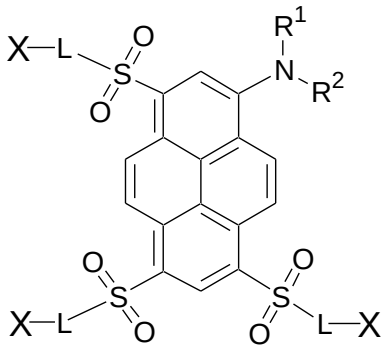


Formula B

wherein R^1 and/or R^2 are independent from each other and may represent:

H, deuterium, alkyl or deutero-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deutero-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3, C_2H_5, a linear or branched C_3-C_{12} alkyl or perfluoroalkyl group, or a substituted C_2–C_{12} alkyl group; in particular, $(CH_2)_nCOOR^3$, where n = 1-12, preferably 1-5, R^3 may be H, alkyl, in particular C_1-C_6, CH_2CN, benzyl, fluorene-9-yl, polyhalogenoalkyl, polyhalogenophenyl, *e.g.* tetra- or pentafluorophenyl, pentachlorophenyl, 2- and 4-nitrophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, and the alkyl chain in $(CH_2)_n$ may be straight or branched; and

R^1---R^2 may form a four-, five, six-, or seven-membered non-aromatic carbocycle with an additional primary amino group NH_2, secondary amino group NHR^a, where R^a = C_1–C_6 alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally R^1---R^2 may form a four-, five, six-, or seven-membered non-aromatic heterocycle with an additional heteroatom such as O, N or S included into this heterocycle;

a hydroxyalkyl group $(CH_2)_mOH$, where m = 1-12, preferably 2–6, with a straight or branched alkyl chain;

one of R^1 or R^2 groups may be a carbonate or carbamate derivative $(CH_2)_mOCOOR^4$ or $(CH_2)_mNHCOOR^4$, where m = 1-12 and R^4 = methyl,

of R^1 or R^2 may be an alkyl azide $(CH)N_3$ or alkyne, in particular propargyl;

wherein the linker L is divalent or polyvalent, comprises at least one carbon atom and is selected from the group consisting of alkyl, heteroalkyl, perfluoroalkyl, alkyloxy, such as CH_2OCH_2, CH_2CH_2O, $CH_2CH_2OCH_2$, ester or urethane moieties, single or multiple difluoromethyl (CF_2), alkene or alkyne moieties in any combinations, at any occurrence, linear or branched, with the length ranging from C_1 to C_{12}, a carbonyl moiety, and a residue of 1,3,5-triazine, thus providing two attachment points for group X;

X denotes a solubilizing and/or ionizable anion-providing moiety, in particular consisting of or including a moiety selected from the group comprising hydroxyalkyl $(CH_2)_nOH$, thioalkyl $((CH_2)_nSH)$, carboxy alkyl $((CH_2)_nCO_2H)$, alkyl sulfonate $((CH_2)_nSO_3H)$, alkyl sulfate $((CH_2)_nOSO_3H)$, alkyl phosphate $((CH_2)_nOP(O)(OH)_2)$ or phosphonate $((CH_2)_nP(O)(OH)_2)$, wherein n is an integer ranging from 0 to 12, or an analog thereof wherein one or more of the CH_2 groups are replaced by CF_2,
further, the anion-providing moieties may be linked by means of non-aromatic O, N and S-containing heterocycles, e. g., piperazines, pipecolines, or, alternatively, one of the groups X may bear any of the moieties listed above for groups R^1 and R^2, also with any type of linkage listed for group L, and independently from other substituents;

with the proviso that in all compounds represented by Formula B three or six negatively charged groups are present in the residues X of Formula B under basic conditions, i.e. $7 < pH < 14$, and these negatively charged groups represent at least

partially deprotonated residues of ionizable groups selected from the following: SH, COOH, SO_3H, OSO_3H, $OP(O)(OH)_2$, $OP(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl, $P(O)(OH)_2$, $P(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl.

In another aspect of the present disclosure, there are provided fluorescent dyes of Formula B as disclosed herein that have a hydroxylamine or a hydrazine moiety as an analyte-reactive group, which is connected directly the dye core or where the hydroxylamine or

hydrazine moieties are connected via a linker group, in particular alkyl, heteroalkyl or a linker L, as defined herein for Formula B.

