

**Sulfonated 2(7)-aminoacridone and 1-aminopyrene dyes
and their use as fluorescent tags, in particular for
carbohydrate analysis**

Background of the invention

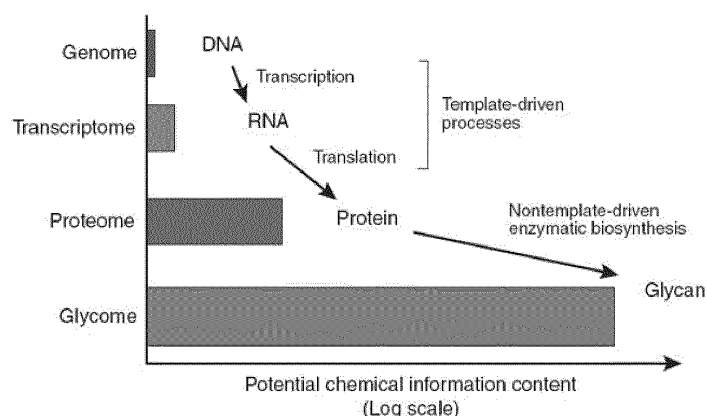
The present invention relates to fluorescent dye compounds which provide novel efficient and advantageous tags for carbohydrate analysis, particularly in those methods that involve reductive amination, followed by chromatographic or electrokinetic separation and fluorescence detection by means of hydrophilic interaction chromatography with fluorescence detection (HILIC-FLR, reversed phase liquid chromatography with fluorescence detection (RPLC-FLR), capillary (gel) electrophoresis with laser-induced fluorescence detection (CE-LIF, respectively CGE-LIF) or other separation based glycoanalytical methods. Chromatographic methods can be operated as high performance or as ultra-high-performance liquid chromatography (HPLC or UHPLC) and all separation-based methods can be coupled to mass-spectrometry, either alternatively or in addition to fluorescence detection. Characteristically, the fluorescent maxima of the dye-carbohydrate conjugates are considerably shifted to the red, relative to that with known dye tags (considered below).

The term "carbohydrate(s)" as used herein is the collective term for monosaccharide(s), like xylose arabinose, glucose, galactose, mannose, fructose, fucose, N-acetylglucoseamine, sialic acids; (homo or hetero) disaccharide(s), like lactose, sucrose, maltose, cellobiose; (homo or hetero) oligosaccharide(s), like glycans (e.g. N- and O-glycans), galactooligosaccharides (GOS), fructooligosaccharides (FOS), milk oligosaccharides (MOS) or even the glycomoiety of glycolipids; and polysaccharide(s), like amylose, amylopektin,

cellulose, glycogen, glycosaminoglycan, or chitin. Oligo- and polysaccharides can either be linear or (multiple) branched.

Glycoconjugates are compounds in which a carbohydrate (the glycone) is linked to a noncarbohydrate moiety (the aglycon). Typically, the aglycone is either a protein (glycoprotein) or a lipid (glycolipid). In a more general sense, glycoconjugate means a carbohydrate covalently linked to any other chemical entity such as protein, peptide, lipid, or even saccharide.

Glycoconjugates represent the structurally and functionally most diverse molecules in nature. They range in complexity from relatively simple glycolipids to extraordinarily complex and multiple glycosylated proteins. Although the carbohydrate moiety is commonly composed of only a few monosaccharides, including *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, galactose, fucose, glucose, and sialic acids, their structural diversity is possibly much larger than that of proteins or DNA. The reasons for this diversity are the presence of the anomers and the ability of monosaccharides to branch and to build different (glycosidic) linkages. Accordingly, an oligosaccharide with a relatively small chain length may have an enormous number of structural isomers. Moreover, unlike protein biosynthesis, which is based on RNA as a template, the information flow from the genome to the *glycome* (see next paragraph for a definition) is a complex and no longer a template-driven process. The co- and post-translational modification of proteins in glycan biosynthesis is provided by enzymatic reactions. This leads to a drastic increase of complexity and structural diversity of *glycans* (as depicted in scheme 1 below). In practice, the term *glycan* refers to the carbohydrate portion of a glycoconjugate. The terms glycan, oligosaccharide and polysaccharide are synonyms and mean "compounds consisting of a medium to large number of monosaccharides linked glycosidically" (a IUPAC definition).

**Scheme 1**

In order to elucidate the structural features of the *glycome*, which means the complete set of glycoconjugates that cells produce under specified conditions, and to understand its functions and its “counterplay” with DNA and protein “machinery”, rapid, robust and high resolution bioanalytical techniques must be available.

Many analytic techniques have been utilized to analyze carbohydrates. Complex samples containing a variety of different oligosaccharides can be separated by chromatographic or electrokinetic techniques. Common chromatographic modes include size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC), reversed phase liquid chromatography (RPLC) and reversed-phase ion-pairing chromatography (RPIPC), as well as porous graphitized carbon chromatography (PGC). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) allow *de novo* structural characterization of complex oligosaccharides. A combination of several techniques is often applied, e.g. LC-NMR, LC-MS or CE-MS. As soon as the structure of a particular glycan is determined, its characteristic properties, such as retention or migration times, are tabulated. By comparing the data obtained for unknown samples with tabulated parameters, the rapid screening and evaluation of unknown samples can be performed.

Electrokinetic separation techniques are based on the motion of charged particles under the influence of an electric field and include various electrophoretic techniques. The most important electrokinetic separation methods for glycoanalysis are capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE). These techniques provide simultaneously high resolution, fast separation, and allow for quantification.

Multiplex capillary gel electrophoresis with laser induced fluorescence detection (xCGE-LIF) has shown to be an especially powerful tool for glycoanalysis (e.g., in R. Hennig, U. Reichl, E. Rapp, *Glycoconjugate J.* **2011**, 28, 331). One advantage is the possibility to utilize a multiplex capillary array setup which offers the potential for very high throughput analysis due to parallelization of separation. Another reason for using xCGE-LIF is the very high sensitivity due to LIF detection (R. Hennig, *Diploma Thesis*, Otto-von-Guericke Universität, Magdeburg, **2010**).

Capillary electrophoresis (CE) is a family of electrokinetic separation methods and is defined by the IUPAC as "separation techniques carried out in capillaries based solely on the differences in the electrophoretic mobilities of charged species (analytes) either in aqueous or non-aqueous background electrolyte solution" (see also M.-L. Riekkola, J. A. Jönsson, R. M. Smith, *Pure Appl. Chem.* **2004**, 76, 443-451). In other words, CE terms the separation of ions by electrophoresis within a very thin capillary.

CGE is defined by the IUPAC as "a special case of capillary sieving electrophoresis when the capillary is filled with a cross-linked gel" (see also M.-L. Riekkola et al., above).

The electrophoretic mobility of a compound depends on the mass to charge ratio, and when employing CGE - due to the gels sieving effect - it depends additionally from the molecular shape.

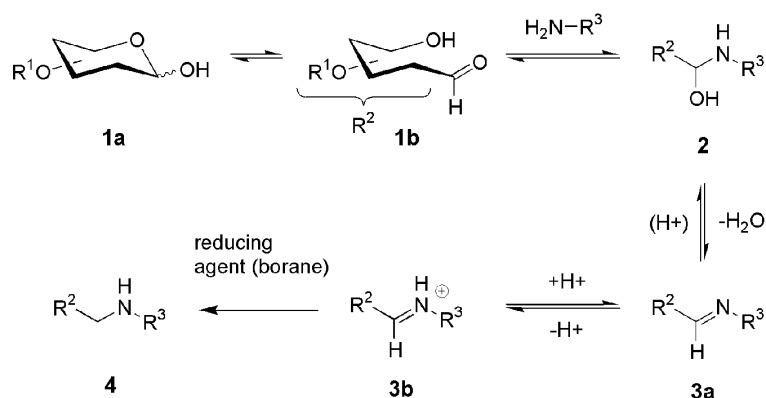
Commonly, native carbohydrates cannot be separated by their mass to charge ratio, because most of them are electroneutral except the ones that contain charged residues like sialic acids, glucuronic acids, or sulfated or phosphorylated monosaccharide units.

Further, native carbohydrates show no fluorescence. Thus, for analysis via chromatographic or electrokinetic separation and fluorescence detection, it is necessary to label the oligosaccharides prior to the analysis (Fig.1). Besides providing proper excitation and emission wavelengths (and (necessary for electrokinetic separation) additional electric charges), the dyes must be suited for coupling to the carbohydrates (see, for example, N. V. Shilova, N. V. Bovin, *Russ. J. Bioorg. Chem.* **2003**, 29 (4), 339-355.

The most common and straightforward method for labeling carbohydrates with fluorescent dyes is the reductive amination. The two-step procedure yields a covalent bond between the reducing end of a carbohydrate (acetal or aldehyde group) and a primary amine. The primary amine is either incorporated into the structure of the fluorophore or bound to a linker, which is connected with the fluorescent dye.

Fig. 1 shows the main steps of separation-based glycan analysis. The glycan analysis procedure can be divided into five steps: sample preparation, glycan release, labeling, sample purification, chromatographic or electrokinetic separation with fluorescent detection and the data evaluation. In the following, the labeling of glycans and detection of labeled products according to the state of art will be described in more detail. Scheme 2 below shows the principal reaction mechanism of the reductive amination of carbohydrates (see, for example, N. Volpi, *Capillary electrophoresis of carbohydrates. From*

monosaccharides to complex polysaccharides, Humana Press, New York, 2011, pp. 1-51).

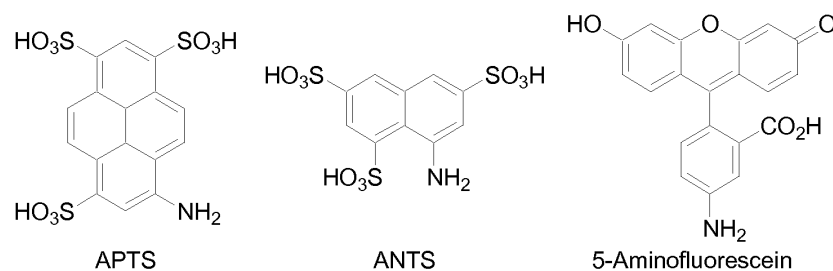


Scheme 2

The first step of the reductive amination involves a nucleophilic addition reaction where the lone electron pair of the amine nitrogen attacks the electrophilic aldehyde carbon atom of the carbohydrate residue in its open-chain form (**1b**). The acid-catalyzed elimination of water from intermediate **2** gives an imine (**3a**). Since the imine formation is reversible, the imine has to be converted into a secondary amine (**4**) via irreversible acid-catalyzed reduction with a hydride source, such as sodium cyanoborohydride, triacetoxy borohydride, 2-picolylnyl borane, or related reagents (reducing agents in Scheme 2). The nature of borane is important, because only iminium ions **3b** need to be reduced, while carbohydrates R^2CHO (**1b**) have to remain unreactive towards the reduction (they react only with amines R^3NH_2 which represent fluorescent tags).

The reaction sequence depicted in Scheme 2 is based on the availability and sufficient reactivity of special reducing agents (boranes) which do not react with aldehydes (or reduce them very slowly), but *under acidic conditions* readily reduce iminium ions (**3b**). Weak or medium strong acids such as acetic

($pK_a = 4.76$), malonic ($pK_a^1 = 2.83$) or citric acid ($pK_a^1 = 3.13$) are frequently used at $pH = 3-6$ to achieve an irreversible and rapid reduction (K.R. Anumula, *Anal. Biochem.* **2006**, 350, 1-23). Therefore, the applied amine (R^3NH_2) has to be a weak base (because only the non-protonated amine can react with aldehyde **1b** in Scheme 2). In proteins, the aliphatic amino groups of lysine, nucleophilic nitrogen atoms in histidine and arginine residues are protonated at $pH = 3-6$ and do not react with carbohydrates according to Scheme 2. Therefore, only aromatic amines with rather low pK_a values of 3-5 (these are values for the conjugated acids) are required and widely used as analytical reagents for reductive amination of natural glycans. Shown below are 3 commercially available aromatic amines applicable for labeling of glycans via reductive amination, chromatographic or electrokinetic separation of conjugates and sensitive detection by fluorescence.



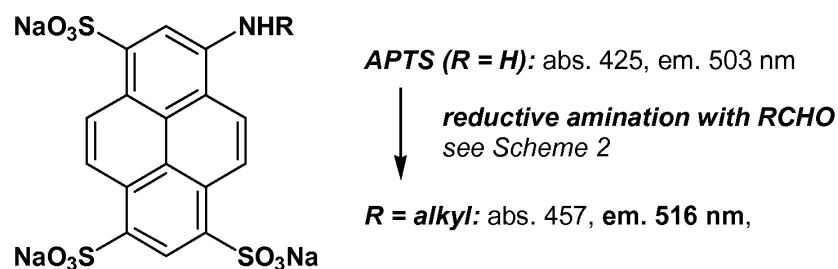
3-Aminopyrene-1,6,8-trisulfonic acid (APTS) is currently the most widely used reagent for carbohydrate labeling. Three strongly acidic residues (sulfonic acid groups) introduce three negative charges at $pH > 2$ (in a very wide pH range). ANTS also has three negative charges, but its absorption maximum is too blue-shifted and, therefore, does not fit for the commercial 488nm laser.

After purification (to remove proteins, excess electrolytes, excess dye, labeling reagents, etc., as also described in the Example 2 of the section "Examples" below and in references

therein), the labeled sample is injected into the chromatographic column, respectively the electrokinetic capillary, and the separation is carried out. Due to their different properties (like hydrophobicity, mass/charge, shape, etc.) the different carbohydrates reach the detector according to their characteristic retention, respectively migration times.

When the labeled carbohydrates reach the fluorescence detector, the fluorescent markers are excited and the emission signal is detected.

Today, "real" high throughput analysis of labeled glycans is typically performed on commercial multiplex CGE-systems. These xCGE-LIF instruments contain a multiplexed capillary gel electrophoresis unit for the separation of charged analytes (e.g., APTS-labeled glycans), a laser and a fluorescence detector.



Scheme 3: Spectral properties of APTS and its *N*-alkylated derivatives in aqueous buffers (according to Z. Sharrett, S. Gamsey, L. Hirayama, B. Villozny, J. T. Suri, R. A. Wessling, B. Singaram, *Org. Biomol. Chem.* **2009**, 7, 1461-1470).

Other dyes than APTS may be used as fluorescent tags for carbohydrates (e.g., ANTS and 5-aminofluorescein) and their derivatives. For example, acridone dyes are described in WO 2002/099424 A3 and WO 2009/112791 A1, but not 7-aminoacridone-2-sulfonamides. WO 2012/027717 A1 describes systems comprising

functionally substituted 1,6,8-trisulfonamido-3-aminopyrenes (APTS derivatives), an analyte-reactive group, a cleavable anchor as well as a porous solid phase. WO 2010/116142 A2 describes a large variety of fluorophores and fluorescent sensors compounds which also encompass aminopyrene-based dyes. However, none of these dyes has been shown or suggested to have superior spectral and electrophoretic properties, in particular as conjugates with carbohydrates, in comparison with APTS.

In view of the drawbacks of the fluorescent dyes of the prior art, the main objective of the present invention was to provide novel fluorescent dyes with improved properties, such as even higher electrophoretic mobility and/or higher brightness or other favorable spectroscopic characteristics, as compared to APTS. These properties are highly demanded from fluorescent tags for carbohydrate analysis based on electrokinetic separation with fluorescence detection and thus would provide a superior performance, here.

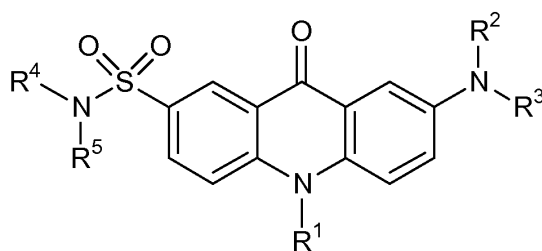
This objective has been achieved by providing novel fluorescent acridone and pyrene dyes with multiple negatively charged groups according to claims 1-14, as well as the use of the disclosed novel fluorescent dyes according to, e.g., claim 18. Further aspects and more specific embodiments of the invention are the subject of further claims.

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

(e.g. -6), provide higher electrophoretic mobility of dye - glycoconjugates, as compared to APTS - glyco-conjugates.

