d'un composé bioconjugué selon l'une quelconque des revendications 1, 2 à 5 et 7, ou d'un bioconjugué selon l'une quelconque des revendications 9 et 10, l'excitation dudit composé avec des photons comprenant des photons de longueur d'onde NIR, et la détection de fluorescence NIR du composé ou du bioconjugué.
12. Procédé de détection d'une cellule ou d'une cible dans une cellule d'un échantillon biologique, comprenant la mise en contact de l'échantillon biologique avec un composé bioconjugué selon l'une quelconque des revendications 1, 2 à 5 et 7, l'excitation dudit composé avec des photons comprenant des photons de longueur d'onde NIR, et la détection de fluorescence NIR du composé.
13. Composé selon la revendication 1, choisi dans le groupe constitué par

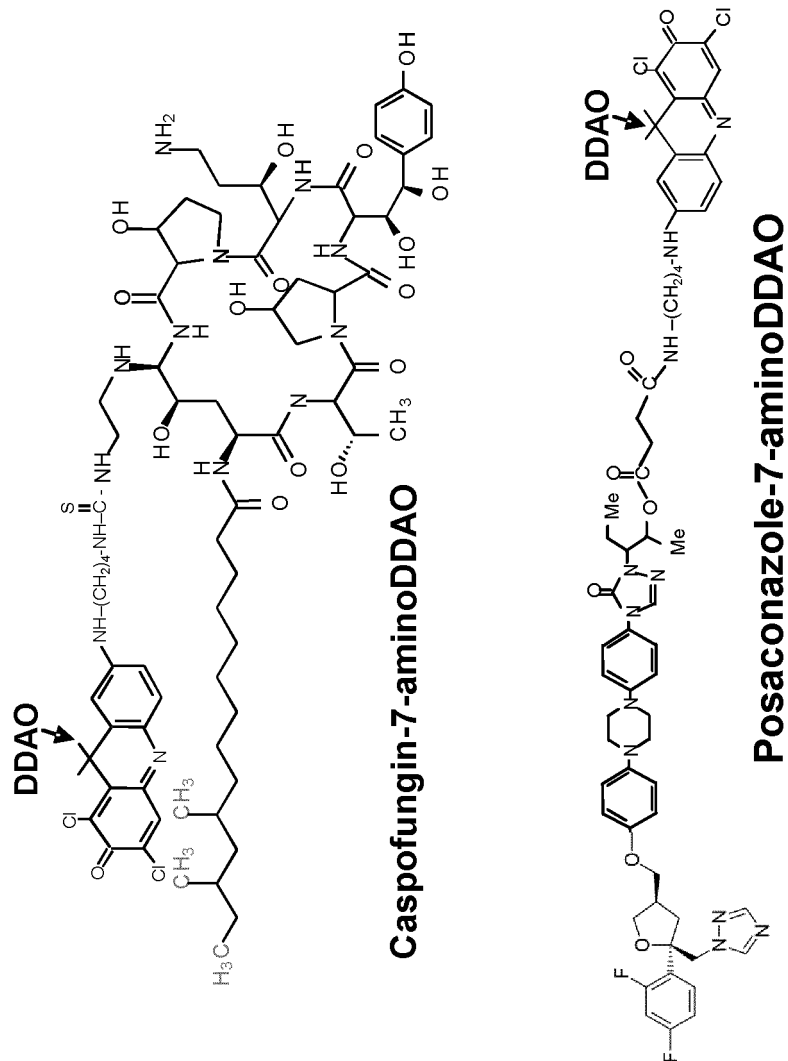


et



14. Composé selon la revendication 8, R étant BrAc-NH-

**FIG. 1**

**FIG.2**

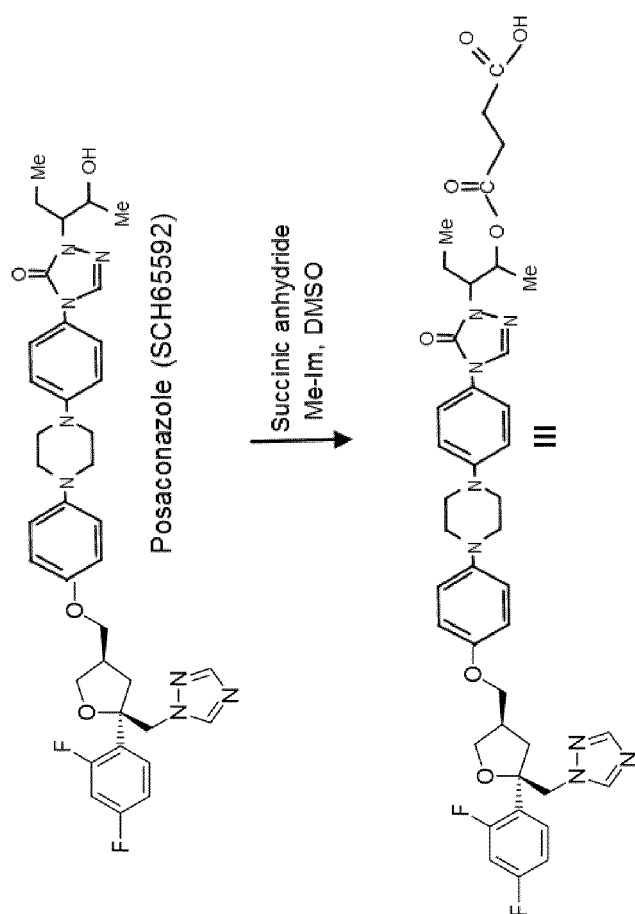


FIG. 3

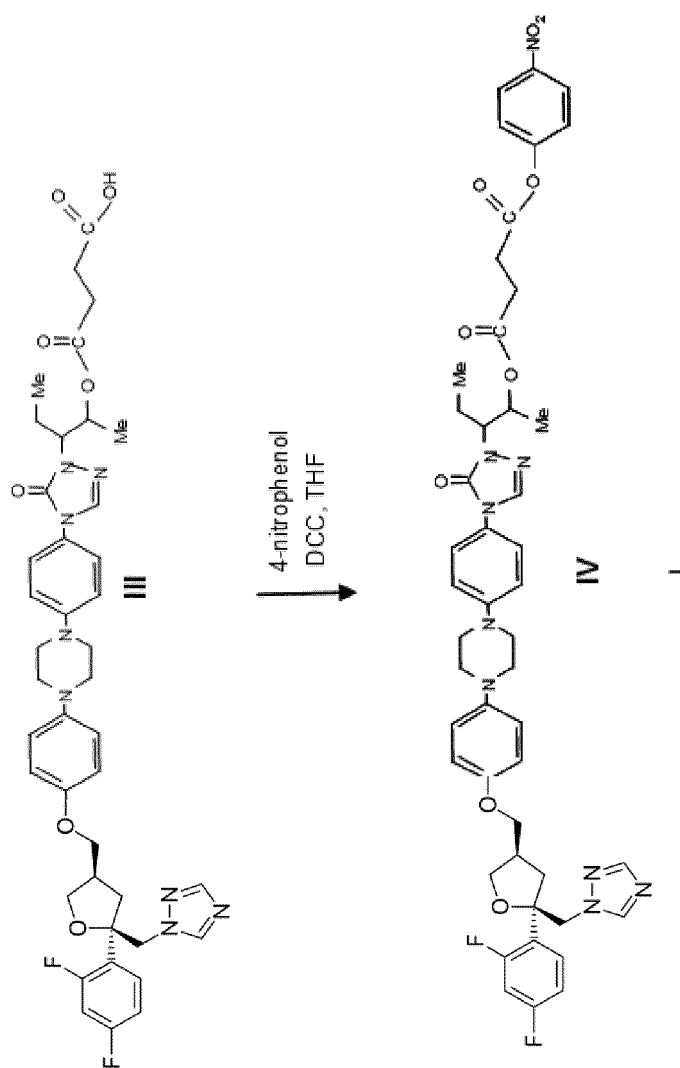


FIG.3
(cont'd)