In a further aspect of the present disclosure, there is provided a compound of Formulae B and C as disclosed herein, which has a net charge z of 0, -1, -3, -6 or -12, preferably -6 or -12, in particular in an aqueous medium at a pH range of 7-13.

In another aspect of the present disclosure, there is provided a compound as disclosed herein and salts thereof for use in the reductive amination and in the condensation with reducing sugars, i.e. monomeric, oligomeric or polymeric carbohydrates possessing an aldehyde group in a free form or as semiacetal, including glycans.

In a further aspect of the present disclosure, there is provided a compound as disclosed herein and salts thereof for use as a fluorescent label for amino acids, peptides, proteins, including primary and secondary antibodies, single-domain antibodies, docetaxel, avidin, streptavidin and their modifications, aptamers, nucleotides, nucleic acids, toxins, lipids, carbohydrates, including 2-deoxy-2-amino glucose and other 2-deoxy-2-aminoaminopyranosides, glycans, biotin, and other small molecules, i.e. having molecular masses of less than 1500 Da, e.g., jasplakinolide and its modifications.

In another aspect of the present disclosure, there is provided a use of the compounds as disclosed herein and salts thereof as fluorescent reagents for conjugation to analytes, wherein the conjugation comprises formation of at least one covalent chemical bond or at least one molecular complex with a chemical

entity or substance, such as amine, carboxylic acid, aldehyde, alcohol, aromatic compound, heterocycle, dye, amino acid, amino acid residue coupled to any chemical entity, peptide, protein, carbohydrate, nucleic acid, toxin and lipid.

In a further aspect of the present disclosure, there are provided carbohydrate-dye conjugates comprising fluorescent dyes as disclosed herein.

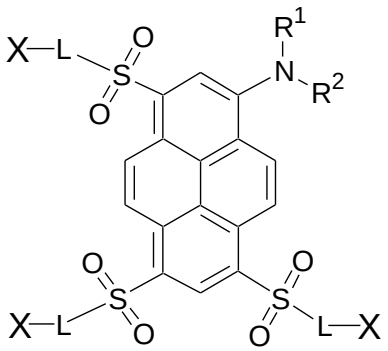
In another aspect of the present disclosure, there is provided a kit or composition comprising one or more of the dyes as disclosed herein or one or more of the carbohydrate conjugates as disclosed herein.

Description of the invention

In their ionized (deprotonated) form, the novel fluorescent dyes of the invention feature multiple negatively charged residues and an aromatic amino or hydrazine group attached to the fluorophore, which is excitable e.g. with an argon ion laser. Importantly, the novel dyes and their carbohydrate conjugates emit light with the maximum that is considerably shifted from that of APTS-labeled analogs. Multiple negative net charges, which are especially high in the phosphorylated derivatives

Claims

1. A fluorescent dye having multiple ionizable and/or negatively charged groups which is selected from the group consisting of compounds of the following general Formulae B and A or of salts thereof:



Formula B

wherein R^1 and/or R^2 are independent from each other and may represent:

H, deuterium, alkyl or deutero-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deutero-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3, C_2H_5, a linear or branched C_3-C_{12} alkyl or perfluoroalkyl group, or a substituted C_2–C_{12} alkyl group; in particular, $(CH_2)_nCOOR^3$, where n = 1-12, preferably 1-5, R^3 may be H, alkyl, in particular C_1-C_6, CH_2CN, benzyl, fluorene-9-yl, polyhalogenoalkyl, polyhalogenophenyl, *e.g.* tetra- or pentafluorophenyl, pentachlorophenyl, 2- and 4-nitrophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, and the alkyl chain in $(CH_2)_n$ may be straight or branched; and

R^1---R^2 may form a four-, five, six-, or seven-membered non-aromatic carbocycle with an additional primary amino group NH_2, secondary amino group NHR^a, where R^a = C_1–C_6 alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally R^1---R^2 may form a four-, five, six-, or seven-