The structures of the novel fluorescent dyes of the invention are selected from the group consisting of compounds of the following general Formulae A-B or salts thereof:



Formula A

wherein

R^1 , R^2 , R^3 , R^4 , R^5 are independent from each other and may represent:

H, CH_3 , C_2H_5 , a straight or branched $\text{C}_3\text{-C}_{12}$, preferably $\text{C}_3\text{-C}_6$, alkyl or perfluoroalkyl group, a phosphonylated alkyl group $(\text{CH}_2)_m\text{P}(\text{O})(\text{OH})_2$, where $m = 1\text{-}12$, preferably $2\text{-}6$, with a straight or branched alkyl chain, $(\text{CH}_2)_n\text{COOH}$, where $n = 1\text{-}12$, preferably $1\text{-}5$, or $(\text{CH}_2)_n\text{COOR}^6$, where $n = 1\text{-}12$, preferably $1\text{-}5$, and R^6 may be alkyl, in particular $\text{C}_1\text{-C}_6$, 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 or other potentially nucleophile-reactive leaving groups, alkyl sulfonate $(\text{CH}_2)_n\text{SO}_3\text{H}$ or alkyl sulfate $(\text{CH}_2)_n\text{OSO}_3\text{H}$ where $n = 1\text{-}12$, preferably $1\text{-}5$, and the alkyl chain in any $(\text{CH}_2)_n$ may be straight or branched;

a hydroxyalkyl group $(\text{CH}_2)_m\text{OH}$ or thioalkyl group $(\text{CH}_2)_m\text{SH}$, where $m = 1\text{-}12$, preferably $2\text{-}6$, with a straight or branched alkyl chain, a phosphorylated hydroxyalkyl group $(\text{CH}_2)_m\text{OP}(\text{O})(\text{OH})_2$,

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 $(CH_2)_mOCOOR^7$ or $(CH_2)_mNHCOOR^7$, where $m = 1-12$ and $R^7 =$ methyl, ethyl, tert-butyl, benzyl, fluoren-9-yl, CH_2CN , *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, phenyl, substituted phenyl group, e.g., 2- or 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, 4-pyridyl, pyrimid-4-yl;

$(CH_2)_mNR^aR^b$, where $m = 1-12$, preferably $2-6$, with a straight or branched alkyl chain; R^a , R^b are independent from each other and represent hydrogen and/or C_1-C_4 alkyl groups, a hydroxyalkyl group $(CH_2)_mOH$, where $m = 2-6$, with a straight or branched alkyl chain, a phosphorylated hydroxyalkyl group $(CH_2)_mOP(O)(OH)_2$, where $m = 2-6$, with a straight or branched alkyl chain;

an alkyl azide $(CH_2)_mN_3$, where $m = 1-12$, preferably $2-6$, with a straight or branched alkyl chain;

R^1 , R^2 , R^3 , R^4 , R^5 may contain a terminal alkyloxyamino group $(CH_2)_mONH_2$, where $m = 1-12$, preferably $2-6$, with a straight or branched alkyl chain, that can include one or multiple alkylamino $(CH_2)_mNH$ or alkylamido $(CH_2)_mCONH$ groups in all possible combinations with $m = 0-12$;

$(CH_2)_nCONHR^8$, with $n = 1-12$, preferably $1-5$; $R^8 = H$, C_1-C_6 alkyl, $(CH_2)_mN_3$, or $(CH_2)_m-N$ -maleimido, $(CH_2)_m-NH-COCH_2X$ ($X = Br$ or I), with $m = 1-12$, preferably $2-6$, and with straight or branched alkyl chains in $(CH_2)_n$, $(CH_2)_m$ and R^6 ;

a primary amino group;

further, one of the residues R^1 , R^2 , R^3 , R^4 , R^5 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;

additionally, R^2---R^3 (R^4---R^5) may form a four-, five, six-, or seven-membered cycle, or a four-, five, six-, or seven-membered cycle with or without a primary amino group NH_2 , secondary amino group NHR^a , where $R^a = C_1-C_6$ alkyl, a hydroxyl group OH , or a

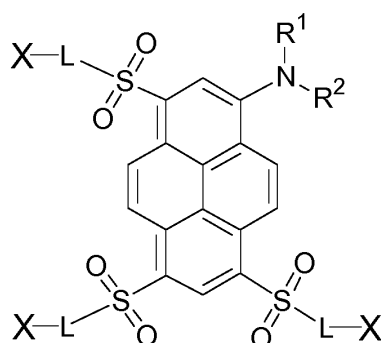
phosphorylated hydroxyl group $-\text{OP}(\text{O})(\text{OH})_2$ attached to one of the carbon atoms in this cycle;

optionally $\text{R}^2\text{---}\text{R}^3$ ($\text{R}^4\text{---}\text{R}^5$) may form a four-, five-, six-, or seven-membered heterocycle with an additional 1-3 heteroatoms such as O, N or S included into this heterocycle;

further, R^1 may represent an unsubstituted phenyl group, a phenyl group with one or several electron-donor substituents chosen from the set of OH, SH, NH_2 , NHR^a , NR^aR^b , R^aO , R^aS , where R^a and R^b are independent from each other and may be $\text{C}_1\text{-C}_6$ alkyl groups with straight or branched carbon chains, a phenyl group with one or several electron-acceptors chosen from the set of NO_2 , CN, COH, COOH, CH=CHCN , $\text{CH=C}(\text{CN})_2$, SO_2R^a , COR^a , COOR^a , CH=CHCOR^a , CH=CHCOOR^a , CONHR^a , $\text{SO}_2\text{NR}^a\text{R}^b$, CONR^aR^b , where R^a and R^b are independent from each other and may be H, or $\text{C}_1\text{-C}_6$ alkyl group(s) with straight or branched carbon chains;

or R^1 may represent a heteroaromatic group;

with the proviso that in all compounds of Formula A above at least two, preferably at least 3, 4, 5 or 6 negatively charged groups are present under basic conditions, i.e. $7 < \text{pH} < 14$, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: SH, COOH, a sulfonic acid residue SO_3H , a primary phosphate group $\text{OP}(\text{O})(\text{OH})_2$, a secondary phosphate group $\text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl, a primary phosphonate group $\text{P}(\text{O})(\text{OH})_2$, a secondary phosphonate group $\text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl;

**Formula B**

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

H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms of the alkyl group may be replaced by deuterium, in particular alkyl or deuterio-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, $(\text{CH}_2)_n\text{COOR}^3$, where $n = 1-12$, preferably 1-5, R^3 may be H, alkyl, in particular C_1 - C_6 , CH_2CN , benzyl, 2- and 4-nitrophenyl, fluorene-9-yl, polyhalogenoalkyl, polyhalogeno-phenyl, e.g. tetra- or pentafluorophenyl, pentachlorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl or other potentially nucleophile-reactive leaving groups, and the alkyl chain in $(\text{CH}_2)_n$ 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 $R^a = \text{C}_1$ - 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 $(\text{CH}_2)_m\text{OH}$, 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 $(\text{CH}_2)_m\text{OCOOR}^4$ or

$(\text{CH}_2)_m\text{NHCOOR}^4$, where $m = 1-12$ and $\text{R}^4 =$ 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-tetrafluoro-phenyl, 2-pyridyl, or 4-pyridyl;

$(\text{CH}_2)_m\text{NR}^a\text{R}^b$, 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 $\text{C}_1\text{-C}_4$ alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(\text{CH}_2)_m\text{N}_3$ with $m = 2-6$ and a straight or branched alkyl chain;

one of R^1 or R^2 may be $(\text{CH}_2)_n\text{SO}_2\text{NR}^5\text{NH}_2$ with $n = 1-12$, while the substituent R^5 can be represented by H, alkyl, hydroxyalkyl or perfluoroalkyl groups $\text{C}_1\text{-C}_{12}$;

one of R^1 or R^2 groups may be a primary amino group to form aryl hydrazines $\text{Ar-NR}^6\text{NH}_2$ where Ar is the entire pyrene residue in Formula B and $\text{R}^6 = \text{H}$ or alkyl;

one of R^1 or R^2 groups may be a primary amino group to form aryl hydrazines $\text{Ar-NR}^6\text{NH}_2$ where Ar is the entire pyrene residue in Formula B and $\text{R}^6 = \text{H}$ or alkyl;

one of R^1 or R^2 groups may be a hydroxy group to form aryl hydroxylamines $\text{Ar-NR}^7\text{OH}$ where Ar is the entire pyrene residue in Formula B and $\text{R}^7 = \text{H}$ or alkyl;

one of R^1 or R^2 groups may be an alkyloxyamino group $(\text{CH}_2)_n\text{ONH}_2$ with $n = 1-12$;

one of R^1 or R^2 groups may be $(\text{CH}_2)_n\text{COOR}^8$, with $n = 1-5$ and a straight or branched alkyl chain $(\text{CH}_2)_n$ and with R^8 selected from H, straight or branched $\text{C}_1\text{-C}_6$ alkyl, CH_2CN , 2- and 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzo-triazolyl;

further, one of R^1 or R^2 may be $(\text{CH}_2)_n\text{CONHR}^9$, with $n = 1-5$ and $\text{R}^9 = \text{H}$, $\text{C}_1\text{-C}_6$ alkyl, $(\text{CH}_2)_m\text{N}_3$, $(\text{CH}_2)_m\text{-N-maleimido}$, $(\text{CH}_2)_m\text{-NHCOCH}_2\text{X}$ ($\text{X} = \text{Br}$ or I), where $m = 2-6$ and with straight or branched alkyl chains in $(\text{CH}_2)_n$ and R^9 ; or one of R^1 or R^2 may represent $\text{CH}_2\text{-}$

$C_6H_4-NH_2$, $COC_6H_4-NH_2$, $CONHC_6H_4-NH_2$ 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;

the linker L (which connects the dye core with the group X and tailors the spectral properties of the dye) comprises at least one carbon atom and may comprise alkyl, heteroalkyl, in particular alkyloxy such as CH_2OCH_2 , $CH_2CH_2OCH_2CH_2OCH_2$, alkylamino or dialkylamino, particularly diethanolamine or *N*-methyl (alkyl) monoethanolamine moieties such as $N(CH_3)CH_2CH_2O-$ and $N(CH_2CH_2O-)_2$, 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} ;

the linker L may also include a carbonyl (CH_2CO , CF_2CO) moiety;

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 1 to 12, or an analogon 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, pipercolines, 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, SO₃H, OSO₃H, OP(O)(OH)₂, OP(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl, P(O)(OH)₂, P(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl.

Compounds of Formulae A and B can exist and be applied in the form of salts that involve all possible types of cations, preferably Na⁺, K⁺, Li⁺, NH₄⁺, organic ammonium (e.g. trialkylammonium) or organic phosphonium cations.

In more specific embodiments, a fluorescent dye salt according to the present invention may comprise negatively charged acid groups, in particular sulfonate and/or phosphate groups, and counterions selected from inorganic or organic cations, preferably alkaline metal cations, ammonium cations or cations of organic ammonium or phosphonium compounds (such as trialkylammonium cations), and/or may comprise a positively charged group or a charge-transfer complex formed at the nitrogen site N(R¹)R² in the dye of Formulae A-D as well as a counterion, in particular selected from anions of a strong mineral, organic or a Lewis acid.

The term "substituted" as used herein, generally refers to the presence of one or more substituents, in particular substituents selected from the group comprising straight or branched alkyl, in particular C₁-C₄ alkyl, e.g. methyl, ethyl, propyl, butyl; isoalkyl, e.g. isopropyl, isobutyl (2-methylpropyl); secondary alkyl group, e.g. sec-butyl (but-2-yl); tert-alkyl group, e.g. tert-butyl (2-methylpropyl). Additionally, the term "substituted" may refer here to alkyl groups having at least one deuterium-, fluoro-, chloro- or bromo substituent instead of hydrogen atoms, or methoxy, ethoxy, 2-(alkyloxy)ethoxy groups (AlkOCH₂CH₂O), and, in a more general case, oligo(ethyleneglycol)

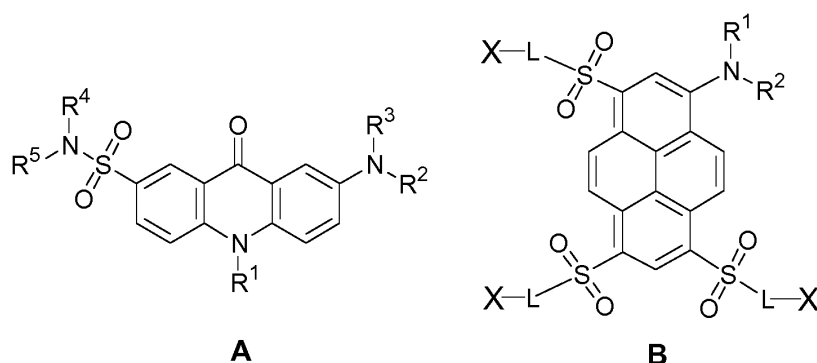
residues of the art $\text{Alk}(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{CH}_2-$, where $\text{Alk} = \text{CH}_3$, C_2H_5 , C_3H_7 , C_4H_{10} , and $n = 1 - 23$.

The term "alkyl" generally may refer to any alkyl group selected from the group comprising straight or branched alkyl, more specifically C_1 - C_{20} alkyl, C_1 - C_{12} alkyl, or C_1 - C_6 alkyl, e.g. methyl, ethyl, propyl, butyl; isoalkyl, e.g. isopropyl, isobutyl (2-methylpropyl); secondary alkyl group, e.g. sec-butyl (but-2-yl); *tert*-alkyl group, e.g. *tert*-butyl (2-methylpropyl) etc.

The terms "aromatic heterocyclic group" or "heteroaromatic group", as used herein, generally refer to an unsubstituted or substituted cyclic aromatic radical (residue) having from 5 to 10 ring atoms of which at least one ring atom is selected from S, O and N; the radical being joined to the rest of the molecule via any of the ring atoms. Representative, but not limiting examples are furyl, thienyl, pyridinyl, pyrazinyl, pyrimidinyl, pyrrolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, quinolinyl and isoquinolinyl.

Compounds of the general structural Formula A above are acridone dyes, compounds of the Formula B above are pyrene dyes.

More specifically, according to the IUPAC rules the compounds of Formula A are 7-aminoacridon-2-sulfonamides, whereas the compounds of Formula B are 1-aminopyrene dyes with functionally substituted sulfonyl groups in positions 3, 6, 8, i.e. (functionally substituted) 1,6,8-trisulfonyl-3-aminopyrenes, as shown in the basic structural formulae in Scheme 4 below.

**Scheme 4**

The novel fluorescent dyes of the present invention exhibit a number of favorable characteristics:

- aromatic amino (ArNH_2), hydrazine (NRNH_2), hydrazide (CONRNH_2), hydroxylamine (RNHOH) or alkoxyamino group (RONH_2) with $pK_b = 11 - 8$ ($pK_a = 3 - 6$) for efficient and clean reductive amination at $pH \sim 2-5$ or direct condensation with carbohydrates; preferably, the aromatic amino group is primary, but it can also be a secondary one; see Scheme 4 for structures
- large net charges in conjugates -- in the range of -3 to -6 at $pH = 7 - 10$
- very good solubility in aqueous media at a wide range of pH; high brightness (which is the overall result of the fluorescence quantum yield and extinction)
- exceptional stability of the dye core against reduction with borane-based reagents and at $pH = 3 - 6$
- the ability to be excited with an argon ion laser (emitting at 488 and 514 nm) with a perfect spectral match and high fluorescence quantum yields
- minimal emission at ca. 520 nm

- The dyes are amenable to purification up to 99%.

The novel fluorescent tags of the invention even allow the detection of "heavy" glycans with very long migration times. Due to these long migration times and peak-broadening, such "heavy" glycans are very difficult to detect electrokinetically; especially if APTS is used as fluorescent tag.

In the following, more specific embodiments of the present invention are described.

In Formula A above, NR^1 and/or $\text{N}(\text{R}^2)\text{R}^3$ comprise carbonyl- or nucleophile-reactive groups. Preferably, R^1 , R^2 , and R^3 are represented by H, linear or branched alkyl, hydroxyalkyl or perfluoroalkyl groups. Substituents R^3 , R^4 and R^5 preferably comprise solubilizing and/or anion-providing groups, particularly hydroxyalkyl $((\text{CH}_2)_n\text{OH})$, thioalkyl $((\text{CH}_2)_n\text{SH})$, carboxyalkyl $((\text{CH}_2)_n\text{CO}_2\text{H})$, alkyl sulfonate $((\text{CH}_2)_n\text{SO}_3\text{H})$, alkyl sulfate $((\text{CH}_2)_n\text{OSO}_3\text{H})$, alkyl phosphate $((\text{CH}_2)_n\text{OP}(\text{O})(\text{OH})_2)$ or alkyl phosphonate $((\text{CH}_2)_n\text{P}(\text{O})(\text{OH})_2)$, wherein n is an integer ranging from 1 to 12.

Alternatively, substituents R^1 , R^2 , R^3 , R^4 and R^5 may be represented by carboxylic acid residues $(\text{CH}_2)_n\text{COOH}$, where $n = 1-12$, and their reactive esters $(\text{CH}_2)_n\text{COOR}^6$ as nucleophile-reactive groups. R^6 can be H, alkyl, (*tert*-butyl including), benzyl, fluorene-9-yl, polyhalogenoalkyl, CH_2CN , polyhalogenophenyl (e. g., tetra- or pentafluorophenyl, pentachlorophenyl), 2- and 4-nitrophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl or other potentially nucleophile-reactive leaving groups. The alkyl chains (or backbones) $(\text{CH}_2)_n$ may be linear or branched.