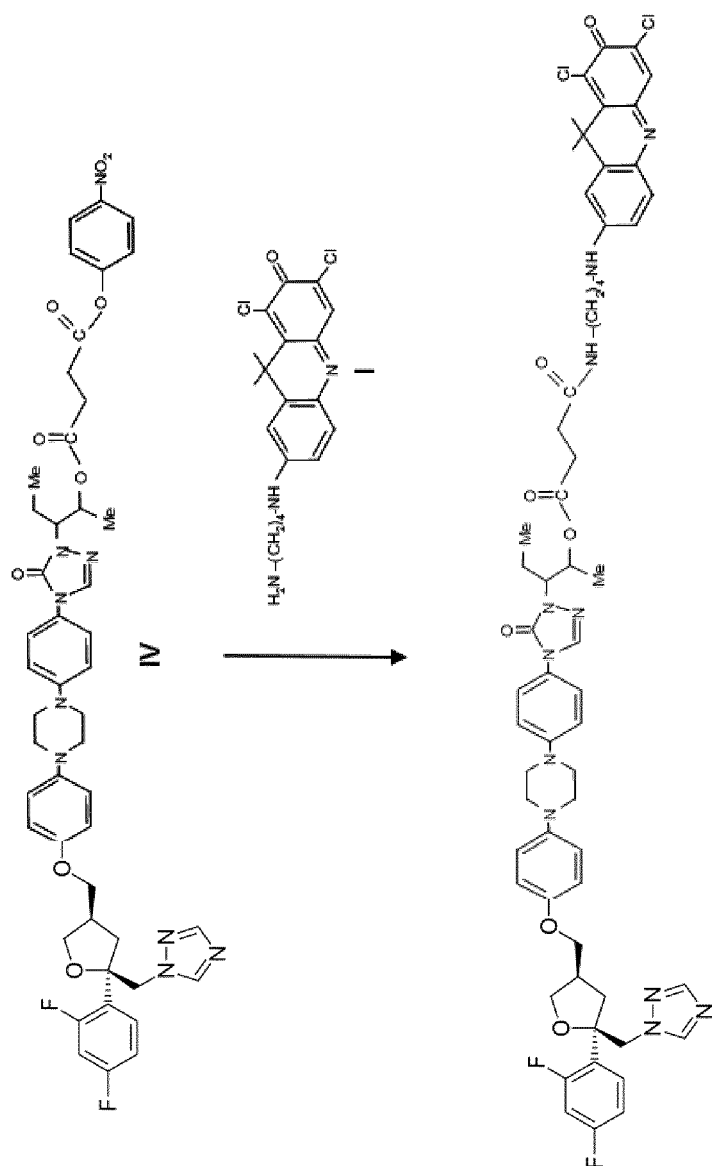
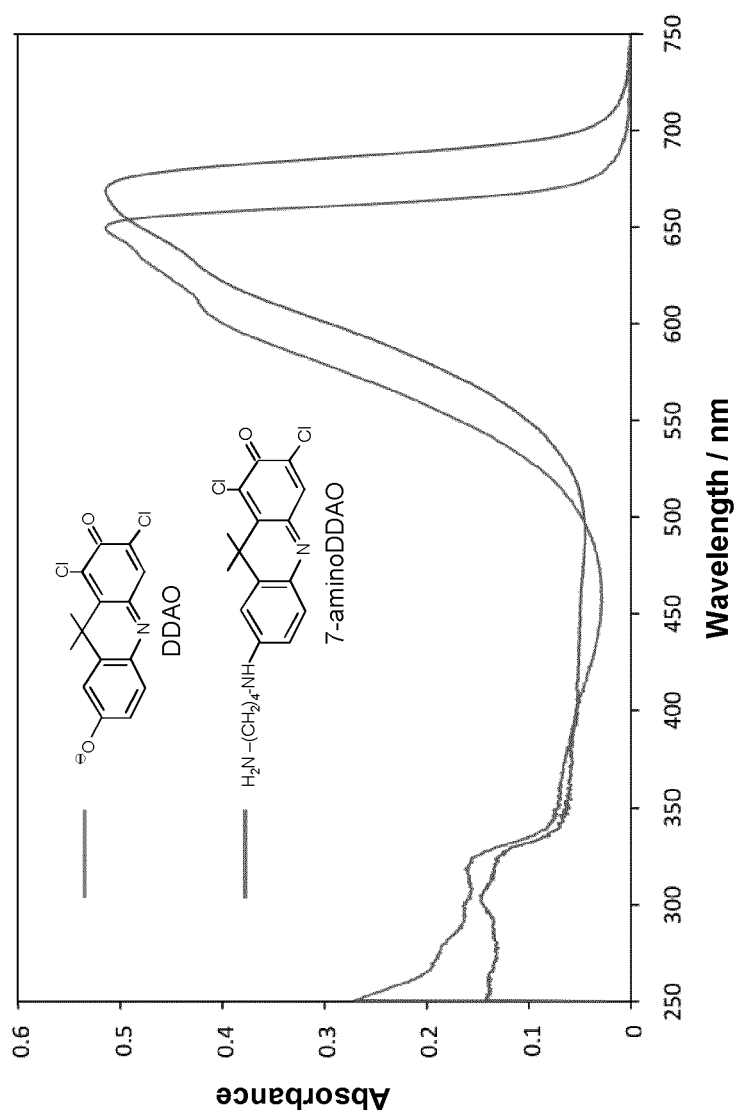


FIG.3
(cont'd)