membered non-aromatic heterocycle with an additional heteroatom such as O, N or S included into this heterocycle;
a hydroxyalkyl group $(CH_2)_mOH$, where m = 1-12, preferably 2-6, with a straight or branched alkyl chain;
one of R^1 or R^2 groups may be a carbonate or carbamate derivative $(CH_2)_mOCOOR^4$ or $(CH_2)_mNHCOOR^4$, where m = 1-12 and R^4 = methyl, ethyl, 2-chloroethyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, a phenyl group or substituted phenyl group, e.g., 2- and 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, or 4-pyridyl;
$(CH_2)_mNR^aR^b$, where m = 1-12, preferably 2-6, with a straight or branched alkyl chain; R^a, R^b are independent from each other and may be H, or optionally substituted C_1-C_4 alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(CH_2)_mN_3$ with m = 2-6 and a straight or branched alkyl chain; one of R^1 or R^2 may be $(CH_2)_nSO_2NR^5NH_2$ with n = 1-12, while the substituent R^5 can be represented by H, alkyl, hydroxyalkyl or perfluoroalkyl groups C_1-C_{12};
one of R^1 or R^2 groups may be a primary amino group to form aryl hydrazines $Ar\text{-}NR^6NH_2$ where Ar is the entire pyrene residue in Formula B and R^6 = H or alkyl;
one of R^1 or R^2 groups may be a hydroxy group to form aryl hydroxylamines $Ar\text{-}NR^7OH$ where Ar is the entire pyrene residue in Formula B and R^7 = H or alkyl;
one of R^1 or R^2 groups may be an alkyloxyamino group $(CH_2)_nONH_2$ with n = 1-12;
one of R^1 or R^2 groups may be $CO(CH_2)_nCOOR^8$, with n = 1-5 and a straight or branched alkyl chain $(CH_2)_n$ and with R^8 selected from H, straight or branched C_1-C_6 alkyl, CH_2CN, 2- and 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl;
further, one of R^1 or R^2 may be $(CH_2)_nCONHR^9$, with n = 1-5 and R^9 = H, C_1-C_6 alkyl, $(CH_2)_mN_3$, $(CH_2)_m$-*N*-maleimido, $(CH_2)_m$-*N*$HCOCH_2X$ (X

= Br or I), where m = 2-6 and with straight or branched alkyl chains in $(CH_2)n$ and R^9;
or one of R^1 or R^2 may represent CH_2-C_6H_4-NH_2, COC_6H_4-NH_2, $CONHC_6H_4$-NH_2 or $CSNHC_6H_4$-NH_2 with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, COC_5H_3N-NH_2 or CH_2-C_5H_3N-NH_2 with C_5H_3N being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl; or one of R^1 or R^2 may be an alkyl azide $(CH)N_3$ or alkyne, in particular propargyl;

wherein the linker L is divalent or polyvalent, comprises at least one carbon atom and is selected from the group consisting of alkyl, heteroalkyl, perfluoroalkyl, alkyloxy, such as CH_2OCH_2, CH_2CH_2O, $CH_2CH_2OCH_2$, ester or urethane moieties, single or multiple difluoromethyl (CF_2), alkene or alkyne moieties in any combinations, at any occurrence, linear or branched, with the length ranging from C_1 to C_{12}, a carbonyl moiety, and a residue of 1,3,5-triazine, thus providing two attachment points for group X;

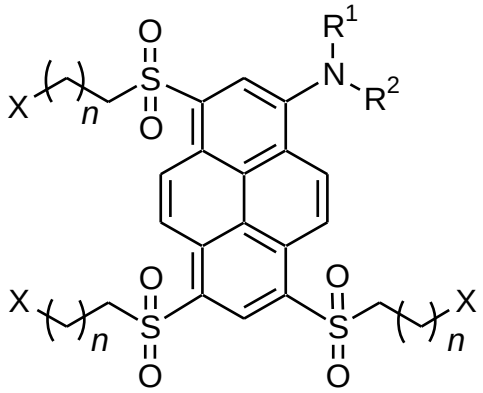
X denotes a solubilizing and/or ionizable anion-providing moiety, in particular consisting of or including a moiety selected from the group comprising hydroxyalkyl $(CH_2)_nOH$, thioalkyl ($(CH_2)_nSH$), carboxy alkyl ($(CH_2)_nCO_2H$), alkyl sulfonate ($(CH_2)_nSO_3H$), alkyl sulfate ($(CH_2)_nOSO_3H$), alkyl phosphate ($(CH_2)_nOP(O)(OH)_2$) or phosphonate ($(CH_2)_nP(O)(OH)_2$), wherein n is an integer ranging from 0 to 12, or an analog thereof wherein one or more of the CH_2 groups are replaced by CF_2,
further, the anion-providing moieties may be linked by means of non-aromatic O, N and S-containing heterocycles, e. g., piperazines, pipecolines, or, alternatively, one of the groups X may bear any of the moieties listed above for groups R^1 and R^2, also with any type of linkage listed for group L, and independently from other substituents;

with the proviso that in all compounds represented by Formula B three or six negatively charged groups are present in the residues X of Formula B under basic conditions, i.e. 7 < pH <

14, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: SH, COOH, SO_3H, OSO_3H, $OP(O)(OH)_2$, $OP(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl, $P(O)(OH)_2$, $P(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl.