Further, the aryl amino groups (NR^1 and NR^2R^3) in Formula A can be connected to an analyte-reactive group via (poly)methylene,

carbonyl, nitrogen or sulfur-containing linear or branched linkers, particularly $(\text{CH}_2)_m\text{CON}(\text{R}^7)$, $\text{CO}(\text{CH}_2)_m\text{N}(\text{R}^7)$, $\text{CO}(\text{CH}_2)_m\text{S}(\text{CH}_2)_n$, $(\text{CH}_2)_m\text{S}(\text{CH}_2)_n\text{CO}$, $\text{CO}(\text{CH}_2)_m\text{SO}_2(\text{CH}_2)_n$, $(\text{CH}_2)_m\text{SO}_2(\text{CH}_2)_n\text{CO}$, their combinations, or linked as a part of nitrogen-containing non-aromatic heterocycles (e.g., piperazines, pipercolines, oxazolines); m and n are integers ranging from 0 to 12 or 1 to 12. The substituent R^7 may be represented by any of the functional groups listed for R^1 , R^2 , R^3 , R^4 and R^5 above.

Substituents R^1 , R^2 and R^3 in Formula A may be also represented by a primary amino group, thus comprising carbonyl-reactive aryl hydrazines, ($\text{R}^2 = \text{H}$, R^1 or $\text{R}^3 = \text{NH}_2$ or $\text{R}^1 = \text{NH}_2$, R^2 , $\text{R}^3 = \text{alkyl}$, perfluoroalkyl or alkyl) conjugated or substituted with with solubilizing and/or anion-providing moieties, listed as possible candidates for R^4 and R^5 , particularly: hydroxyalkyl $(\text{CH}_2)_n\text{OH}$, thioalkyl $((\text{CH}_2)_n\text{SH})$, carboxyalkyl $((\text{CH}_2)_n\text{CO}_2\text{H})$, alkyl sulfonate $((\text{CH}_2)_n\text{SO}_3\text{H})$, alkyl sulfate $((\text{CH}_2)_n\text{OSO}_3\text{H})$, alkyl phosphate $((\text{CH}_2)_n\text{OP}(\text{O})(\text{OH})_2)$ or phosphonate $((\text{CH}_2)_n\text{P}(\text{O})(\text{OH})_2)$, wherein n is an integer ranging from 0 to 12 or 1 to 12. Alternatively, hydrazine derivatives might be represented by sulfonyl hydrazides, where $\text{R}^4 = \text{NH}_2$, while R^5 are alkyl, perfluoroalkyl or alkyl groups decorated with solubilizing and/or anion-providing groups of the types mentioned above.

Alternatively, aryl amino groups (NR^1 and/or NR^2R^3) in Formula A can be connected to an acyl hydrazine or alkyl hydrazine moiety indirectly, via linkers, thus comprising hydrazides (ZCONHNH_2) or hydrazines (ZNHNH_2), respectively. Here Z denotes the dye residue of Formula A that includes aryl amino groups and linkers. In particular, R^1 and R^2 may be represented by: $(\text{CH}_2)_m\text{CON}(\text{R}^7)$, $\text{CO}(\text{CH}_2)_m\text{N}(\text{R}^7)$, $\text{CO}(\text{CH}_2)_m\text{S}(\text{CH}_2)_n$, $(\text{CH}_2)_m\text{S}(\text{CH}_2)_n\text{CO}$, $\text{CO}(\text{CH}_2)_m\text{SO}_2(\text{CH}_2)_n$, $(\text{CH}_2)_m\text{SO}_2(\text{CH}_2)_n\text{CO}$ and their combinations; m and n are integers ranging from 0 to 12. Substituent R^7 can be represented by any of the functional groups for R^1 , R^2 , R^3 , R^4 and R^5 that are listed

above as candidates for functional groups R^1 - R^5 , particularly: hydroxyalkyl $(CH_2)_nOH$, thioalkyl $((CH_2)_nSH)$, carboxyalkyl $((CH_2)_nCO_2H)$, 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 1 to 12. Linkers may also be represented by non-aromatic O, N and S-containing heterocycles (e. g., piperazines, pipercolines).

Further, R^1 , R^2 and R^3 may be represented by $CH_2-C_6H_4-NH_2$, $COC_6H_4-NH_2$, $CONHC_6H_4-NH_2$ 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 pyridine-2,4-diyl, pyridine-2,5-diyl, pyridine-2,6-diyl, pyridine-3,5-diyl.

The analyte-reactive group at variable positions R^1 , R^2 , R^3 , R^4 and R^5 may be represented by an aromatic or heterocyclic amine, carboxylic acid, ester of the carboxylic acid (e.g., N-hydroxysuccinimidyl or another amino reactive ester); or represented by alkyl azide $(CH_2)_nN_3$, alkyne (propargyl), amino-oxyalkyl $(CH_2)_nONH_2$, maleimido ($C_4H_3NO_2$ with a nucleophile-reactive double bond) or halogeno ketone function ($COCH_2X$; $X=Cl$, Br and I), as well as halogeno amide group ($NRCOCH_2X$, $R=H$, $C1-C6$ -alkyl, $X=Cl$, Br , I) connected either directly or indirectly via carbonyl, amido, nitrogen, oxygen or sulfur-containing linkers listed for hydrazine derivatives where $n=1-12$.

According to some more preferred embodiments of the present invention, the substituent R^1 in the above Formula A is defined as follows:

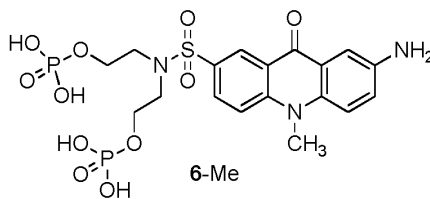
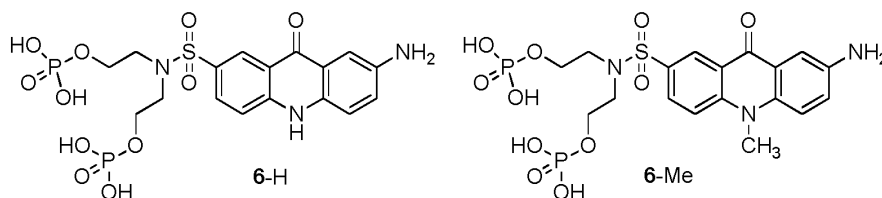
R^1 in Formula A represents hydrogen, a lower alkyl group (C_1-C_4), an unsubstituted phenyl group, a phenyl group with one or several electron-donor substituents chosen from the set of OH , SH , NH_2 , NHR^a , NR^aR^b , R^aO , R^aS , $OP(O)(OR^a)(OR^b)$ where R^a and R^b are

independent from each other and may be C₁-C₁₂, preferably C₁-C₆, alkyl groups with linear or branched chains, a phenyl group with one or several electron-acceptors chosen from the set of NO₂, CN, COH, COOH, CH=CHCN, CH=C(CN)₂, SO₂R^a, SO₃R^a, COR^a, COOR^a, CH=CHCOR^a, CH=CHCOOR^a, CONHR^a, SO₂NR^aR^b, CONR^aR^b, P(O)(OR^a)(OR^b) where R^a and R^b are independent from each other and may be H, or C₁-C₆ alkyl group(s) with straight or branched carbon chains;

alternatively, R¹ may represent an aromatic heterocyclic group, in particular, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl, pyrimidin-4-yl, pyrimidin-2-yl, pyrimidin-5-yl, or other electron acceptor groups derived from aromatic heterocycles, such as 4-pyridyl-*N*-oxides, *N*-alkylpyridinium salts, or betaines, in particular, *N*-(ω-sulfoalkyl)-4-pyridinium, *N*-(ω-sulfoalkyl)-2-pyridinium, *N*-(1-hydroxy-4,4,5,5-tetrafluoro-cyclopent-1-en-3-on-2-yl)-4-pyridinium, *N*-(1-hydroxy-4,4,5,5-tetrafluorocyclopent-1-en-3-on-2-yl)-2-pyridinium.

In particular, R¹ may represent a positively charged heterocyclic group derived from 2-pyridyl, 3-pyridyl, or 4-pyridyl precursors with an 7-aminoacridon-2-sulfonamide backbone and alkylating agents (e.g. alkyl halides, alkyl sulfonates, alkyl triflates, 1,3-propanesultone, 1,4-butanedisulfonate) or electrophiles (e.g., perfluorocyclopentene).

Especially preferred are compounds of the structural Formula A above that have one of the following formulae:



In the Formula B, L is a divalent linker that connects the dye core with solubilizing and/or ionizable moieties and also tailors the spectral properties.

Typically, its presence results in considerable bathoflorig and bathochromic shifts accompanied by a better match to the 488 nm commercial lasers, as compared to APTS dye tag, where fragment L is absent and X is OH.

The linker L in the Formula B comprises or consists of at least one carbon atom and can represent alkyl, heteroalkyl (e. g., alkyloxy: CH_2OCH_2 , $\text{CH}_2\text{CH}_2\text{O}$ $\text{CH}_2\text{CH}_2\text{OCH}_2$), difluoromethyl (CF_2), alkene or alkyne moieties in any combinations, at any occurrence, linear or branched, with the length ranging from C1 to C12. The linker can also include a carbonyl (CH_2CO , CF_2CO) and Sulfonamides are the case when L is an alkylamino or a dialkylamino group, particularly diethanolamine or N-methyl (alkyl) monoethanolamine moieties (i.e., $\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{O}-$ and $\text{N}(\text{CH}_2\text{CH}_2\text{O}-)_2$), which allow further connection to a solubilizing and/or ionizable moieties X. Certain embodiments of this invention represent the combination of moieties L and X according to the formulae $(\text{CH}_2)_3\text{OP}(\text{O})(\text{OH})_2$ and $\text{N}(\text{CH}_3)(\text{CH}_2)_2\text{OP}(\text{O})(\text{OH})_2$. The sulfonamides of this type thus have general formula $\text{SO}_2\text{NR}^3\text{R}^4$, where R^3 and R^4 are independent from each other and can be represented by H, alkyl, heteroalkyl (e. g., alkyloxy: CH_2OCH_2 , $\text{CH}_2\text{CH}_2\text{O}$, $\text{CH}_2\text{CH}_2\text{OCH}_2$), difluoromethyl (CF_2) in any combinations, linear or branched, with the length ranging from C1 to C12, also bearing terminal OH groups.

$\text{N}(\text{R}^1)\text{R}^2$ in the Formula B preferably comprises a carbonyl- or nucleophile-reactive group. Substituents R^1 and R^2 are independent from each other and can be both represented by hydrogen. One of those can be a linear or branched alkyl (perfluoroalkyl) group $\text{C}_1\text{-C}_{12}$. At the same time, one of R^1 and R^2 may be represented by carboxylic acid residues $(\text{CH}_2)_n\text{COOH}$ and

their regular or reactive esters $(\text{CH}_2)_n\text{COR}^3$ where n is an integer ranging from 1 to 12. The residue R^3 is H, alkyl, (*tert*-butyl including), benzyl, fluorene-9-yl, polyhalogenoalkyl, CH_2CN , polyhalogenophenyl (e. g., tetra- or pentafluoro phenyl, pentachlorophenyl), 2- and 4-nitrophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl or other potentially nucleophile-reactive leaving groups. The alkyl chains (or backbones) $(\text{CH}_2)_n$ may be linear or branched. Particularly, the formula can be depicted as $\text{Z-NR}^1(\text{CH}_2)_n\text{COR}^4$, where Z is the rest of the molecule in Formula B that also includes groups L and X.

Further, the nucleophile-reactive group COR^5 can be connected to the aryl amino group $\text{N(R}^1\text{)R}^2$ via (poly)methylene, oxymethylene (CH_2OCH_2 , $\text{CH}_2\text{CH}_2\text{OCH}_2$, PEG) carbonyl, carbonate, carbamate, urethane, nitrogen or sulfur-containing linkers (spacers) branched or linear, particularly $(\text{CH}_2)_m\text{CON(R}^6\text{)}$, $\text{CONH(CH}_2\text{)}_n$, $(\text{CH}_2)_m\text{OCONH(CH}_2\text{)}_n$, $\text{CO(CH}_2\text{)}_n$, CO(O)NR^6 , $(\text{CH}_2)_m\text{SO}_{2m}\text{N(R}^6\text{)}$, $\text{CO(CH}_2\text{)}_m\text{S(CH}_2\text{)}_n$, $(\text{CH}_2)_m\text{S(CH}_2\text{)}_n\text{CO}$, $\text{CO(CH}_2\text{)}_m\text{SO}_2(\text{CH}_2\text{)}_n$, $(\text{CH}_2)_m\text{SO}_2\text{NR}^6$, and their combinations; m and n are integers ranking from 0 to 12, R^6 = alkyl. The reactive group R^5 can be linked by means of non-aromatic O, N and S-containing heterocycles (e. g., piperazines, pipercolines, oxazolines). Substituent R^6 might be represented by H, alkyl, hydroxyalkyl or perfluoroalkyl groups $\text{C}_1\text{-C}_{12}$.

One of the substituents R^1 and R^2 in Formula B may be represented by a primary amino group, thus comprising carbonyl-reactive aryl hydrazines ($\text{R}^1 = \text{NH}_2$, R^2 = alkyl, perfluoroalkyl) or by a hydroxy group to form aryl hydroxylamines (ArNHOH). Alternatively, the alkyl hydrazine or hydroxylamine reactive moiety in Formula B can be connected to aryl amino group $\text{N(R}^1\text{)R}^2$ via linkers listed above for the reactive group R^4 . Sulfonyl hydrazides constitute a special case when R^1 or $\text{R}^2 = (\text{CH}_2)_n\text{SO}_2\text{NR}^6\text{NH}_2$ with $n = 1\text{-}12$, while the substituent R^6 can be represented by H, alkyl, hydroxyalkyl or perfluoroalkyl groups $\text{C}_1\text{-C}_{12}$. The sulfonylamide (sulfonamide,

sulfamide) group can be also attached via diverse linkers listed above for the case with the reactive groups R^4 , R^3 and R^5 .

Further, R^1 and R^2 may be represented by $CH_2-C_6H_4-NH_2$, $COC_6H_4-NH_2$, $CONHC_6H_4-NH_2$ 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 pyridine-2,4-diyl, pyridine-2,5-diyl, pyridine-2,6-diyl, pyridine-3,5-diyl.

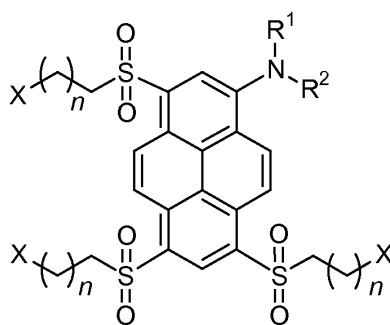
Substituents R^1 and R^2 may be also represented by alkyl azide $(CH_2)_nN_3$, alkine (propargyl), maleimido ($C_4H_3NO_2$ with a nucleophile-reactive double bond) or halogeno-ketone function ($COCH_2X$; $X = Cl, Br$ and I) connected either directly or via carbonyl, amido, nitrogen or sulfur-containing linkers listed for hydrazine derivatives; $n = 1-12$.

Group X in Formula B denotes solubilizing and/or ionizable anion-providing moieties, particularly the ones that provide enhanced electrophoretic mobility. Group X can include 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. Alternatively, the CH_2 group can be replaced by CF_2 . The anion-providing moieties can be also linked by means of non-aromatic O, N and S-containing heterocycles (e.g., piperazines, pipercolines). Alternatively, one of the groups X can bear any of the carbonyl- or nucleophile-reactive moieties listed for groups R^1 and R^2 , also with any type of linkage listed for group L, and independently from other substituents.

As already mentioned above, compounds of Formula B can exist and be applied in the form of salts that involve all possible types of cations, preferably Na^+ , Li^+ , K^+ , NH_4^+ and organic ammonium or organic phosphonium cations.

According to one specific embodiment of the invention, the anion-providing group(s) X may represent, at each occurrence in Formula B, one to four groups SO_3H attached to the respective linker group L at one or more sites (and for each type of linkage as defined for L above).