**FIG.4**

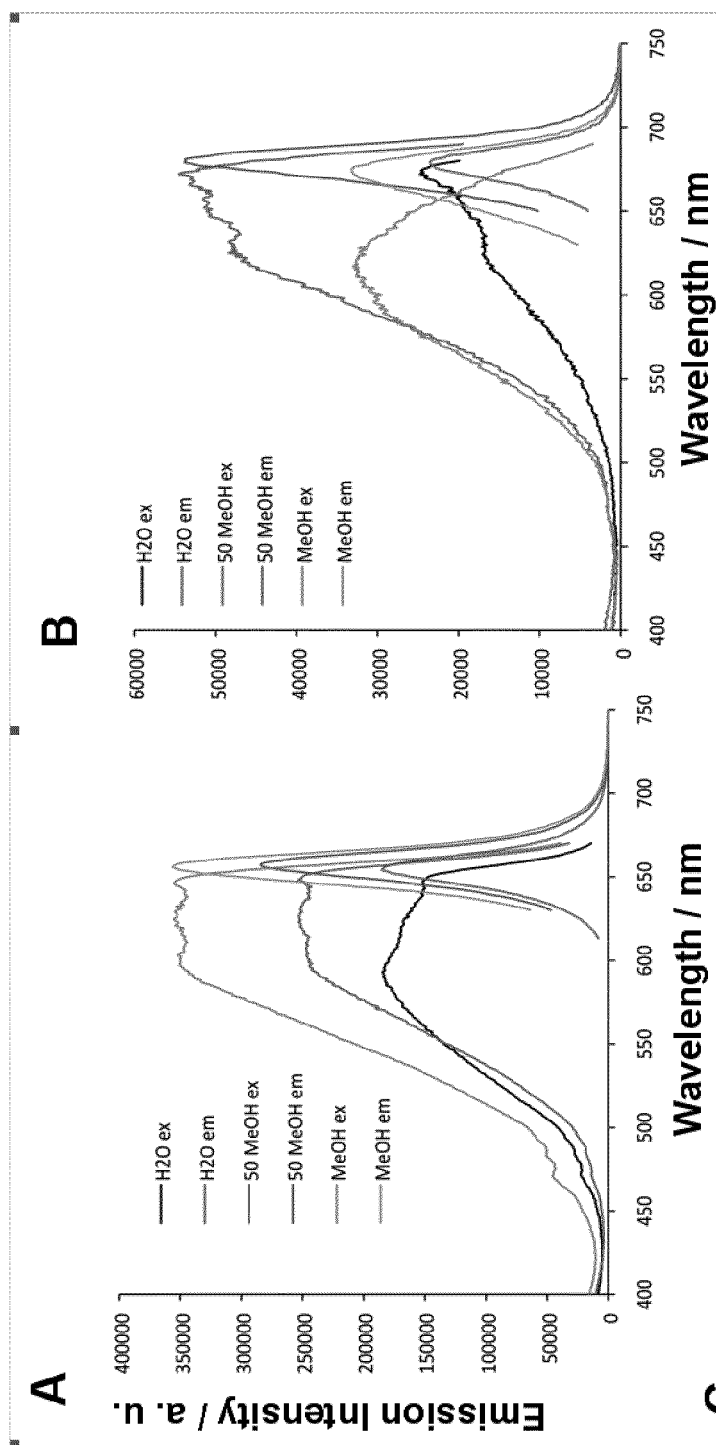
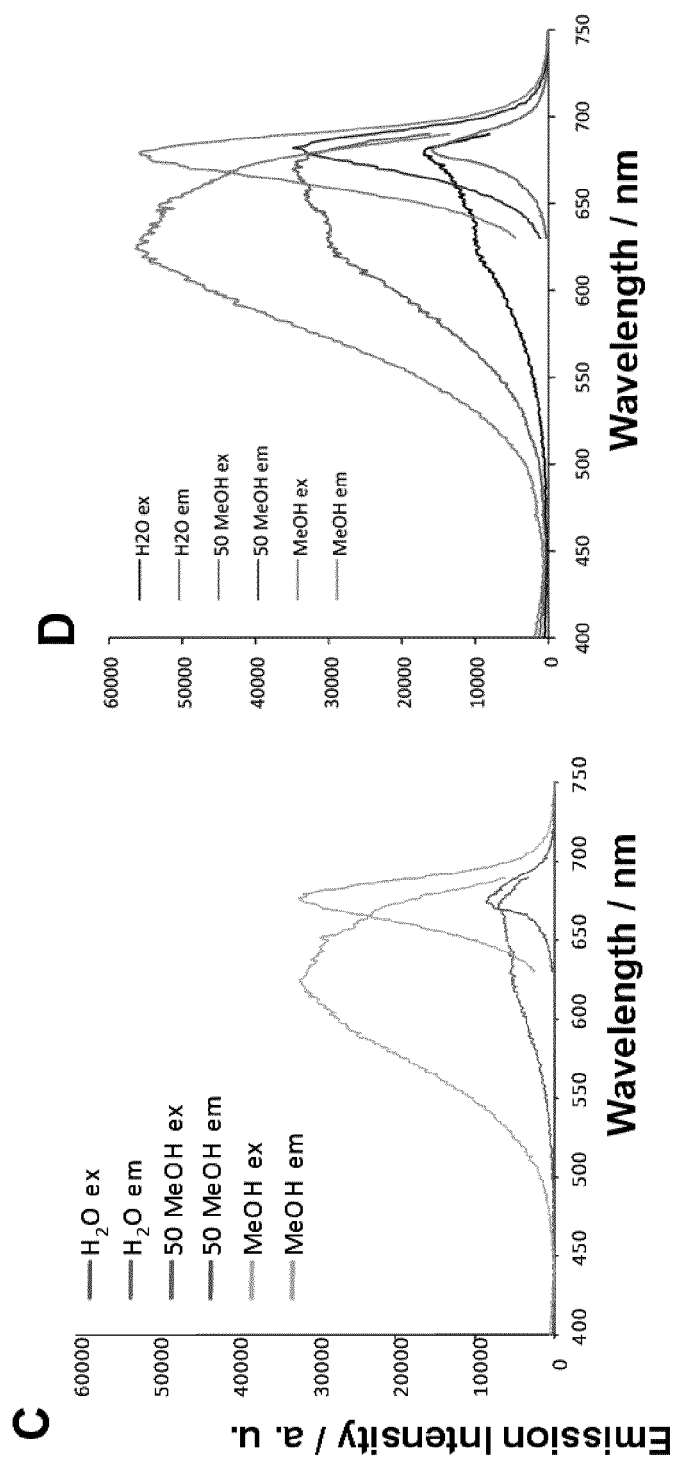