2. A fluorescent dye according to claim 1 having Formula B wherein X at each occurrence may represent 1 to 4 groups SO_3H attached to the respective linker group L at one or more sites.

3. A fluorescent dye according to claim 1 having Formula C



Formula C

or a salt thereof,
wherein
n = 1-12, preferably 1-6;
R^1 and/or R^2 are independent from each other and may represent: H, deuterium, alkyl or deutero-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deutero-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3, C_2H_5, a straight or branched C_3-C_{12}, preferably C_3-C_6, alkyl group, or a substituted C_2–C_{12}, preferably C_2-C_6, alkyl group; in particular, $(CH_2)_nCOOR^3$, where n = 1-12, preferably 1-5, R^3 may be H, CH_2CN, 2- and 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl,

pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, and the alkyl chain in $(CH_2)_n$ may be straight or branched; and

R^1---R^2 may form a four-, five, six-, or seven-membered non-aromatic carbocycle with an additional primary amino group NH_2, secondary amino group NHR^a, where R^a = C_1–C_6 alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally R^1---R^2 may form a four-, five, six-, or seven-membered non-aromatic heterocycle with an additional heteroatom such as O, N or S included into this heterocycle;

a hydroxyalkyl group $(CH_2)_mOH$, where m = 2-12, preferably 2–6, with a straight or branched alkyl chain; one of R^1 or R^2 groups may be a carbonate or carbamate derivative $(CH_2)_mOCOOR^4$ or $(CH_2)_mNHCOOR^4$, where m = 1-12 and R^4 = methyl, ethyl, 2-chloroethyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, a phenyl group or substituted phenyl group, e.g., 2- and 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, or 4-pyridyl;

$(CH_2)_mNR^aR^b$, where m = 2-12, preferably 2–6, with a straight or branched alkyl chain; R^a, R^b are independent from each other and may be H, or optionally substituted C_1–C_4 alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(CH_2)_mN_3$ with m = 2-6 and a straight or branched alkyl chain; one of R^1 or R^2 groups may be $(CH_2)_nCOOR^5$, with n = 1-5 and a straight or branched alkyl chain $(CH_2)_n$ and with R^5 selected from H, straight or branched C_1-C_6 alkyl, CH_2CN, 2- and 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl;

one of R^1 or R^2 groups may be a primary amino group to form aryl hydrazines, as described for Formula B;

one of R^1 or R^2 groups may be a hydroxy group to form aryl hydroxylamines, as described for Formula B;

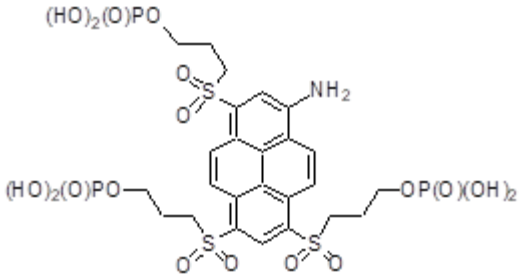
one of R^1 or R^2 groups may be an alkyloxyamino group $(CH_2)_nONH_2$ with n = 1–12;
further, one of R^1 or R^2 may be $(CH_2)_nCONHR^6$, with n = 1-12, preferably 1–5, and R^6 = H, C_1-C_6 alkyl, $(CH_2)_mN_3$, $(CH_2)_m$-*N*-maleimido, $(CH_2)_m$-*N*$HCOCH_2X$ (X = Br or I), where m = 2-6 and with straight or branched alkyl chains in (CH_2)n and R^6; or one of R^1 or R^2 may represent CH_2-C_6H_4-NH_2, COC_6H_4-NH_2, $CONHC_6H_4$-NH_2 or $CSNHC_6H_4$-NH_2 with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, COC_5H_3N-NH_2 or CH_2-C_5H_3N-NH_2 with C_5H_3N being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl;
the (CH_2)n-CH_2 linker, with n = 1-12, preferably 1-5, between the SO_2 fragment and the residue X in Formula C may represent a straight-chain, branched or cyclic group having 2–6 carbon atoms;
X = SH, COOH, SO_3H, OSO_3H, $OP(O)(OH)_2$, $OP(O)(OH)R^a$, where R^a = optionally substituted C_1–C_4 alkyl, $P(O)(OH)_2$, $P(O)(OH)R^a$, where R^a = optionally substituted C_1–C_4 alkyl;
with the proviso that in all compounds represented by Formula C three or six negatively charged groups are present in the residues X of Formula B under basic conditions, i.e. 7 < pH < 14, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: SH, COOH, SO_3H, OSO_3H, $OP(O)(OH)_2$, $OP(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl, $P(O)(OH)_2$, $P(O)(OH)R^a$, where R^a = C_1–C_4 alkyl or substituted C_1–C_4 alkyl.