According to another specific embodiment of the invention, the compounds of the structural Formula B above are alkylsulfonyl derivatives as depicted in Formula C



Formula C

wherein

R^1 and/or R^2 are independent from each other and may represent: H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3 , C_2H_5 , a straight or branched $\text{C}_3\text{-C}_{12}$, preferably $\text{C}_3\text{-C}_6$, alkyl group, or a substituted $\text{C}_2\text{-C}_{12}$, preferably $\text{C}_2\text{-C}_6$, alkyl group; in particular, $(\text{CH}_2)_n\text{COOR}^3$, where $n = 1\text{-}12$, preferably $1\text{-}5$, 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 $(\text{CH}_2)_n$ may be straight or branched; and

$\text{R}^1\text{---}\text{R}^2$ 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 $\text{R}^a = \text{C}_1\text{-C}_6$ alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally $\text{R}^1\text{---R}^2$ 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 $(\text{CH}_2)_m\text{OH}$, where $m = 1\text{-}12$, 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 where one of R^1 or R^2 groups is $(\text{CH}_2)_m\text{OCOOR}^4$ or $(\text{CH}_2)_m\text{NHCOOR}^4$, where $m = 1\text{-}12$ and $\text{R}^4 =$ methyl, ethyl, 2-chloroethyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, a phenyl group or substituted phenyl group, e.g., 2- and 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluoro-phenyl, 2-pyridyl, or 4-pyridyl;

$(\text{CH}_2)_m\text{NR}^a\text{R}^b$, where $m = 1\text{-}12$, 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 $\text{C}_1\text{-C}_4$ alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(\text{CH}_2)_m\text{N}_3$ with $m = 2\text{-}6$ and a straight or branched alkyl chain;

one of R^1 or R^2 groups may be $(\text{CH}_2)_n\text{COOR}^5$, with $n = 1\text{-}5$ and a straight or branched alkyl chain $(\text{CH}_2)_n$ and with R_3 selected from H, straight or branched $\text{C}_1\text{-C}_6$ alkyl, CH_2CN , 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, 1-oxybenzotriazolyl;

further, one of R^1 or R^2 may be $(\text{CH}_2)_n\text{CONHR}^6$, with $n = 1\text{-}12$, preferably 1-5, and $\text{R}^6 = \text{H}$, $\text{C}_1\text{-C}_6$ alkyl, $(\text{CH}_2)_m\text{N}_3$, $(\text{CH}_2)_m\text{-N-maleimido}$, $(\text{CH}_2)_m\text{-NHCOCH}_2\text{X}$ ($\text{X} = \text{Br}$ or I), where $m = 2\text{-}6$ and with straight or branched alkyl chains in $(\text{CH}_2)_n$ and R^6 ; or one of R^1 or R^2 may represent $\text{CH}_2\text{-C}_6\text{H}_4\text{-NH}_2$, $\text{COC}_6\text{H}_4\text{-NH}_2$, $\text{CONHC}_6\text{H}_4\text{-NH}_2$ or $\text{CSNHC}_6\text{H}_4\text{-NH}_2$ with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, $\text{COC}_5\text{H}_3\text{N-NH}_2$ or $\text{CH}_2\text{-C}_5\text{H}_3\text{N-NH}_2$, with $\text{C}_5\text{H}_3\text{N}$ being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl;

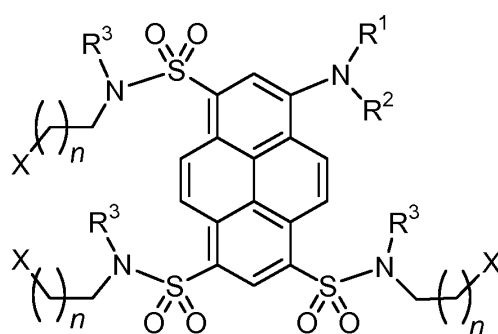
the $(\text{CH}_2)_n\text{-CH}_2$ linker, with $n = 1\text{-}5$, between the SO_2 fragment and the residue X in Formula C may represent a straight-chain, branched or cyclic group having 2-6 carbon atoms;

$X = \text{SH}, \text{COOH}, \text{OSO}_3\text{H}, \text{SO}_3\text{H}, \text{OP}(\text{O})(\text{OH})_2, \text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ optionally substituted $\text{C}_1\text{-C}_4$ alkyl, $\text{P}(\text{O})(\text{OH})_2, \text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ optionally substituted $\text{C}_1\text{-C}_4$ 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 C under basic conditions, i.e. $7 < \text{pH} < 14$, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: $\text{SH}, \text{COOH}, \text{OSO}_3\text{H}, \text{SO}_3\text{H}, \text{OP}(\text{O})(\text{OH})_2, \text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl, $\text{P}(\text{O})(\text{OH})_2, \text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl.

According to a more specific embodiment, of the invention, the fluorescent dye of the invention is represented by Formula C wherein X at each occurrence is SO_3H and n is 1-12, preferably 1-6, or a salt thereof.

According to another specific embodiment of the invention, the compounds of the structural Formula B above are sulfamide derivatives as depicted in Formula D



Formula D

wherein

R^1 and/or R^2 are independent from each other and may represent H, deuterium, alkyl or deuterio-substituted alkyl, wherein one,

several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH₃, C₂H₅, or a straight or branched, optionally substituted, C₃-C₁₂, preferably C₃-C₆, alkyl group; in particular, (CH₂)_nCOOR⁴, where n = 1-12, preferably 1-5, R⁴ may be H, CH₂CN, 2- and 4-nitrophenyl, 2,3,5,6-tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, N-succinimidyl, N-sulfosuccinimidyl, and the alkyl chain in (CH₂)_n may be straight or branched; and

R¹---R² may form a four-, five-, six-, or seven-membered non-aromatic carbocycle with an additional primary amino group NH₂, secondary amino group NHR^a, where R^a = optionally substituted C₁-C₆ alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; or optionally R¹---R² may form a four-, five-, six-, or seven-membered non-aromatic heterocycle with a heteroatom such as O, N or S included into this heterocycle;

R¹ and/or R² may further represent:

a hydroxyalkyl group (CH₂)_mOH, where m = 1-12, preferably 2-6, with a straight or branched, optionally substituted alkyl chain; one of R¹ or R² groups may be a carbonate or carbamate derivative (CH₂)_mOCOOR⁵ or (CH₂)_mNHCOOR⁵, where m = 1-12 and R⁵ = methyl, ethyl, 2-chloroethyl, CH₂CN, N-succinimidyl, sulfo-N-succinimidyl, 1-oxybenzotriazolyl, a phenyl group or substituted phenyl group, such as 2- and 4-nitrophenyl, pentachlorophenyl, pentafluoro-phenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, 4-pyridyl;

(CH₂)_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 represent hydrogen and/or optionally substituted C₁-C₄ alkyl groups;

(CH₂)_mN₃, m = 1-12, preferably 2-6, with a straight or branched alkyl chain;

-(CH₂)_nCOOR⁴, with n = 1-12, preferably 1-5 and a straight or branched alkyl chain (CH₂)_n and with R⁴ selected from H, straight or branched C₁-C₆ alkyl, CH₂CN, 2- and 4-nitrophenyl, 2,3,5,6-

tetrafluorophenyl, pentachlorophenyl, pentafluorophenyl, *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl;

$(\text{CH}_2)_n\text{CONHR}^5$, where $n = 1-12$, preferably 1-5 and $\text{R}^5 = \text{H}$, substituted or unsubstituted C_1-C_6 alkyl, $(\text{CH}_2)_m\text{N}_3$, $(\text{CH}_2)_m\text{-N-maleimido}$, $(\text{CH}_2)_m\text{-NHCOCH}_2\text{Y}$ ($\text{Y} = \text{Br}, \text{I}$) where $m = 1-12$, preferably 2-6, with straight or branched alkyl chains in $(\text{CH}_2)_n$ and R^5 ;

further, R^1 or R^2 may represent $\text{CH}_2-\text{C}_6\text{H}_4-\text{NH}_2$, $\text{COC}_6\text{H}_4-\text{NH}_2$, $\text{CONHC}_6\text{H}_4-\text{NH}_2$ or $\text{CSNHC}_6\text{H}_4-\text{NH}_2$ with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, $\text{COC}_5\text{H}_3\text{N}-\text{NH}_2$ or $\text{CH}_2-\text{C}_5\text{H}_3\text{N}-\text{NH}_2$, with $\text{C}_5\text{H}_3\text{N}$ being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl;

$\text{R}^3 = \text{H}$, $(\text{CH}_2)_q\text{CH}_2\text{X}$, C_2H_5 , a straight or branched C_3-C_6 alkyl group, $\text{C}_m\text{H}_{2m}\text{OR}$, where $m = 2-6$, with a straight or branched alkane-diyl chain C_mH_{2m} , and $\text{R}^3 = \text{H}$, CH_3 , C_2H_5 , C_3H_7 , $\text{CH}_3(\text{CH}_2\text{CH}_2\text{O})_k\text{CH}_2\text{CH}_2$; with $k = 1-12$; while the $(\text{CH}_2)_q\text{CH}_2$ linker may represent a straight-chain, branched or cyclic group having 2-6 carbon atoms;

in Formula D, the $(\text{CH}_2)_n-\text{CH}_2$ linker, with $n = 1-12$, preferably 1-5, between the sulfonamide fragment SO_2N and the residue X may represent a straight-chain, branched or cyclic group having 2-6 carbon atoms;

$\text{X} = \text{SH}$, COOH , SO_3H , OSO_3H , $\text{OP}(\text{O})(\text{OH})_2$, $\text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ substituted or unsubstituted C_1-C_4 alkyl, $\text{P}(\text{O})(\text{OH})_2$, $\text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ substituted or unsubstituted C_1-C_4 alkyl;

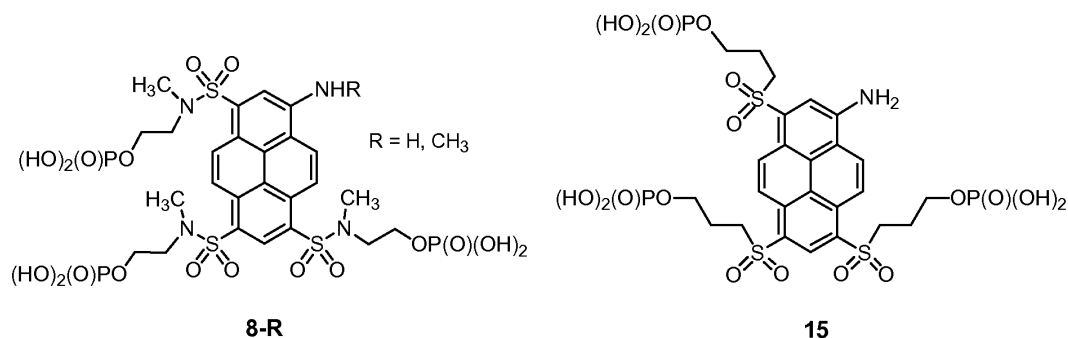
with the proviso that in all compounds represented by Formula D three, six, nine or twelve negatively charged groups are present in the residues X of Formula C under basic conditions, i.e. $7 < \text{pH} < 14$, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: SH , COOH , SO_3H , OSO_3H , $\text{OP}(\text{O})(\text{OH})_2$, $\text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1-\text{C}_4$ alkyl or substituted C_1-C_4 alkyl, $\text{P}(\text{O})(\text{OH})_2$, $\text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1-\text{C}_4$ alkyl or substituted C_1-C_4 alkyl.

According to preferred embodiments of the invention, the substituents R^1 and R^2 in the above Formulae B, C and D are defined as follows:

R¹ and/or R² in Formula B represent H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-alkyl with 1-12 C atoms, preferably 1-6 C atoms, such as methyl, ethyl, propyl etc., 4,6-dihalo-1,3,5-triazinyl (C₃N₃X₂) where halogen X is preferably chlorine, 2-, 3- or 4-aminobenzoyl (COC₆H₄NH₂), 2-, 3- or 4-aminophenylureyl (NCONHC₆H₄NH₂), 2-, 3- or 4-aminophenylthioureyll (NCSNHC₆H₄NH₂) or linked carbonic acid residues and their reactive esters of the general formulae (CH₂)_{m1}COOR³, (CH₂)_{m1}OCOOR³ (CH₂)_{n1}COOR³ or (CO)_{m1}(CH₂)_{m2}(CO)_{n1}(NH)_{n2}(CO)_{n3}(CH₂)_{n4}COOR³ 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 (CH₂)_{m/n} being straight, branched, saturated, unsaturated, partially or completely deuterated, and/or or included into a carbo- or heterocycle containing N, O or S, whereas R³ 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.

Compounds of Formulae C and D can exist and be applied in the form of salts that involve all possible types of cations, in particular as mentioned above for Formula B, preferably Na⁺, Li⁺, K⁺, NH₄⁺ and organic ammonium or organic phosphonium cations.

Especially preferred compounds of the general structural Formulae B, C and D above have one of the following formulae:



One preferred embodiment of the present invention relates to compounds Formulae A-B or A-D above, where the negative charges are provided by several primary phosphate groups, in particular, doubly *O*-phosphorylated 7-aminoacridon-2-sulfonamides (two phosphate groups), triple *O*-phosphorylated 1,6,8-tris[(ω -hydroxyalkyl)sulfonyl]-pyrene-3-amines (three phosphate groups), and 1,6,8-tris[*N*-(ω -hydroxyalkyl)sulfonylamido]pyrene-3-amines. These compounds possess superior brightness and considerably better electrophoretic mobilities, compared to APTS.

The novel compounds of the invention have small molecular size and, in preferred embodiments, a drastically increased high negative net charge (z) is provided (such as, at least, $z = -4$ for phosphorylated acridones and $z = -6$ for phosphorylated pyrene dyes). These two requirements are equivalent to a low hydrodynamic radius and a low mass to charge ratio (m/z), respectively. As a result, high velocities and fast separations at good analytical resolution can be achieved in electrokinetic measurements for these compounds and the corresponding labeled carbohydrates.

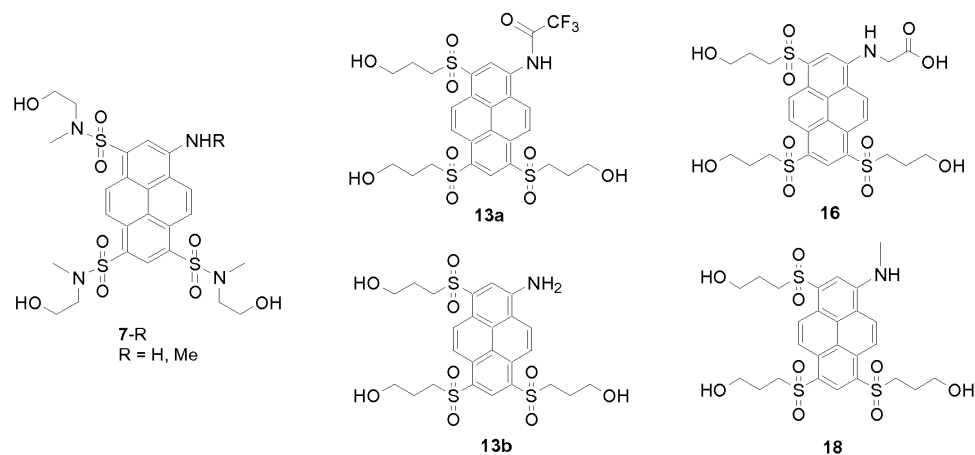
The negative charges are provided by acidic groups which can be deprotonated in basic or even neutral media. Phosphate groups are preferred for this purpose, because primary alkyl phosphates ($R-OPO_3H_2$) have pK_a values for the first and the second acidic protons in the range of 1-2 and 6-7, respectively. As a

consequence, one single phosphate group can introduce two negative charges in buffer solutions under basic conditions (e.g., at pH 8, $R-OPO_3^{2-}$ is present). To achieve the negative charge of -4, the attachment of two phosphate groups is necessary, etc. However other acidic groups, in particular selected from the groups X as defined in Formulae A-B above are also suitable.

Generally, the compounds of Formulae A-B above are suitable and advantageous for the 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-aminoglucose and other 2-deoxy-2-aminoaminopyranosides, glycans, glucans, biotin, and other small molecules, i.e. having molecular masses of less than 1500 Da, e.g., jasplakinolide and its modifications.

Compounds **7-R** (R = H, Me), **13a**, **13b**, **16** and **18** (Scheme 5 below) possess free hydroxyl groups and are suitable as precursors for obtaining phosphorylated pyrene dyes of the general Formula B. In particular, compounds **7-R** (R = H, Me) were phosphorylated directly ($POCl_3$ in trimethyl phosphate) and afforded dyes **8-R** (R = H, Me). Analogously, dyes **13a,b** and **18** were phosphorylated using $POCl_3$ in trimethyl phosphate. Thus, both precursor dyes **13a** and **13b** gave (after the basic work-up of the reaction mixture) compound **15**. Compound **16** has a free carboxyl group which can be used a reactive center for bioconjugation. Thus, compound **16** represents a fluorescent label for amino acids, peptides, proteins, including primary and secondary antibodies, single-domain antibodies, docetaxel, avidin, streptavidin and their modifications, aptamers, modified nucleotides, modified nucleic acids containing an amino group, toxins, lipids, carbohydrates, including 2-deoxy-2-aminoglucose and other 2-deoxy-2-aminoaminopyranosides, modified biotin (e.g., biocytin), and

other small molecules, i.e. having molecular masses of less than 1500 Da (e.g., jasplakinolide and its modifications).



Scheme 5

Consequently, a closely related aspect of the present invention relate to the use of compounds of the structural Formulae A-B as fluorescent reagents for conjugation to a broad range of 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.

The claimed compounds are suitable for and may be used in a method for fluorescent labelling and detecting of target molecules. Typically, such a method implies reacting a compound according to any one of Formulae A-D above with a target molecule selected from the group comprising amino acids, peptides, proteins, including primary and secondary antibodies, single-domain antibodies, docetaxel, avidin, streptavidin and their modifications, aptamers, (modified) nucleotides, (modified)

nucleic acids, toxins, lipids, carbohydrates, including 2-deoxy-2-aminoglucose and other 2-deoxy-2-aminoaminopyranosides, glycans, glucans, (modified) biotin (e.g., biocytin), and other small molecules, i.e. having molecular masses of less than 1500 Da (e.g., jasplakinolide and its modifications). The labeling is followed by separation, detection, quantification and/or isolation of the labeled fluorescent derivatives by means of chromatographic and/or electrokinetic techniques.

Further the present invention encompasses also carbohydrate-dye conjugates comprising a fluorescent dye according to Formulae A-B or A-D above.