FIG.5B

FIG.5A



¶

FIG.5C**FIG.5D**

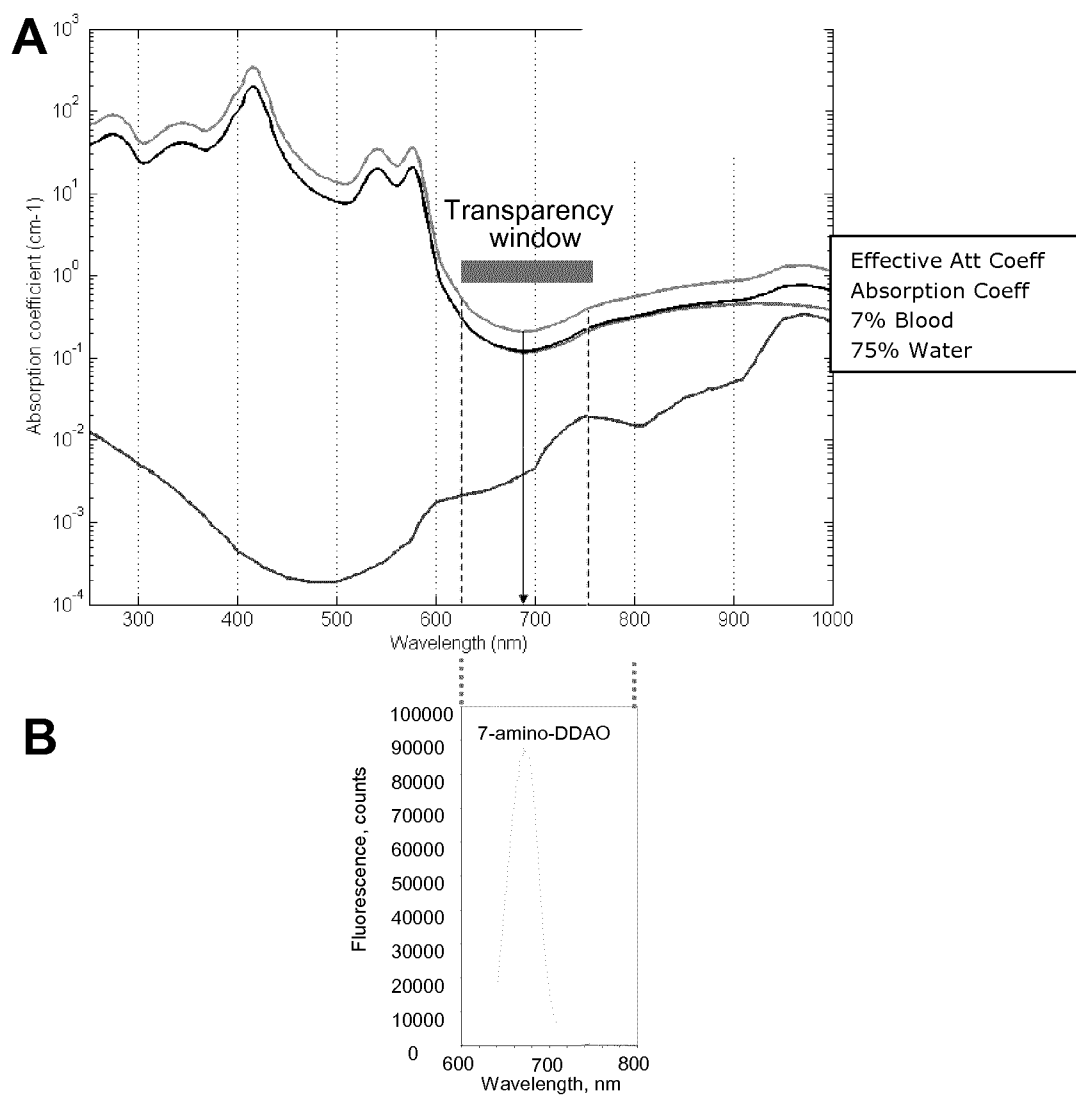


FIG.6A & 6B