4. A fluorescent dye having Formula C according to claim 3 wherein X at each occurrence is SO_3H and n is 1-12, preferably 1-6.

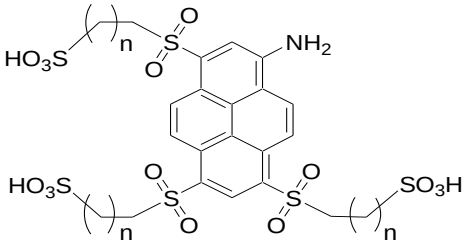
5. A fluorescent dye according to claims 1-4 wherein R^1 and/or R^2 in Formula B or C represents H, deuterium, alkyl or deutero-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deutero-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, 4,6-dihalo-1,3,5-triazinyl ($C_3N_3X_2$) where halogen X is preferably chlorine, 2-, 3- or 4-aminobenzoyl ($COC_6H_4NH_2$), N-[(2-, N-[(3- or N-[(4-aminophenyl)ureido group ($NHCONHC_6H_4NH_2$), N-[(2-, N-[(3- or N-[(4-aminophenyl)thioureido group ($NHCSNHC_6H_4NH_2$) or linked carboxylic acid residues and their reactive esters of the general formulae $(CH_2)_{m1}COOR^3$, $(CH_2)_{m1}OCOOR^3$, $(CH_2)_{n1}COOR^3$ or $(CO)_{m1}(CH_2)_{m2}(CO)_{n1}(NH)_{n2}(CO)_{n3}(CH_2)_{n4}COOR^3$ where the integers m1,m2 and n1,n2,n3,n4 independently range from 1 to 12 and from 0 to 12, respectively, with the chain $(CH_2)_{m/n}$ being straight, branched, saturated, unsaturated, partially or completely deuterated, and/or or included into a carbo- or heterocylcle containing N, O or S, whereas R^3 is H, D or a nucleophile-reactive leaving group, preferably including but not limited to *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, cyanomethyl, polyhalogenoalkyl, polyhalogenophenyl, *e.g.* tetra- or pentafluorophenyl, 2- or 4-nitrophenyl.

6. Fluorescent dyes of Formula B as shown in claim 1 that have a hydroxylamine or a hydrazine moiety as an analyte-reactive group, which is connected directly the dye core or where the hydroxylamine or hydrazine moieties are connected via a linker group, in particular alkyl, heteroalkyl or a linker L, as defined in claim 1 for Formula B.

7. A compound according to one of the general formulae B and C of claims 1-5 having one of the following formulae:



15



23a (n = 2)
23b (n = 1)

or a salt thereof.

8. A compound of Formulae B and C as shown in claims 1 and 3, respectively, which has a net charge z of 0, -1, -3, -6 or -12, preferably -6 or -12, in particular in an aqueous medium at a pH range of 7-13.

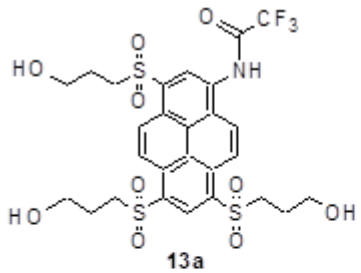
9. A fluorescent dye salt according to any one of claims 1-8, comprising negatively charged sulfonate and/or phosphate groups and counterions selected from inorganic or organic cations, preferably alkaline metal cations, ammonium cations or cations of organic ammonium and phosphonium compounds, and/or comprising a positively charged group or a charge-transfer complex formed at the nitrogen site $N(R^1)R^2$ in the dye of formulae B and C as shown in claims 1 and 3, respectively, with a counterion selected from anions of a strong mineral, organic or a Lewis acid.