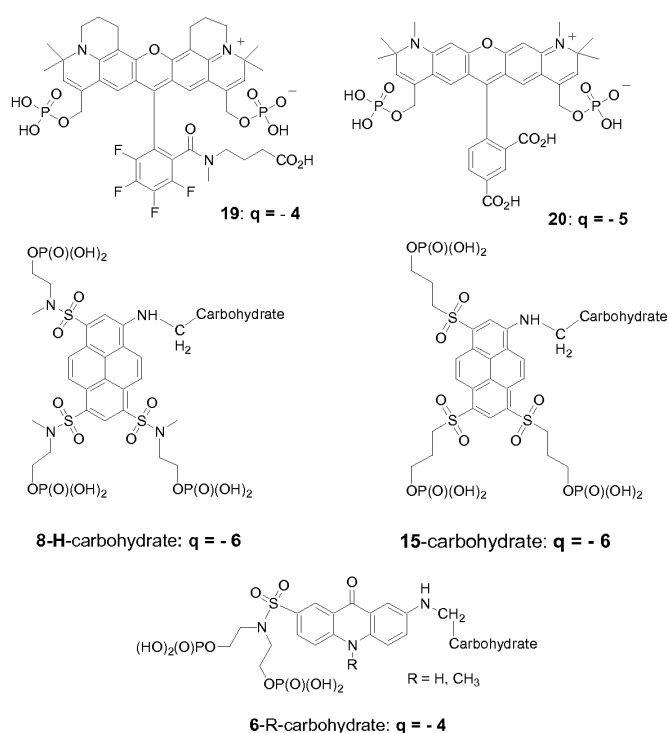
More specifically, the dye in said conjugates, in particular carbohydrate-conjugates, is selected from the compounds of the formulae **6-H**, **6-Me**, **8-H**, **15** or **19** and **20** as shown in Scheme 6 below.

The compounds of Formulae A-B or A-D above are suitable and advantageous for the use in the reductive amination of reducing sugars, i.e. monomeric, oligomeric or polymeric carbohydrates possessing an aldehyde group in a free form or protected form, e.g. as semiacetal, including glycans (as shown in Scheme 2 and 6).

Consequently, closely related aspects of the present invention relate to this use and to a method for the reductive amination of reducing sugars comprising reacting a compound of Formulae A-D above with a monomeric, oligomeric or polymeric carbohydrate possessing an aldehyde group in a free form or as semiacetal, including a glycan, for a sufficient time to effect the reductive amination and chromatographic or electrokinetic separation of the labeled fluorescent derivatives optionally followed by detection of analytes by means of optical spectroscopy, including fluorescence detection and/or mass spectrometric

detection. Examples of dye-conjugate structures are given in Scheme 6.

The compounds of Formulae A-D and the carbohydrate-dye conjugates comprising the same make up a dye set that features high negative net charges, accompanied by favorable absorption/emission properties (see Table 2) and electrophoretic mobilities.

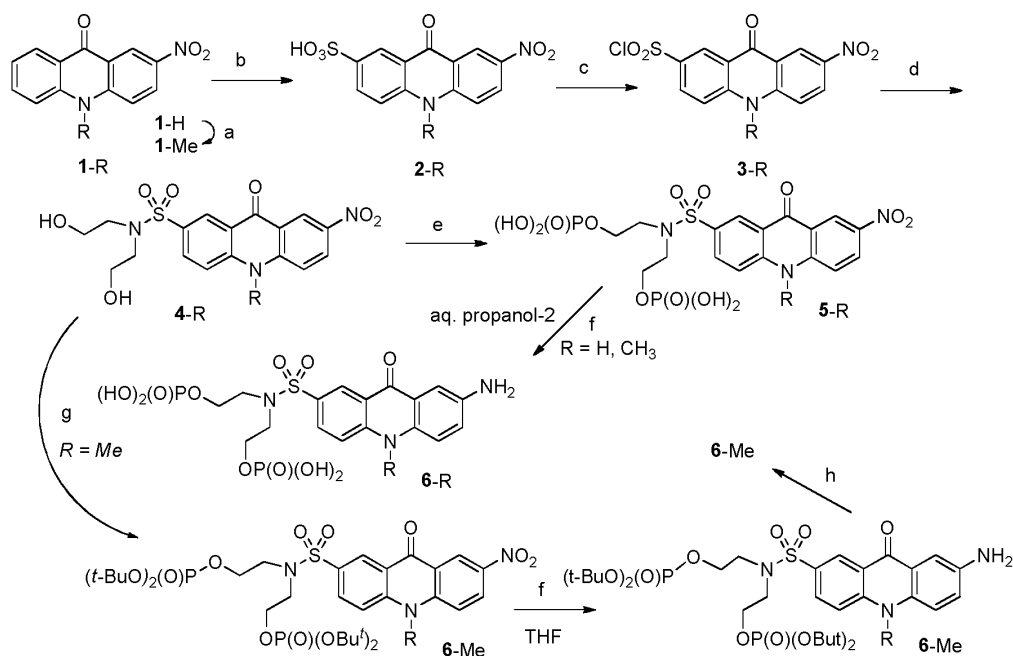


Scheme 6

General synthesis of the novel fluorescent dyes of the invention

Synthetic routes to the new sulfonated 2(7)-aminoacridone and 1-aminopyrene dyes are given in schemes 7-10 below.

Scheme 7 depicts the preparation of the double *O*-phosphorylated 7-aminoacridone-2-[*N,N'*-bis-(2-hydroxyethyl)]-sulfonamides **6-H** and **6-Me**. The known reaction between 2-chloro-5-nitrobenzoic acid and aniline in the presence of K_2CO_3 and catalytic amounts of $Cu(NO_2)_2$ (185 °C, 2-4 h) afforded 4-nitro-*N*-phenylanthranilic acid (not shown in Scheme 5) according to the procedures described in WO 2007/049057 and US2012/0015373. 4-Nitro-*N*-phenylanthranilic acid was cyclized to 2-nitroacridin-9(10*H*)-one (**1-H**) ($POCl_3$, reflux, 3 h). Experimental details of the further syntheses are given in a special section with examples.

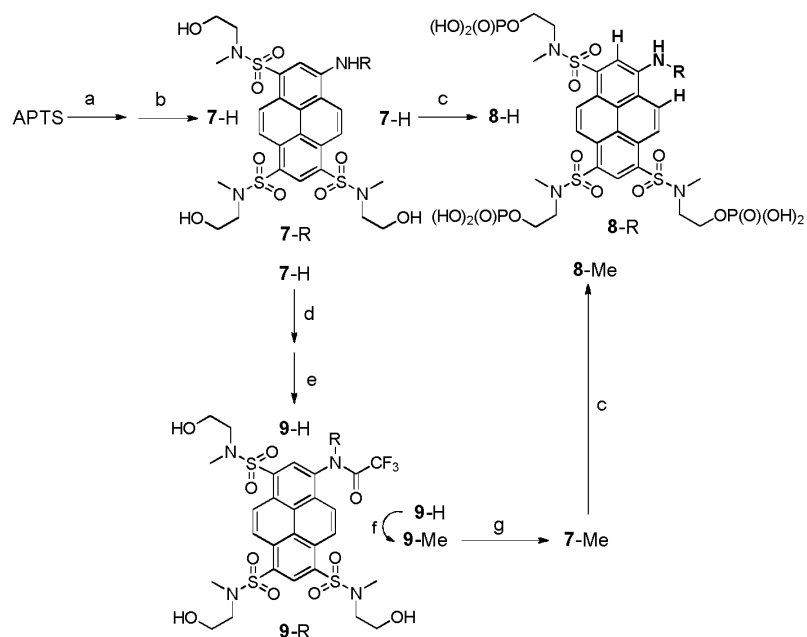


Scheme 7. Preparation of the doubly *O*-phosphorylated 7-aminoacridon-2-[*N,N'*-bis-(2-hydroxyethyl)]sulfonamides.

Reagents and conditions: a) NaH, DMSO, 50 – 60 °C, then CH_3I , r. t., overnight; b) 20% SO_3 in H_2SO_4 , 100 °C, 1.5 h; c) $ClSO_3H$, 50°C, 2 h, then r. t., overnight; d) diethanolamine, aq THF or aq. MeCN, r. t., overnight; e) $POCl_3$, $(MeO)_3PO$, r. t., then aq. $Et_3N \cdot H_2CO_3$ buffer (pH 8); f) H_2 , Pd/C (10% Pd, oxidized form), aq. propanol-2, overnight, r. t. and ambient pressure; g) $(tBuO)_2PN(i-C_3H_7)_2$, 1*H*-tetrazol, DMF, 40 °C, 1.5 h; then 5-6 M

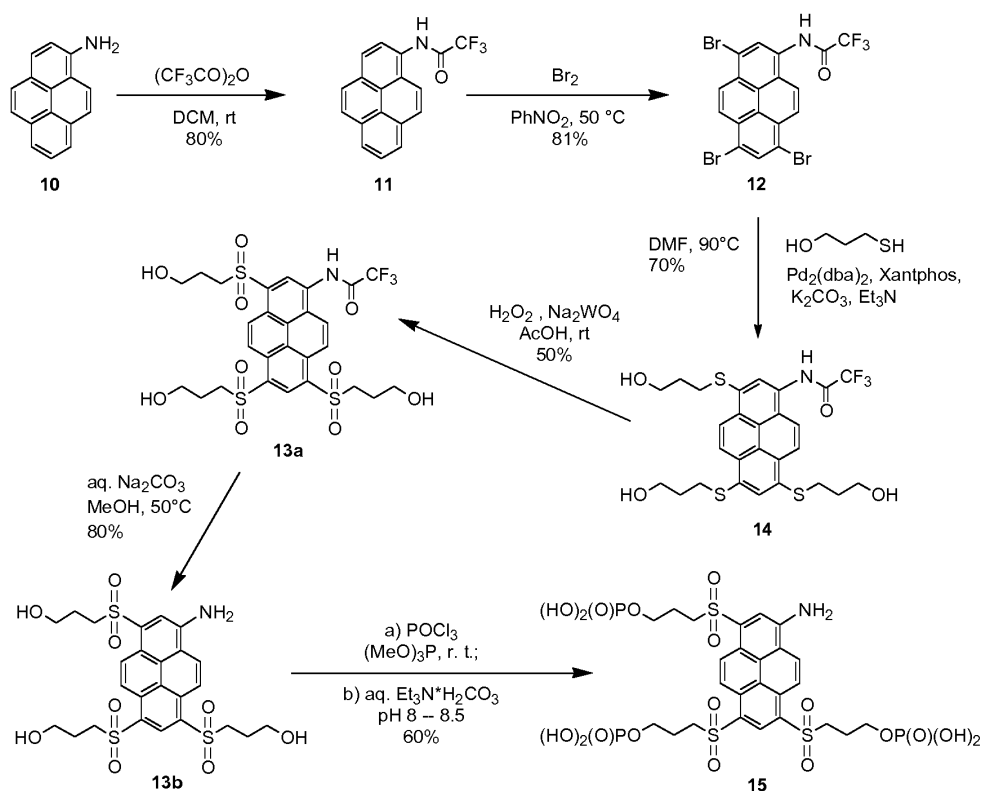
*t*BuOOH in decane, 0 °C - r. t.; h) TFA, aq. MeCN, room temp; then aq. Et₃N*H₂CO₃ buffer (pH 8).

The synthetic route to the triple *O*-phosphorylated 1,6,8-tris[[*N*-(2-hydroxyethyl)-*N*-methyl]sulfonylamido]pyrene-3-amines **8-R** is shown in Scheme 8. Detailed experimental procedures are given in the section with examples.



Scheme 8. Preparation of the triple *O*-phosphorylated 1,6,8-tris[[*N*-(2-hydroxyethyl)-*N*-methyl]sulfonylamido]pyrene-3-amines. Reagents and conditions: a) ClSO₃H, 65 °C, 3 h overnight; b) CH₃NHCH₂CH₂OH, aq. MeCN, r. t., overnight; c) POCl₃, (MeO)₃PO, r. t., then aq. Et₃N*H₂CO₃ buffer (pH 8 - 8.5); d) (CF₃CO)₂O, CH₂Cl₂, Et₃N, r. t.; e) MeOH, NaHCO₃; f) CH₃I, Cs₂CO₃, DMF, 70 °C, 40 min; g) Na₂CO₃, aq. MeOH; r. t.

The synthetic route to the triple *O*-phosphorylated 1,6,8-tris[(3-hydroxypropyl)sulfonyl]-pyrene-3-amine **15** is shown in Scheme 9. Detailed experimental procedures are given in the Example Section.



Scheme 9. Preparation of the triple O-phosphorylated 1,6,8-tris[(3-hydroxypropyl)-sulfonyl]-pyrene-3-amine **15**.

A priori, pyrene derivatives **7-R/8-R** and **15** promised better photophysical properties than 7-aminoacridon-2-[*N,N*-bis-(2-hydroxyethyl)]sulfonamides **6-H** and **6-Me**. In particular, higher molar extinction coefficients and higher fluorescence quantum yields (higher "brightness") could be expected. For example, in order to prepare pyrene dye **15**, 1-aminopyrene (**10**) – a cheap commercially available starting material – was used as a building block (Scheme 9). APTS was also synthesized by the inventors starting from 1-aminopyrene (**10**) and using the reported method (Sharrett et al., *Org. Biomol. Chem.* **2009**, 7, 1461–1470). 1-Aminopyrene (**10**) has an amino group which acts as an electron-donor residue and a reactive site. Shifting the

absorption spectrum to the red is necessary, as compound **10** has an absorption maximum at 336 nm and the emission maximum at 445 nm (in MeOH). The bathochromic and bathofluoric shifts can be achieved by introduction of electron-acceptor groups into the pyrene fluorophore followed by the mono or double alkylation of the amino group. The later modifications take place in the course of reductive amination of glycans and convert the primary amino group to a more electron-donating secondary or tertiary amine. The electronic effects of the substituents can be illustrated, and their relative strengths can be compared by the magnitudes and signs of the *Hammett* constants (see Table 1).

If one assumes that the amino group is attached to C-1, then the substituents can be introduced to C-3, C-6 and C-8 (in the course of electrophilic substitution reaction). The introduction of acceptor groups into the "active" positions 6 and 8 of pyrene system (corresponding to the *para*-position in benzene ring), as well to C-3 (corresponds to the *meta*-position in benzene ring) leads to the formation of a donor-acceptor dye with red-shifted bands. This red-shift is due to a "push-pull" electronic effect between the electron-donating amino group and the electron-withdrawing groups in positions 3, 6 and 8. Valuable precursor groups are, for example, sulfonic acid residues in APTS (convertible to sulfonamides; see Scheme 8) and thioethers in compound **14**, which can be oxidized to sulfones and become much more powerful electron-withdrawing residues (Scheme 9). Alkyl sulfones (represented by compounds **13b**, **15**, **16** and **18**) have highest values of the *Hammett* σ -constants in Table 1 and this indicates that they are more powerful acceptors than sulfonamides (compounds **7-H**, **7-Me**, **8-H**, **8-Me**). Another reason for choosing sulfonamides and alkyl sulfones in the dye design is that they can be combined with hydroxyl residues required for attaching primary phosphate or other ionizable groups.

Table 1. *Hammett constants for different electron-donor and electron-acceptor groups in meta and para positions. Positive values correspond to electron-acceptors; negative values to electron-donor groups. Hydrogen atom has, by definition, the zero values of "sigma" constants. The data are taken from Hansch, A. Leo, R.W. Taft, A survey of Hammett substituent constants and resonance and field parameters. Chem. Rev. 1991, 91, 165-195.*

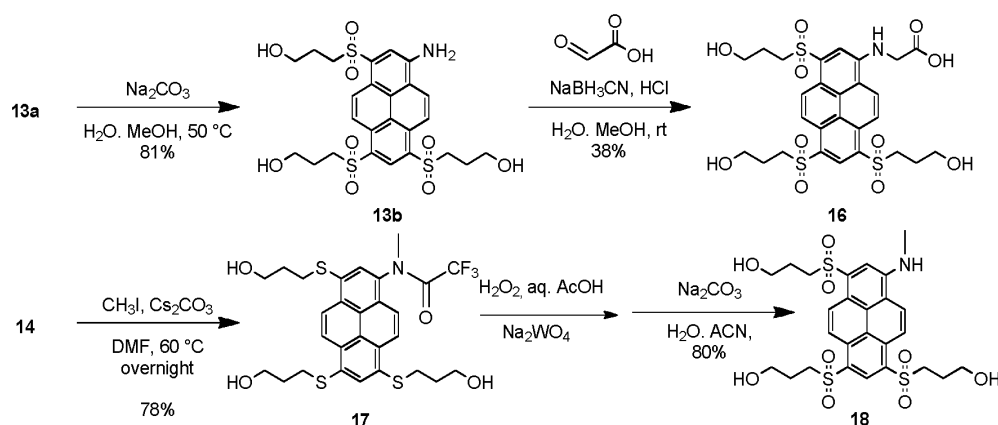
Name	Substituent	σ_m	σ_p
Amino	-NH ₂	- 0.16	- 0.66
Alkyl amino	-NHMe	- 0.21	- 0.70
Dialkyl amino	-NMe ₂	- 0.16	- 0.83
Alkylthio	-SMe	0.15	0.00
Alkyl sulfon	-SO ₂ Me	0.60	0.72
Alkyl sulfoxide	-SOMe	0.52	0.49
Sulfonamide	-SO ₂ NH ₂	0.53	0.60

Before starting to introduce any groups into the 1-amino pyrene scaffold (the first reaction would be always an aromatic electrophilic substitution), in some cases it was necessary to protect the amino group of 1-aminopyrene **10** (Scheme 9). The inventors used acetylation with trifluoroacetic anhydride (quite a new option) and obtained the acetylated amine **11**. In this case, the trifluoroacetyl group is a better choice than acetyl group

(which was used, for example, by *Li et al.*, *J. Am. Chem. Soc.* **1994**, *116*, 9890–9893) because it is acid-stable, but can be easily cleaved under mild basic conditions. After the protection of the amino group by trifluoroacetylation, the electrophilic substitution in positions 3, 6 and 8 of compound **11** led to the tribromo aminopyrene (**12**). This tribromide represented a very important intermediate, as the aromatic bromine residues can be readily converted to a number of other functionalities. For example, a convenient synthetic way for the introduction of the thioether groups was published by *Mispelaere-Canvivet et al.*, *Tetrahedron* **2005**, *61*, 5253–5259, who reported a Pd-catalyzed cross-coupling of thiols with aryl bromides. It was found that unprotected 3-mercapto-1-propanol can be directly used to obtain triol **14**. The straightforward oxidation of **14** to the trisulfonyl derivative **13a** was performed using concentrated hydrogen peroxide in acetic acid and sodium tungstate as a catalyst (compare: *Xu et al.*, *Molecules* **2010**, *15*, 766–779). Compound **13a** was deprotected in methanol by treatment with aq. Na₂CO₃ to give the free amine **13b** which was, in turn, phosphorylated to compound **15**.