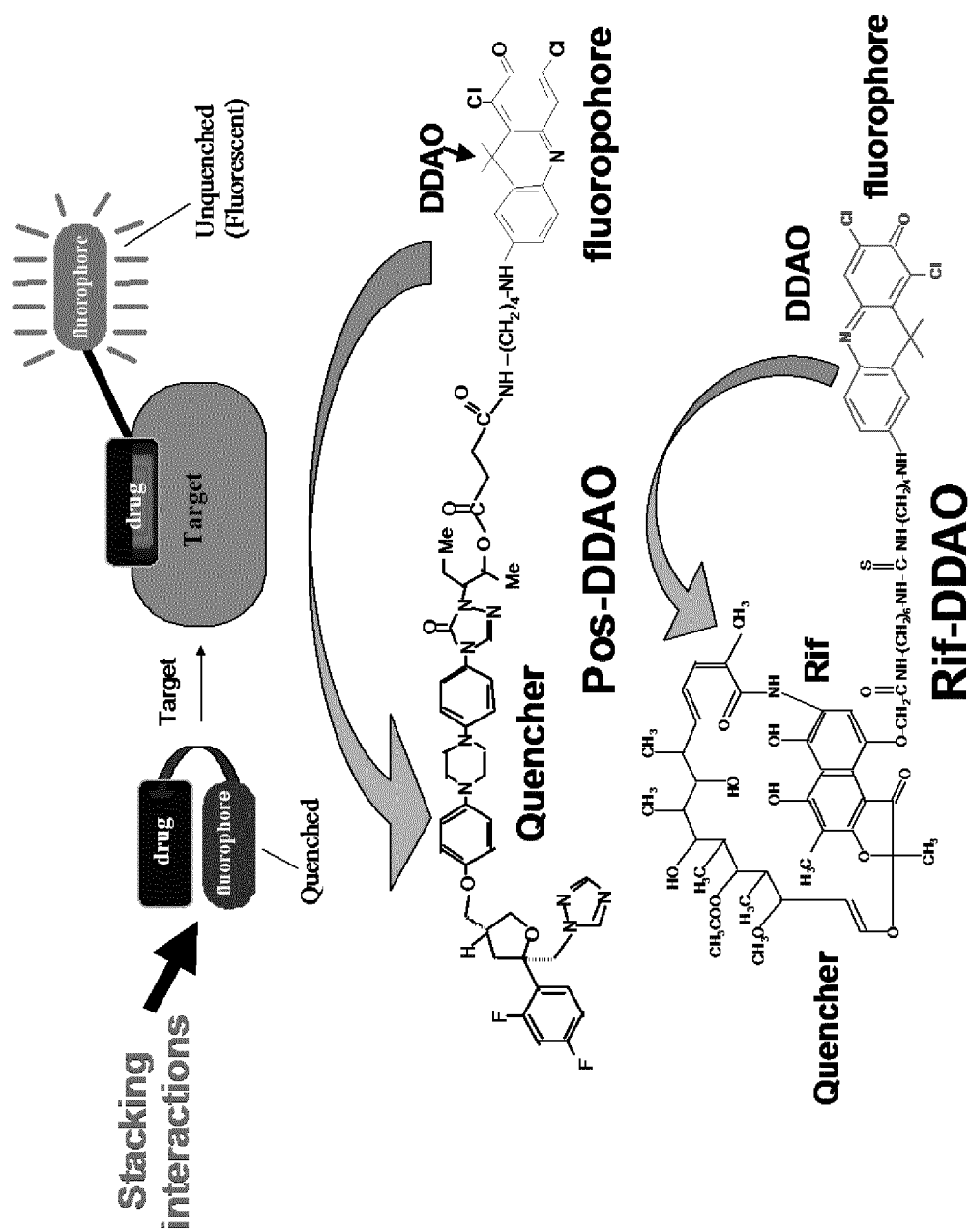
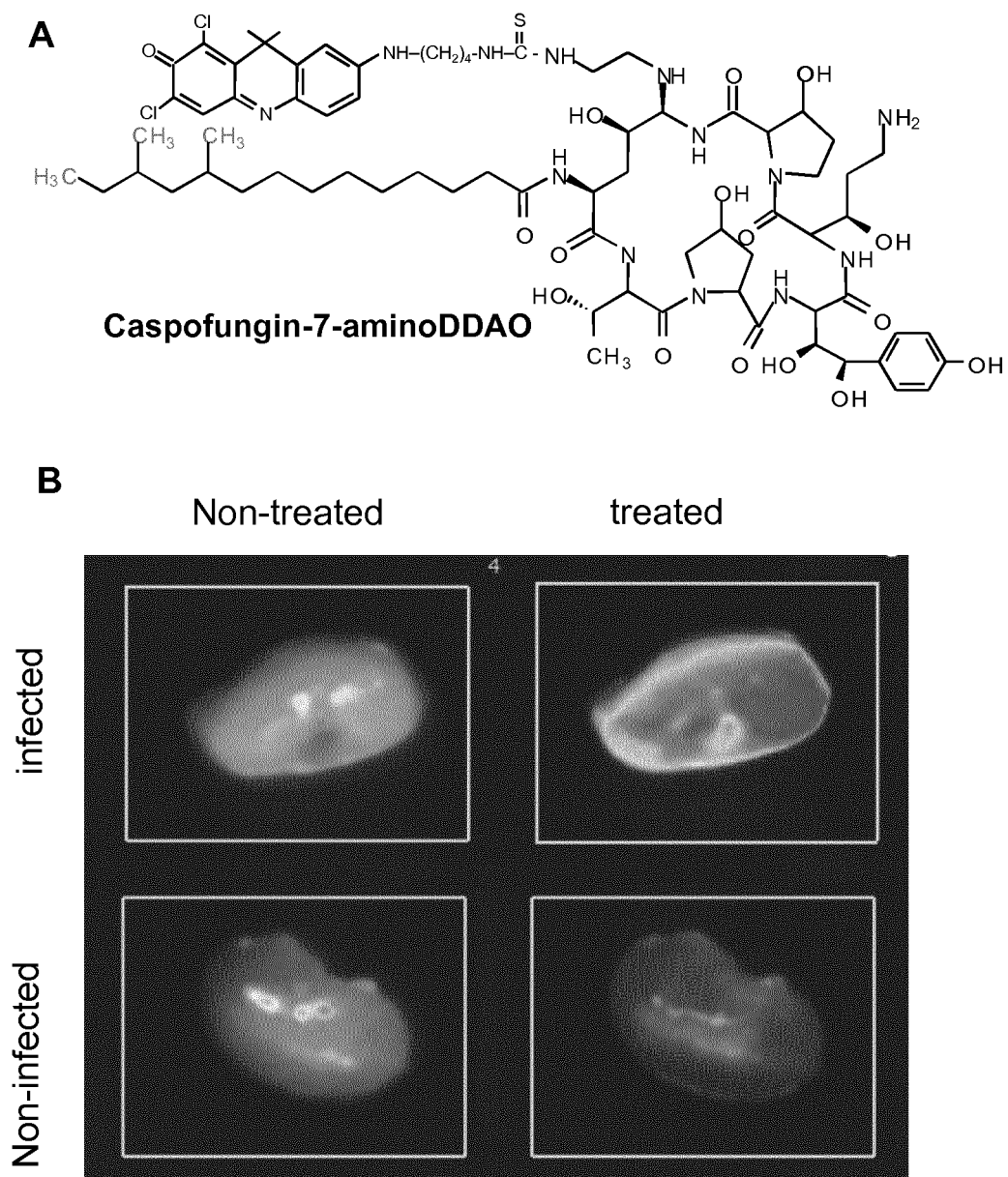