10. A fluorescent dye according to any one of claims 1-9, wherein hydrazine $N(R)NH_2$, hydroxylamine N(R)OH or aminooxy ONH_2 groups with R being H, alkyl, heteroalkyl, alkenyl or perfluoroalkyl are connected to the nitrogen site $N(R^1)R^2$ in the dye of formula B via all types of linkers listed for group L in formula B.

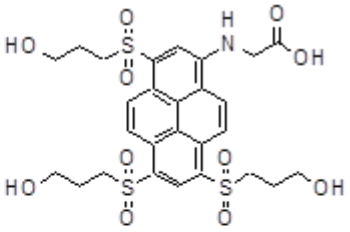
11. A compound according to any one of claims 1–10 and salts thereof for use in the reductive amination and in the condensation with reducing sugars, i.e. monomeric, oligomeric or polymeric carbohydrates possessing an aldehyde group in a free form or as semiacetal, including glycans.

12. A compound according to any one of claims 1–11 and salts thereof for use as a fluorescent label for amino acids, peptides, proteins, including primary and secondary antibodies, single-domain antibodies, docetaxel, avidin, streptavidin and their modifications, aptamers, nucleotides, nucleic acids, toxins, lipids, carbohydrates, including 2-deoxy-2-amino glucose and other 2-deoxy-2-aminoaminopyranosides, glycans, biotin, and other small molecules, i.e. having molecular masses of less than 1500 Da, e.g., jasplakinolide and its modifications.

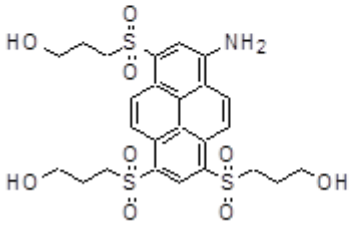
13. Compounds according to claim 12 which belong to the following compound set: **13a**, **13b**, **16** and **18**.



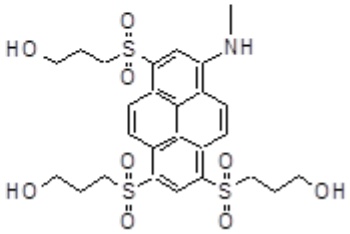
13a



16



13b

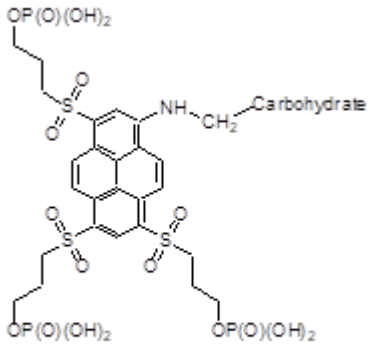


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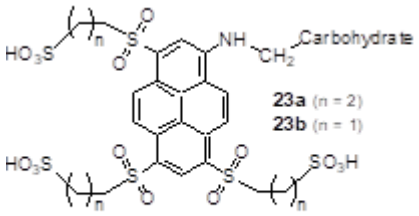
14. Use of the compounds according to any one of claims 1-10 and salts thereof as fluorescent reagents for conjugation to analytes, wherein the conjugation comprises formation of at least one covalent chemical bond or at least one molecular complex with a chemical entity or substance, such as amine, carboxylic acid, aldehyde, alcohol, aromatic compound, heterocycle, dye, amino acid, amino acid residue coupled to any chemical entity, peptide, protein, carbohydrate, nucleic acid, toxin and lipid.

15. Carbohydrate-dye conjugates comprising fluorescent dyes according to any one of claims 1-10.

16. The carbohydrate-dye conjugates according to claim 15 wherein the dye is selected from the compounds of the formulae **15, 23a** and **23b,** as shown below



15-carbohydrate: **q** = - 6



23a,b-carbohydrate: **q** = - 3

17. A kit or composition comprising one or more of the dyes of claims 1-10 or one or more of the carbohydrate conjugates of claims 15 and 16.