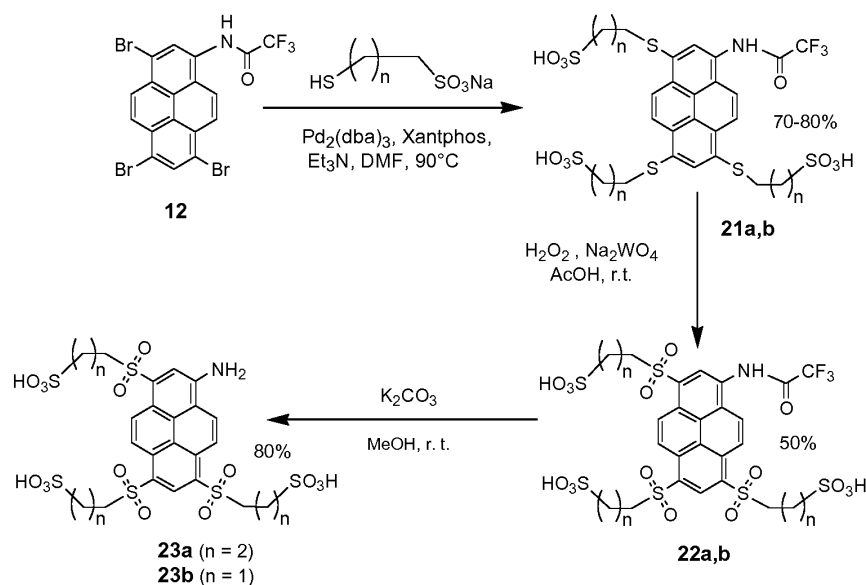
Direct phosphorylation of aromatic amines with unprotected amino groups (**7-R** to **8-R** in Scheme 8 and **13b** to **15** in Scheme 9) using POCl₃ in trimethyl phosphate followed by treatment with aqueous Et₃N*H₂CO₃ buffer proved to be possible. Aromatic amino groups also underwent phosphorylation, but due to very poor electron density, these amino groups readily lost the phosphate residues in aqueous solutions (under slightly acidic or basic conditions).

On the contrary, primary phosphate groups in dyes **8-R** (R=H, CH₃) and **15** are hydrolytically stable at the pH = 3 (reductive amination) and the pH = 8 (electrophoresis).



Scheme 10. Aminopyrene-based fluorescent dyes (**16** and **18**) with a derivatized nitrogen atom.

The amino group in tri-sulfone **13a** was deprotected by gentle heating in aqueous-methanolic solution of sodium carbonate, and aminosulfone **13b** was isolated in a good yield. Reductive amination of aminosulfone **13b** with glyoxylic acid monohydrate in the course of *Borch reaction* (Borch et al., *J. Am. Chem. Soc.* **1969**, *91*, 3996–3997) led to the pyrene dye 500P (**16**) with the yield of 38%. The reaction was carried out by addition of sodium cyanoborohydride in portions, glyoxylic acid monohydrate and concentrated aqueous HCl to compound **13b** over several days. The moderate yield could be explained by the low reactivity of the amino group deactivated by three electron-withdrawing alkylsulfonyl groups. Compound **16** represents a fluorescent dye with a versatile reactive center – carboxyl group. The amino group in **16** is carboxymethylated, and it was interesting to see what bathochromic and bathofluoric shifts this kind of derivatization (with a less efficient donor than an alkyl group) will induce. *N*-Methylated pyrene dye **18** was also prepared. Methyl group is a more powerful electron donor than carboxymethyl group, and in this respect, compound **18** probably represents a better model for the product of the reductive amination of carbohydrates than compound **16**.



Scheme 11. Preparation of the alkyldisulfonate-substituted fluorescent dyes **23a,b**.

The synthesis of alkyldisulfonate-substituted (particularly, sulfoalkyldisulfonate-substituted) 1-aminopyrene dyes **23a** and its homolog **23b** requires no protection of the sulfonic acid group (SO_3H) in the corresponding bifunctional synthone, as depicted in Scheme 11. This greatly simplifies the whole process, compared to that for preparation of its "phosphorylated counterpart" - dye **15** (see Scheme 9), especially considering that some useful sulfoalkyl-substituted synthones, e. g., sodium 3-mercapto-1-propanesulfonate or sodium 2-mercaptoethanesulfonate, are commercially available. The reaction leads to a highly water-soluble dye, which has three ionizable groups connected to the dye core via thioether functions. Oxidation of the thioether to the sulfone brings a big bathochromic and bathofluoric shifts relative to the thioether-containing intermediate **21a,b** and even to APTS, a well-established benchmark dye (see Schemes 11 and 12 for structures and Table 2 for spectral properties). The cleavage of the protective group at the amino site of the dye leads to the carbohydrate-reactive compounds that represent a case of

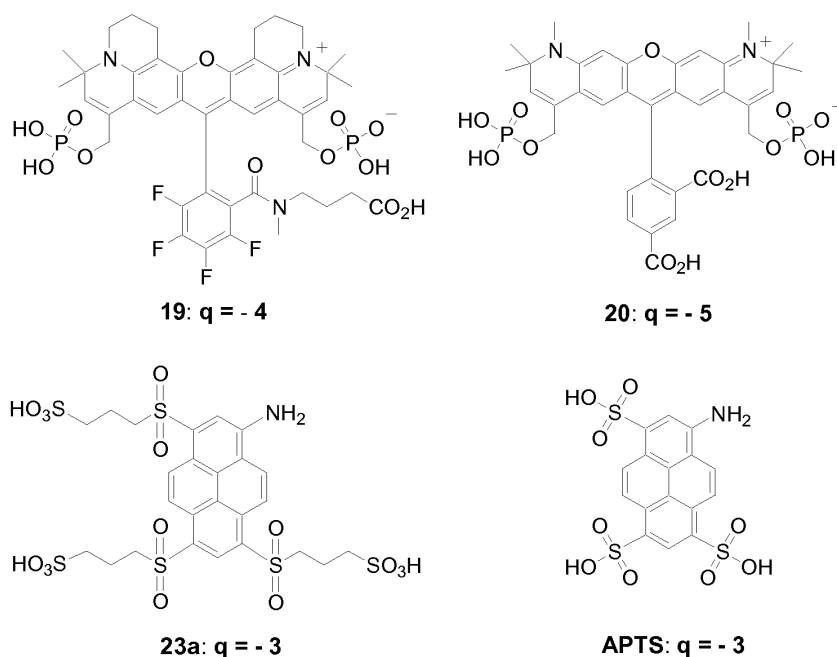
general Formula C. The whole synthesis sequence is depicted in Scheme 11 above.

Spectral properties of the new dyes

Table 2. Spectral properties of the phosphorylated aminoacridones **6-H** and **6-Me**, sulfonylamidopyrenes **8-R** (R=H, Me), alkylsulfonyl-modified pyrene dyes **15**, **16**, **18**, **23a**, some of their precursors and related compounds (see Schemes 9, 10, 11 and 12 for structures). For selected actual UV/Vis absorbance and fluorescence spectra see also Fig. 2A and Fig. 2B.

Dye	Absorption, $\lambda_{\max}$, nm (ϵ , M ⁻¹ cm ⁻¹)	Emission $\lambda_{\max}$, nm (Φ_{fl}^a)	Solvent	Lifetime of excited state (τ , ns)
6-H	217 (13500), 260 (26000) 295 (28000), 420 (3700)	485 (excit. 405 nm), 586 (all excit. λ ; ~ 0.05)	H ₂ O	22.3 (485 nm em.) 3.7 (585 nm em.)
6-Me	219 (10300), 263 (18600) 299 (18500), 430 (2900)	485 and 585 (all excit. 300–470 nm, ~ 0.06)	TEAB ^b	8.8 (470 nm em.) ^c 2.9 (590 nm em.) ^c
7-H	477 (22400)	535 (0.96) ^a	MeOH	5.6
7-Me	493 (23000)	549 (0.97)	MeOH	5.9
8-H	465 --	544 (0.88)	H ₂ O	5.9
8-Me	502 --	563 (0.85)	H ₂ O	3.6
13b	486 (21000)	534 (0.80) ^{c,d}	MeOH	4.9
15	477 (19600)	542 (0.92)	TEAB ^{b,h}	5.8
16	499 (18000)	553 (0.71) ^d	MeOH	4.9
18	502 (23400)	550 (0.88)	MeOH	6.3 ^f
	509 (19500)	563 (0.67)	H ₂ O	6.4 ^f
APTS^e	425 (22000)	457 (0.95)	PBS	
19	635 (75000)	655 (0.62)	PBS	
20	581 (120000)	607 (0.74)	PBS	
23a	486 ^g (21000)	542 (0.86) ^h	TEAB ⁱ	

a - absolute values of the fluorescence quantum yields (if not stated otherwise); b - TEAB is aqueous $\text{Et}_3\text{N}^+\text{H}_2\text{CO}_3^-$ buffer with pH = 8 - 8.5; c - excitation at 375 nm; d - relative value, with Rhodamine 6G as a reference dye with $\Phi_{\text{F1}} = 0.9$; e - for mono *N*-alkylated APTS derivatives abs. and emiss. maxima are 457 and 516 nm, respectively ($\epsilon \sim 19000 \text{ M}^{-1} \text{ cm}^{-1}$); f - excitation at 515 nm; g - compound **23a** is spectrally identical to its homolog **23b**; h - relative value with fluorescein as a reference dye with $\Phi_{\text{F1}} = 0.9$ in 0.1 M NaOH under excitation at 496 nm; i - switching from TEAB to PBS buffer with pH 7.4 virtually does not affect the spectral properties, i.e., Φ_{F1} .



Scheme 12. Phosphorylated fluorescent dyes **19**, **20**, sulfonated dyes **23a** and APTS as reference compounds possessing large net charges and different spectral properties.

The structural features and spectral data in Table 2 demonstrate that the doubly phosphorylated aminoacridones **6-H** and **6-Me**, triple phosphorylated pyrene dyes **8-H**, **8-Me**, and **15** meet the criteria to the fluorescent tags defined above. Additionally, it was necessary to prove if they could be used in reductive amination of glycans, and if the emission of their conjugates would not interfere with the emission of glycans labeled with APTS (for structure and spectral data, see Schemes 9-12 and Table

2). For example, compounds **6-R** (R = H, Me) have m/z ratios equal to 134 and 138, respectively (APTS has m/z = 151). They have several absorption maxima and emit orange light (with two emission maxima at 485 nm and 585 nm and relative intensities of ca. 1:2; see Fig. 2A). Though their absorption at 488 nm is relatively low, the red-shifted emission is a remarkable feature and is accompanied with a Stokes shift of ca. 160 nm. The absolute values of the fluorescence quantum yields for compounds **6-R** are 5-6%. Therefore, in spite of their relatively low brightness, yellow-orange emitting acridone dyes **6-R** are potentially at least as useful as the yellow-green emitting aminopyrene dyes **8-R** and **15**, which are brighter. The dyes of both types represent new tags which can either be used for labelling of glycans, including "heavy" and "exotic" glycans which could not yet been detected due to limitations posed by APTS with its relatively low net charge (-3) and low mobility of the "heavy" carbohydrates decorated with an APTS label. Indeed, due to the presence of four negative charges and extremely low m/z ratio, phosphorylated dyes introduced here are able to provide better electrophoretic mobility of conjugates, reduce their migration times and thus reveal and highlight bulky and massive carbohydrates.

All pyrene dyes listed in Table 2 are highly fluorescent. The non-phosphorylated pyrenes **7-R** (R = H, Me), **13b**, **16** and **18** allow to estimate the extinction coefficients with higher accuracy. The extinction coefficients of the most long-wavelength bands are in the range of 18000 - 23000, while the positions of the maxima vary from 465 to 507 nm. Therefore, the fluorescence can be readily induced by the argon ion laser emitting at 488 nm. Emission maxima are found in the range from 535 to 563 nm, and the fluorescence quantum yields are always high (71 - 97%). Therefore, sulfonated 1-aminopyrenes (general Formulae B-D) represent much brighter dyes than 2-sulfonamido-7-aminoacridones (Formula A). The brightness is proportional to the product of

the extinction coefficient (at 488 nm) and fluorescence quantum yield. We can assume that for acridone dyes this value is ca. $1500 \times 0.06 = 90$, and for pyrenes - $20000 \times 0.9 = 18000$. This rough estimation means that trisulfonated 1-aminopyrenes are ca. 200 times brighter dyes than 2-sulfonamido-7-aminoacridones. This property makes 1-aminopyrene dyes of the present invention (Formulae B-D) to be superior tags than 2-sulfonamido-7-aminoacridones (Formula A) and APTS. If one assumes that for APTS conjugates the extinction coefficient at the maximum (457 nm) is 19000 (Scheme 3), and the absorption at 488 nm is typically ca. 35% of the maximal absorption at 457 nm, then one obtains the relative brightness of 6000 (assuming the same fluorescence quantum yield). Therefore, the dyes of the present invention are ca. 3 times brighter than APTS under excitation with the 488 nm laser (in conjugates with glycans). Pyrene dyes of the present invention, in particular, compounds **8-H**, **15** and **23a,b** represent new tags which can be used for labelling of glycans, including "heavy" and "exotic" glycans which could not yet been detected due to limitations posed by APTS its relatively low net charge (-3) and low brightness. Also very importantly, none of those aminopyrene dyes (i. e., **8-H**, **13b**, **15**, **23a,b**) showed significant changes in the fluorescence quantum yields while switching from neutral (PBS buffer, pH = 7.4) to basic (TEAB buffer, pH = 8.0 - 8.5) medium.

In order to shift the emission band to the red spectral region the *N*-methylated derivative **8-Me** was prepared. This dye possesses a *N*-methylamino group and therefore, it represents a fluorophore which is very similar to the product of the reductive amination formed from glycans and the parent dye **8-H** (compare with compound **6** in Scheme 2). The absorption maximum has been shifted to the red (+37 nm; **8-H** $\rightarrow$ **8-Me**), but the emission maximum underwent the bathofluoric shift of "only" 19 nm (see Table 2). Thus, the Stokes shift reduced from 79 nm to 61 nm.

There is another tool for increasing bathochromic and bathofluoric shifts in the series of aromatic fluorescent dyes, provided that they possess electron-donor and electron-acceptor groups having the so-called "push-pull" electronic interactions between them (direct polar conjugation). In the case of 1-aminopyrene dyes, the donor group is fixed (and its electron donating properties cannot be enhanced), but the electron-withdrawing groups in positions 3, 6 and 8 may be varied. For example, thioethers in compound **14** (Scheme 9) can be oxidized to the corresponding sulfones and acquire more powerful electron-withdrawing residues than the sulfonamide in compounds **7-R**, **8-R** and **9-R** (Scheme 8). Indeed, alkyl sulfone groups ($R-SO_2$, that is present in compounds **13b**, **15**, **16**, **18** and **23a,b**; see Schemes 8, 9 and 11) have the highest values of the *Hammett* σ -constants, as seen in Table 1, and this indicates that they are even more powerful acceptors than sulfonamide moieties (that is present in compounds **7-H**, **7-Me**, **8-H**, **8-Me**; see Schemes 8 and 9). However, after preparing compounds **8-H** and **15** and comparing their spectral properties in aqueous solutions (Table 2), it was determined that, as expected, the bathochromic shift was 12 nm, but the position of the emission maximum and the band form are the same (as seen in Fig 2B). The simplest explanation for that is based on the assumption that the single amino group (as a donor) is "at its limit" and not capable to provide more electron density to the π -system decorated with three very powerful acceptor groups, however strong they are. Fortunately, upon the reductive alkylation of the nitrogen atom (see Scheme 2), further bathochromic and bathofluoric shifts occurred (compare the spectral data for compounds **8-H** and **8-Me** discussed above), and compound **15** afforded bright conjugates with glycans.