FIG. 7

**FIG. 8A & 8B**

12/13

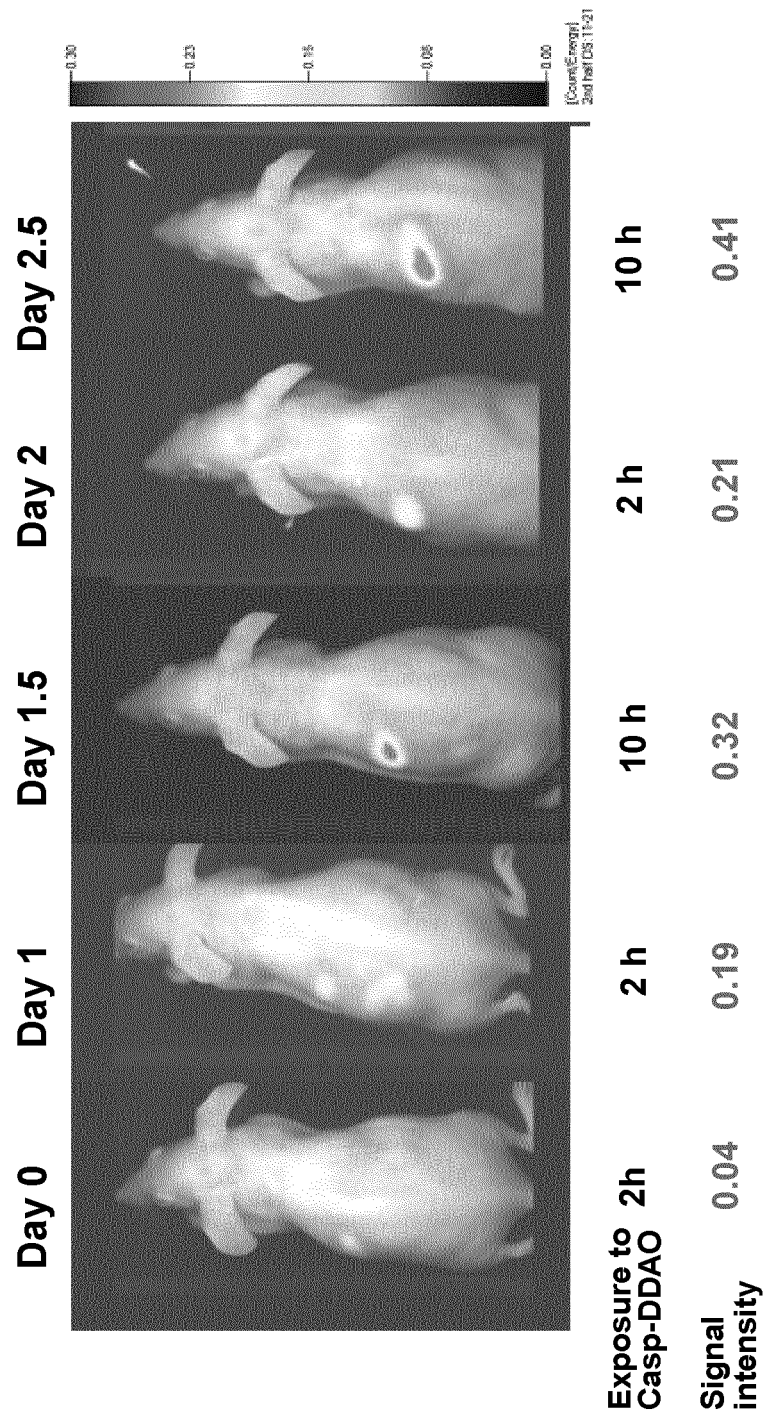


FIG. 9

Antibacterial activity of Rif-DAO compound

Compound	MIC, μM
<i>S. aureus</i>	
Rif	0.03
Rif-DDAO	0.008
<i>B. subtilis</i>	
Rif	0.1
Rif-DDAO	0.2

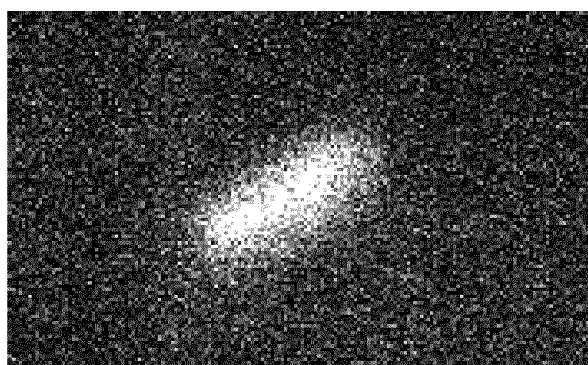
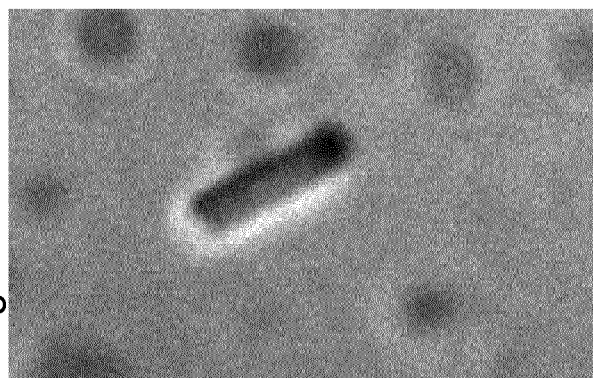
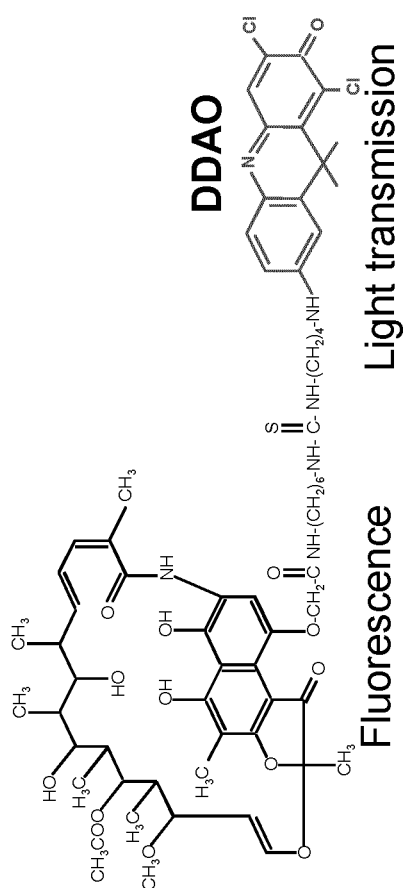


FIG. 10

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 61821187 [0001]
- US 2013040182 W [0001]
- US 61644283 [0001]
- WO 2013169932 A [0007]
- US 20050222427 A, Sharpless [0094]

Non-patent literature cited in the description

- **TUNG et al.** Imaging of b-Galactosidase Activity Using Far Red Fluorescent Switch. *Cancer Research*, 2004, vol. 64, 1579-1583 [0008]
- **MALMBERG, E., W. ; HAMILTON, C., S. J.** *Am. Chem. Soc.*, 1948, vol. 70, 2415 [0116]
- **WILLENZ, J. J.** *Chem. Soc.*, 1955, 2049 [0116]

专利名称(译)	近红外标签及其使用方法		
公开(公告)号	EP2999408A4	公开(公告)日	2017-03-15
申请号	EP2014794148	申请日	2014-05-08
[标]申请(专利权)人(译)	新泽西理工学院		
申请(专利权)人(译)	罗格斯新泽西州立大学 新泽西理工大学		
当前申请(专利权)人(译)	罗格斯新泽西州立大学 新泽西理工大学		
[标]发明人	MUSTAEV ARKADY PERLIN DAVID S WIRPSZA LAURA		
发明人	MUSTAEV, ARKADY PERLIN, DAVID, S. WIRPSZA, LAURA		
IPC分类号	A61B5/1455 A61K49/00 A61B5/00		
CPC分类号	A61B5/0071 A61K49/0021		
优先权	61/821187 2013-05-08 US		
其他公开文献	EP2999408A1 EP2999408B1		
外部链接	Espacenet		

摘要(译)

本发明提供可用作新型近红外标记的化合物，含有这些化合物的组合，以及使用近红外标记物在体外，原位和体内鉴定靶标的方法。本发明还提供靶向剂和NIR标记之间的小分子或大分子缀合物，以及其方法和试剂盒，其可用于诊断和治疗与哺乳动物中的微生物有关的疾病。