A further aspect of the present invention relates to a general and straightforward method for synthesizing fluorescent pyrene dyes of Formula C, wherein X at each occurrence is SO_3H and n is 1-12, preferably 1-6, which involves an exchange reaction of

aryl halogen with bifunctional reagents that have pre-synthesized thioalkyl and unprotected sulfonic acid functions, optionally followed by oxidation of the thioether to the sulfone. Thus, the synthesis yields sulfoalkylsulfonyl-substituted dyes where sulfonic acid group (SO_3H) is connected to the aromatic dye core via an alkylsulfonyl (alkanesulfonyl) linker $\text{SO}_2(\text{CH}_2)_n$.

More specifically, such a method comprises at least the following steps: 1) protection of 1-aminopyrene with an appropriate protective group; 2) halogenation, usually bromination, as depicted in Scheme 9; 3) cross-coupling reaction, usually catalyzed with palladium or copper salts or complexes, of the trihalogeno-substituted pyrene derivative with a bifunctional reagent with a thiol and sulfonic acid (sulfonate) functions, as depicted in Scheme 11; 4) oxidation of the thioether to the sulfone; 5) deprotection of the amino group.

A closely related aspect of the invention relates to a method for synthesizing fluorescent aminopyrene dyes with absorbance maxima of 477 - 510 nm, net charge of 0 or down to -6 and larger, emission maxima of 535 - 560 nm in water, having the general Formulae B and C, particularly compounds **7-R**, **13a**, **13b**, **15**, **16**, **18**, **23a** and **23b**, which involves an exchange reaction of the aryl halogen to a substituted thioalkyl function, followed by oxidation to the sulfone and, optionally, by phosphorylation at the hydroxyl site.

The present invention further relates to a method for synthesizing phosphorylated fluorescent aminopyrene or aminoacridone dyes with net charges of -4, -6 and larger, that have the general Formulae A and D, particularly compounds **6-R** and **8-R**, which involves sulfamidation of amino alcohols, followed by phosphorylation at the hydroxyl site.

Brief Description of the Figures

Fig. 1 Principal steps of separation-based glycan analysis.

Fig. 2A shows actual normalized absorbance and fluorescence spectra of the new aminoacridone dyes: **6-H**, **6-Me** in TEAB buffer (pH 8-8.5)

Fig. 2B shows actual normalized absorbance and fluorescence spectra of the new phosphorylated aminopyrene dyes **8-H** and **15** in TEAB buffer (pH 8-8.5)

The following Examples illustrate the present invention in more detail.

General Materials and Methods

1.1.1 Preparative Methods

If not otherwise stated, reactions were carried out under an argon atmosphere. Solvents met *Reag. PhEur* purity standard (p.a.). Commercially available substances were used without further purification and purchased from *Sigma Aldrich*, *Acros Organics*, *Alfa Aesar*, *ABCR*, *Merck Millipore*, *Carbolution Chemicals*, etc.

1.1.2 Chromatographic Methods

1.1.2.1 Thin Layer Chromatography (TLC)

Normal phase TLC was performed on *silica gel 60 F₂₅₄* (*Merck Millipore*). For TLC on reversed phase *silica gel 60 RP-18 F₂₅₄* (*Merck Millipore*) was used. Preparative TLC was performed on *HPTLC Silica gel 60 F₂₅₄* with concentrating zone 10 × 2.5 cm (*Merck Millipore*). Compounds were detected by exposing TLC plates to UV-light (254 or 366 nm). Amines were detected by using ninhydrin solution (0.5% in EtOH).

1.1.2.2 Column Chromatography

Silica gel 60 with a particle size of 40-63 μm was purchased from *Merck Millipore*. Reversed phase column chromatography was performed on *POLYGOPREP® 60-50 C₁₈* (*Macherey Nagel*). Deactivated *silica gel 60* was purchased from *MP Biomedical*. Routine separation was performed with an automated *Isolera™ One* system (*Biotage GmbH*) with commercially available cartridges.

1.1.3 Analytical instruments

1.1.3.1 Absorption Spectroscopy

Absorption spectra were recorded with a double-beam UV-vis spectrophotometer (*Varian, series 4000*). For the determination of the absorption spectra, quartz cells with a 1 cm path length were used. Emission spectra and fluorescence quantum yield were obtained on a *Quantaaurus-QY Absolute PL* quantum yield spectrometer C11347 (*Quantaaurus QY*) or on a *Cary Eclipse* fluorescence spectrometer (*Varian*).

1.1.3.2 Nuclear Magnetic Resonance (NMR)

NMR spectra were recorded on an *Agilent 400MR DD2* spectrometer. All spectra are referenced to tetrametylsilane as an internal standard ($\delta = 0.00$ ppm) using the signals of the residual protons of CHCl_3 (7.26 ppm) in CDCl_3 , CHD_2OD (3.31 ppm) in CD_3OD , $\text{CHD}_2\text{COCD}_3$ (2.05 ppm) in $(\text{CD}_3)_2\text{CO}$ or $\text{DMSO}-d_5$ in $\text{DMSO}-d_6$. Multiplicities of the signals are described as follows: s = singlet, br. s = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants $^nJ_{x,y}$ are given in Hz, where n is the number of bonds between the coupled nuclei x and y. For ^{13}C -signals, which were revealed by indirect detection by HSQC, only resonances of the carbon atoms linked to H-atoms were recorded.

1.1.3.3 Mass-Spectrometry (MS)

Low resolution mass spectra (ESI-MS) with electro-spray ionization (ESI) were obtained on a *Varian 500-MS* spectrometer. High resolution mass spectra (ESI-HRMS) were obtained on a *Bruker micro TOF (ESI-TOF-MS)* spectrometer.

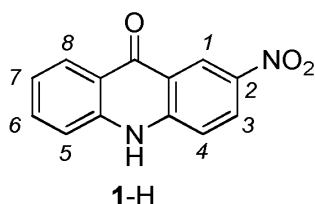
1.1.3.4 High-Performance Liquid Chromatography (HPLC)

HPLC system (Knauer): Smartline pump 1000 (2x) with 10 mL pump-head, UV detector 2500, column thermostat 4000, mixing chamber, injection valve with a 20 or 50 μ L loop for the analytical and 500 μ L loop for preparative columns; 6-port-3-channel switching valve; analytical column: *Eurospher-100 C18* 5 μ m (if not stated otherwise), or *Kinetex C18 100*, 5 μ m, 250x4.6 mm, flow 1.2 mL/min; preparative column: *Kinetex C18 100*, 5 μ m, 250x21 mm, flow 10 mL/min, solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA (if not stated otherwise.) For isolation and purification of phosphorylated dyes on preparative scales, acetonitrile - water systems containing 0.05-0.1 M of $\text{Et}_3\text{N} \cdot \text{H}_2\text{CO}_3$ buffer (pH = 8-8.9; Sigma-Aldrich, or self-prepared from 1 M aq. Et_3N and CO_2 gas obtained by evaporation of solid CO_2).

EXAMPLE 1

Synthesis of fluorescent acridone dyes and their precursors

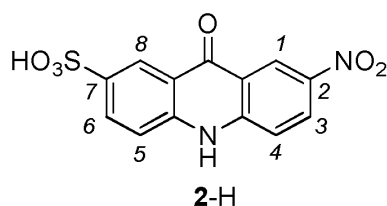
2-Nitroacridine-9(10H)-one (1-H)



The reaction between 2-chloro-5-nitrobenzoic acid and aniline in the presence of K_2CO_3 and catalytic amounts of $\text{Cu}(\text{NO}_2)_2$ (185 °C, 2-4 h) afforded 4-nitro-*N*-phenylanthranilic acid according to the procedure described in WO 2007/049057 (03.05.2007) by R. Ramage, B. Maltman, G. Cotton, S. C. M. Couturier, and R. A. S. McMordie; see also: US2012/0015373 (19.01.2012) by J. A. Smith

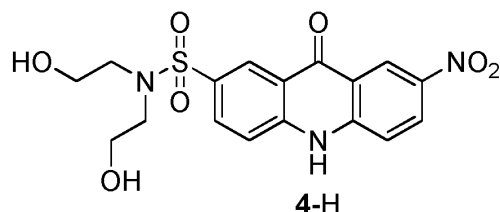
and R. M. West. 4-Nitro-*N*-phenylanthranilic acid was cyclized to 2-nitroacridine-9(10*H*)-one (**1-H**) (POCl₃, reflux, 3 h). ¹H NMR (400 MHz, DMSO-*d*₆) δ = 12.4 (s, 1 H, NH), 8.96 (d, *J* = 2.8, 1 H, H-1), 8.46 (dd, *J* = 9.2, 2.7 Hz, 1 H, H-3), 8.23 (dd, *J* = 8.1, 1.6 Hz, 1 H, H-8), 7.81 (ddd, *J* = 8.5, 7.0 and 1.6 Hz, 1 H, H-6/7), 7.66 (d, *J* = 9.1 Hz, 1 H, H-4), 7.58 (dm, 1 H, H-5), 7.37 (ddd, *J* = 8.1, 7.0 and 1.1 Hz, 1 H, H-7/6)ppm.

2-Nitro-9-oxo-9,10-dihydroacridine-7-sulfonic acid (2-H)



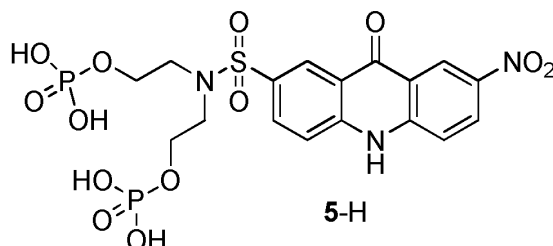
Compound **1-H** was dried and sulfonated with a 20% oleum (100 °C, 1.5 h) to afford the title compound **2-H** (US2012/0015373). For example, to 530 mg (2.21 mmol) of 2-nitro-10*H*-acridine-9-one (**1-H**), 20% SO₃ in H₂SO₄ (25 mL) was added dropwise and the reaction mixture was stirred at 100 °C for 90 minutes. Then the reaction mixture was very carefully and slowly poured onto ice (>30 g), and 4 mL of conc. aq. HCl was added. The brown precipitate was separated by centrifugation, washed with 4 M aq. HCl (2×5 mL) and lyophilized. Yield - 463 mg (65%) of brown solid. HPLC: *t*_R = 5.6 min; H₂O/ACN (+0.1% TFA): 80/20 → 50/50 in 25 min, detection at 254 nm. ¹H NMR (400 MHz, D₂O) δ = 7.61 (dd, *J* = 8.0 and 2.6 Hz, 1 H, H-3), 7.59 (br. s, 1 H, H-1), 7.57 (d, *J* = 2.6 Hz, 1 H, H-8), 7.33 (dd, *J* = 8.6 and 2.1 Hz, H-6), 6.53 (dd, *J* = 8.8 and 1.0 Hz, 1 H, H-4), 6.47 (d, *J* = 8.6 Hz, 1 H, H-5)ppm. ESI-MS (C₁₃H₈N₂O₆S, M = 320), negative mode: *m/z* (rel. int., %) = 319 (100) [M-H]⁻.

***N,N*-(2-Hydroxyethyl)-2-nitro-9-oxo-9,10-dihydroacridine-7-sulfonamide (4-H)**



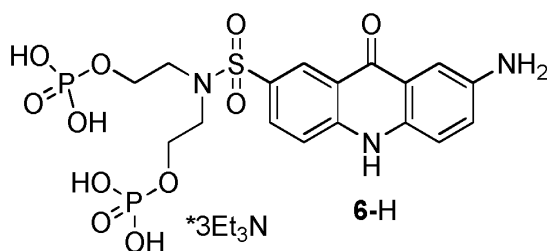
Compound **2-H** (55 mg, 0.17 mmol) was cooled down to 0°C and chlorosulfonic acid (1.7 mL, d = 1.75 g/mL, 3.0 g, 26 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 6 h and poured onto ice (7–10 g). The beige precipitate was separated by centrifugation, washed with ice-water (2×5 mL) and dried *in vacuo*. The obtained sulfonyl chloride was added to a solution of *N*-methyl ethanolamine (86 mg, 0.80 mmol) in MeCN/H₂O (1:1) at 0°C. The reaction mixture was warmed-up to room temperature and stirred at 50°C overnight. The precipitate dissolved gradually. Yield – 33 mg (48%) of a yellow solid which was obtained upon concentration of the reaction mixture *in vacuo* (in the course of evaporation of acetonitrile). HPLC: t_R = 16.7 min H₂O/CAN (+0.1% TFA): 80/20 → 50/50 in 25 min, 254 nm. ¹H NMR (400 MHz, DMSO-*d*₆) δ = 12.7 (br. s, 1 H, NH), 8.95 (d, J = 2.6 Hz, 1 H, H-1), 8.55 (d, J = 2.2 Hz, 1 H, H-8), 8.51 (dd, J = 9.2, 2.7 Hz, 1 H, H-6), 8.13 (dd, J = 8.8, 2.2 Hz, 1 H, H-3), 7.72 ("t", J = 8.9 Hz, 2 H, H-4,5), 4.83 (t, J = 5.5 Hz, 2 H, OH), 3.51 (q, J = 6.3 Hz, 4 H, CH₂), 3.18 (t, J = 6.4 Hz, 4 H, CH₂) ppm. C₁₇H₁₇N₃O₇S, M = 407 g/mol); ESI-MS, negative mode: m/z (rel. int., %) = 406 (100) [M-H]⁻.

***N,N'*-(2-Hydroxyethyl)-2-nitro-9-oxo-9,10-dihydroacridine-7-sulfonamide *O,O'*-diphosphate (5-H)**



Compound **4-H** (50 mg, 0.12 mmol) was dissolved in 10 mL MeCN, and this solution was added under Ar to 5 mL of freshly distilled POCl₃. The suspension was stirred at 30°C for 2 h. The solvent and the excess of POCl₃ were removed *in vacuo*, the residue was dissolved in aqueous TEAB buffer (1 M), and the title compound was isolated on RP-18 using MeCN - 0.05 M aq. TEAB buffer (1:3) as an eluent. Yield - 27 mg (40%) of a yellow solid (after lyophilization). HPLC: t_R = 3.5 min; H₂O/ACN (+0.1% TFA): 70/30 → 0/100 in 25 min, 254 nm. HPLC: t_R = 9.2 min, H₂O/ACN (+0.1% TFA): 80/20 → 50/50 in 25 min, 254 nm. ¹H NMR (400 MHz, D₂O) δ = 8.72 (d, J = 2.6 Hz, 1 H, H-1), 8.35 (d, J = 2.2 Hz, 1 H, H-8), 8.24 (dd, J = 9.3, 2.6 Hz, 1 H, H-6), 7.95 (dd, J = 8.9, 2.2 Hz, 1 H, H-3), 7.47 (d, J = 8.9 Hz, 1 H, H-4/5), 7.39 (d, J = 9.2 Hz, 1 H, H-5/4), 3.84-3.78 (m, 4 H, CH₂), 3.37 (t, J = 5.9 Hz, 4 H, CH₂). ³¹P NMR (81 MHz, D₂O): δ = 1.0 ppm. C₁₇H₁₉N₃O₁₃P₂S, M = 567 g/mol). ESI-MS, negative mode: m/z (rel. int., %) = 566 (100) [M-H]⁻.

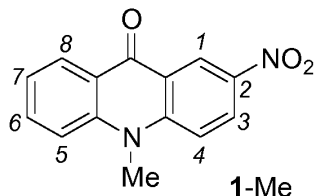
2-Amino-*N,N'*-(2-hydroxyethyl)-9-oxo-9,10-dihydroacridine-7-sulfonamide *O,O'*-diphosphate (6-H)



Pd/C (the oxidized form (VWR International, 10% Pd, 7 mg) was mixed under argon with propanol-2 (attention: Pd/C causes ignition when gets dry in air during the reaction workup, especially filtration!), water (1 mL) was added; the mixture was flushed with hydrogen, and stirred for 30 min. Compound **5-H** (27 mg, 0.48 mmol) was dissolved in propanol-2 (0.5 mL), and this solution was added to the suspension of the pre-reduced catalyst. The reaction mixture was stirred overnight in the atmosphere of hydrogen. HPLC control indicated nearly full conversion to a new substance with yellow fluorescence and $t_R = 2.9$ min (HPLC area 92%), 8.8 min (7.5%; starting material) H_2O/ACN (+0.1% TFA): 80/20 $\rightarrow$ 50/50 in 25 min, 254 nm. The reaction mixture was flushed with argon, the catalyst was separated by centrifugation, washed with aqueous propanol-2, and the combined supernatant was concentrated *in vacuo*. The title compound was isolated by preparative HPLC (see below). $C_{17}H_{21}N_3O_{11}P_2S$ (exact mass 537.0372), ESI-MS, negative mode: m/z (rel. int., %) = 536 (100) $[M-H]^-$. ESI-HRMS: 536.0278 (found $[M-H]^-$), calculated: 536.0299. Final purification (for reductive amination of sugars) was achieved by preparative HPLC using an aqueous 0.05 M TEAB buffer (pH 8) and Kinetex column (5 μm C18 100, 250 $\times$ 10 mm), ACN/H_2O : 5/95 – 30/70 in 20 min, 4 mL/min; $t_R \sim 10$ –11 min (strongly depends on concentration of the analyte). Purity control: 0.05 M TEAB aqueous buffer (pH 8) and Kinetex column (5 μm C18 100, 250 $\times$ 4.6 mm), ACN/H_2O : 5/95 – 50/50 in 20 min, 1.2 mL/min; $t_R = 8.1$ min. 1H NMR (400 MHz, D_2O) δ = 8.35 (d, $J = 2.2$ Hz, 1 H, H-8), 7.82 (dd, $J = 9.3$ and 2.6 Hz, 1 H, H-6), 7.29 (d, $J = 9.3$, 1 H, H-5), 7.12 (d, $J = 2.2$ Hz, 1 H, H-1), 7.04 (dd, $J = 2.4$ and 8.9 Hz, 1 H, H-3), 7.01 (d, $J = 8.9$ Hz, 1 H, H-4), 3.87 (q, $J = 6.0$, 4 H, $(OH)_2POCH_2$), 3.40 (t, $J = 5.9$ Hz, 4 H, NCH_2), 3.05 (q, ~ 16 H, CH_2N in Et_3N), 1.13 (t, ~ 23 H, CH_3 in Et_3N) ppm. λ_{max} (absorption, H_2O (ϵ , $M^{-1} cm^{-1}$) = 217 (13500), 260 (26000), 295 (28000), 420 (3700) nm; λ_{max} (emission, H_2O) = 485 nm (excitation at 405 nm), 586 nm (excitation at 300 nm, 420 nm or 470 nm);

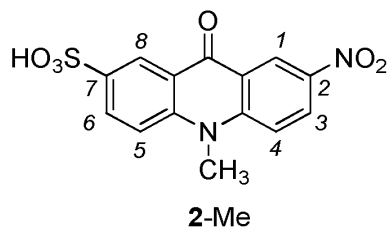
fluorescence lifetimes – 22.3 ns (excitation at 405 nm, emission detected at 485 nm) and 3.7 ns (excitation at 470 nm, emission detected at 585 nm).

10-Methyl-2-nitroacridin-9(10H)-one (1-Me)



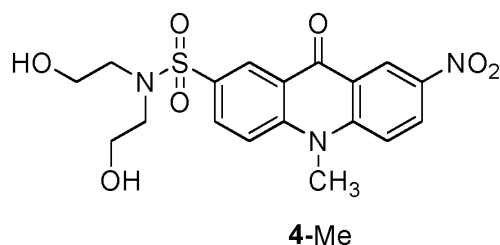
NaH (600 mg of a 60% suspension in mineral oil; 360 mg, 15 mmol) was washed with dry pentane (3 × 15 mL) under argon atmosphere, suspended in dry DMSO, and then 2-nitroacridine-9(10H)-one (**1-H**, 2.5 g, 10.4 mmol) was added to this suspension in small portions. The organic substance dissolves gradually, and the solution turned magenta (violet). It was warmed-up to 50–60°C, stirred for 30 min, cooled to room temperature, and then MeI (4 mL, 64 mmol) was added to the reaction mixture. In the course of stirring overnight at room temperature, the suspension gradually turned yellow. Then the reaction mixture was poured onto ice with water (75 mL). The precipitate was collected by filtration, washed with water, and recrystallized from AcOH (200 mL). Yield – 2.1 g (80%) of a yellow solid. HPLC: t_R = 13.9 min A/B: 70/30 → 0/100 in 25 min, 254 nm. $C_{14}H_{10}N_2O_3$, M = 254 g/mol). ESI-MS, positive mode: m/z (rel. int., %) = 277.0579 (100) [found $M+Na$]⁺; 277.0584 (calculated). ¹H NMR (400 MHz, DMSO- d_6) δ = 9.01 (d, J = 2.9, 1 H, H-1), 8.50 (dd, J = 9.6, 2.9 Hz, 1 H, H-3), 8.32 (ddd, J = 7.9, 1.6, 0.7 Hz, 1 H, H-8), 8.02 (d, J = 9.6 Hz, 1 H, H-4), 7.95–7.85 (m, 2 H, H-6/7), 7.44 (ddd, J = 8.0, 6.2, 1.7 Hz, 1 H, H-5), 3.98 (s, 3 H, CH₃)ppm.

**10-Methyl-2-nitro-9-oxo-9,10-dihydroacridine-7-sulfonic acid
(2-Me)**



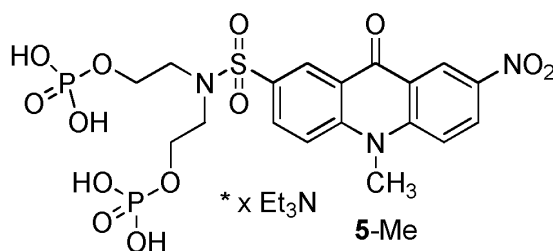
To compound **1-Me** (400 mg, 1.57 mmol), 20% SO₃ in H₂SO₄ (7.2 mL, 16.8 mmol) was added dropwise, and the reaction mixture was stirred at 100°C for 90 min. Then the reaction mixture was poured very carefully and slowly onto ice (>30 g), and 4 mL of conc. HCl was added. The brown precipitate was separated by centrifugation, washed with cold 4 M HCl (2×5 mL) and lyophilized. Yield - 376 mg (72%) of the title compound as a brown solid. HPLC: t_R = 7.9 min A/B: 80/20 → 50/50 in 25 min, 254 nm. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 9.03 (d, J = 2.9 Hz, 1 H, H-1), 8.55 (d, J = 2.1 Hz, 1 H, H-8), 8.52 (dd, J = 9.5, 2.9 Hz, 1 H, H-3), 8.08–8.02 (m, 2 H, H-4, H-6), 7.90 (d, J = 9.1 Hz, 1 H, H-5), 4.00 (s, 3 H, CH₃) ppm. C₁₄H₁₀N₂O₆S, M = 334 g/mol. ESI-MS, negative mode: m/z (rel. int., %) = 333 (100) [M-H]⁻. ¹H NMR (100 MHz, DMSO-*d*₆): δ = 176.7 (CO), 146.2, 143.3, 142.6, 141.2, 132.8, 128.1, 123.8, 123.3, 121.4, 121.0, 118.8, 117.3, 35.3 (N-Me) ppm.

***N,N*-(2-Hydroxyethyl)-10-methyl-2-nitro-9-oxo-9,10-dihydroacridine-7-sulfonamide (4-H)**



To compound **2-Me** (62 mg, 0.19 mmol), chlorosulfonic acid (3.5 mL, 53 mmol) was added dropwise at 0°C. The reaction mixture was stirred at 50°C for 30 min and at room temperature overnight. Then the reaction mixture was dropwise poured into ice (10 g), and the beige precipitate separated by centrifugation. It was washed with ice-water (2×10 mL) and lyophilized. The obtained sulfonyl chloride was added to the solution of diethanolamine (185 mg, 1.76 mmol) in THF (2 mL) at 0°C. The reaction mixture was stirred at room temperature overnight. The precipitate was separated by centrifugation, washed with ice water and lyophilized. Yield - 57 mg (70%) of the title product as a yellow solid. HPLC: t_R = 16.9 min A/B: 80/20 → 50/50 in 25 min, 254 nm. ESI-MS, positive mode: m/z (rel. int., %) = 444 (100) $[M+Na]^+$. $C_{18}H_{19}N_3O_7S$, $M = 421$ g/mol. ESI-MS, positive mode: m/z = 422.1005 [found $M+H$]⁺; 422.1016 (calculated); 444.0829 [found $M+Na$]⁺; 444.0836 (calculated). 1H NMR (400 MHz, DMSO- d_6): δ = 8.99 (d, J = 2.8 Hz, 1 H, H-1), 8.61 (d, J = 1.6 Hz, 1 H, H-8), 8.56 (dd, J = 9.5 and 2.9 Hz, 1 H, H-3), 8.19 (dd, J = 9.1, 2.4 Hz, 1 H, H-6), 8.10 ("d", J = 9.4 Hz, 2 H, H-4,5), 4.82 (t, J = 5.5 Hz, 2 H, OH), 4.02 (s, 3 H, NMe), 3.52 (q, J = 6.0 Hz, 4 H, OCH₂), 3.20 (t, J = 6.3 Hz, 4 H, NCH₂) ppm.

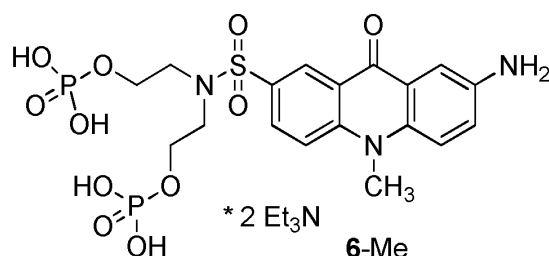
***N,N*-(2-Hydroxyethyl)-9,10-dihydro-9-oxo-10-methyl-2-nitroacridine-7-sulfonamide *O,O'*-diphosphate (5-Me)**



To a solution of **4-Me** (31 mg, 73 μ mol) in trimethyl phosphate (0.5 mL) POCl₃ (0.2 mL) was added under Ar at 0°C. The suspension was stirred at room temperature for 2 hours. Then the solvent and the excess of POCl₃ were removed in vacuo the residue was

diluted with an excess of aq. Et₃N-H₂CO₃ buffer (pH 7.5) and kept at 4°C overnight. The title compound was isolated on RP-18 using MeCN – 0.1 M aq. Et₃N-H₂CO₃ buffer (1:5) as an eluent, lyophilized and used in the final reduction step (see below). C₁₈H₂₁N₃O₁₃P₂S, M = 581 g/mol. ESI-MS, negative mode: *m/z* (rel. int., %) = 580.0191 (100) [found *M-H*]⁻; 580.0198 (calculated).

2-Amino-*N,N'*-(2-hydroxyethyl)-9,10-dihydro-9-oxo-10-methylacridine-7-sulfonamide *O,O'*-diphosphate (6-Me)



Pd/C (VWR International, the oxidized form, 10% Pd) 7 mg of was mixed under argon with propanol-2, water (1 mL) was added; the mixture was flushed with hydrogen, and stirred for 30 min. Compound **5-Me** (27 mg) was dissolved in propanol-2 (0.5 mL), and this solution was added to the suspension of the pre-reduced catalyst. The reaction mixture was stirred overnight in the atmosphere of hydrogen. HPLC control indicated nearly full conversion to a new substance with yellow fluorescence and *t_R* = 16.1 min (HPLC area 95%), 19.2 min (5%; starting material?) H₂O/ACN (+0.05 M Et₃N*H₂CO₃ buffer in aqueous phase): 99/1 → 80/20 in 20 min, dioden array detector, Kinetex column 5 μm C18 100, 4.6 × 250 mm, 20°C, 1.2 mL/min. The reaction mixture was flushed with argon, the catalyst was separated by centrifugation, washed with aqueous propanol-2, and the combined supernatant was concentrated in vacuo. The title compound was isolated by preparative HPLC using an aqueous 0.05 M TEAB buffer (pH 8) and Kinetex column (5 μm C18 100, 250 × 10 mm), ACN/H₂O: 5/95 – 30/70 in 20 min, 4 mL/min; *t_R* ~ 11.4 min. C₁₈H₂₃N₃O₁₁P₂S (exact mass

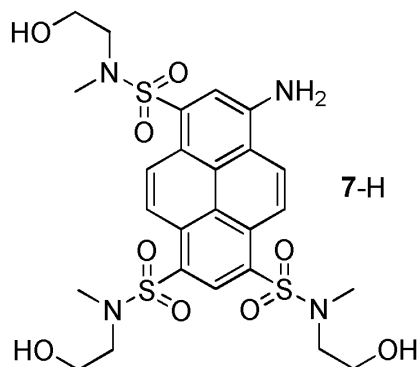
551.0529). ESI-MS, negative mode: m/z (rel. int., %) = 550 (100) $[M-H]^-$. ESI-HRMS: 550.0441 (found $[M-H]^-$), calculated: 550.0456. 1H NMR (400 MHz, D_2O): δ = 8.29 (d, J = 2.4 Hz, 1 H, H-8), 7.84 (dd, J = 9.3 and 2.4 Hz, 1 H, H-6), 7.43 (d, J = 9.3, 1 H, H-5), 7.12 (d, J = 9.3 Hz, 1 H, H-3), 7.03 (d, J = 2.8 Hz, 1 H, H-1), 6.97 (dd, J = 9.2 and 2.8 Hz, 1 H, H-4), 3.85 (q, J = 6.1, 4 H, $POCH_2$), 3.49 (s, 3 H, NMe), 3.37 (t, J = 5.9 Hz, 4 H, NCH_2), 3.04 (q, ~14 H, CH_2N in Et_3N), 1.13 (t, ~20 H, CH_3 in Et_3N) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = 1.1 ppm. λ_{max} (0.05 M aq $Et_3N \cdot H_2CO_3$ buffer, pH 8, ϵ , $M^{-1} cm^{-1}$) = 219 (10 300), 263 (18 600), 299 (18 500), 430 (2900) nm; λ_{max} (emission, 0.05 M aq $Et_3N \cdot H_2CO_3$ buffer, pH 8) = 485 nm and 585 nm (two maxima in ca. 1:2 ratio, independent from excitation wavelength which may be at 300 nm, 420 nm or 470 nm); fluorescence quantum yield: 5–6 % (absolute value).

EXAMPLE 2

Synthesis of fluorescent 1-aminopyrene dyes and their precursors

3-Aminopyrene-1,6,8-trisulfonic acid trisodium salt (APTS) was prepared from 1-aminopyrene and a 20% oleum in the presence of Na_2SO_4 as described by Z. Sharrett, S. Gamsey, L. Hirayama, B. Villozny, J. T. Suri, R. A. Wessling, B Singaram, *Org. Biomol. Chem.* **2009**, 7, 1461–1470.

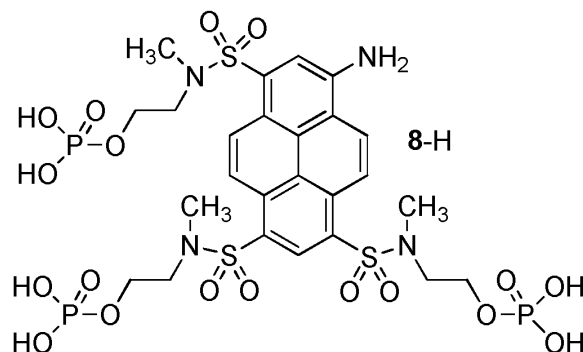
3-Amino-*N,N',N''*-tris(2-hydroxyethyl)-*N,N',N''*-trimethylpyrene-1,6,8-trisulfonamide (7-H)



446 mg (0.98 mmol) of APTS (trisodium salt) was cooled down to 0 °C in an ice bath, and then chlorosulfonic acid (7.5 mL, 0.11 mol) was added dropwise with stirring. The reaction mixture was stirred at 65 °C for 3 h. After cooling down to room temperature, the reaction mixture was transferred onto crushed ice. The red precipitate of trisulfonyl chloride was isolated by centrifugation, washed with ice-water (2 × 10 mL) and added to a solution of *N*-methylethanolamine (1.0 g, 13 mmol) in aqueous acetonitrile (1:1, 25 mL) at 0 °C. The reaction mixture was vigorously stirred at room temperature, until it became homogeneous, and then lyophilized. The title compound was isolated by chromatography on SiO₂ (100 g) with CHCl₃/MeOH/H₂O (80:18:2) mixture as an eluent. Yield - 252 mg (41%) of a brown-orange solid obtained after the second chromatographic purification. HPLC: t_R = 15.8 min, ACN/H₂O: 20/80 - 50/50 in 25 min, 1.2 mL/min, 254 nm. C₂₅H₃₂N₄O₉S₃ (exact mass 628.1331); ESI-HRMS: 651.1212 (found [M+Na]⁺), calculated: 651,1224. ¹H NMR (400 MHz, CD₃OD) δ = 9.19 (d, J = 9.8 Hz, 1 H), 9.06 (s, 1 H), 8.99 (d, J = 9.6 Hz, 1 H), 8.82 (d, J = 9.8 Hz, 1 H), 8.74 (d, J = 9.7 Hz, 1 H), 8.18 (s, 1 H), 3.69 (m, 6 H, CH₂), 3.42 (m, 6 H, CH₂), 3.04 (s, 3 H, NCH₃), 3.01 (s, 6 H, 2×NCH₃) ppm; λ_{max} (absorption) = 477 nm (ϵ = 22 400 M⁻¹ cm⁻¹, MeOH), λ_{max} (emission) = 535 nm (MeOH, excitation at 470 nm); fluorescence lifetime 5.6

ns (MeOH); fluorescence quantum yield (0.96; absolute value in MeOH).

3-Amino-*N,N',N''*-tris(2-hydroxyethyl)-*N,N',N''*-trimethylpyrene-1,6,8-trisulfonamide *O,O',O''*-triphosphate tris(triethylammonium salt) (8-H)

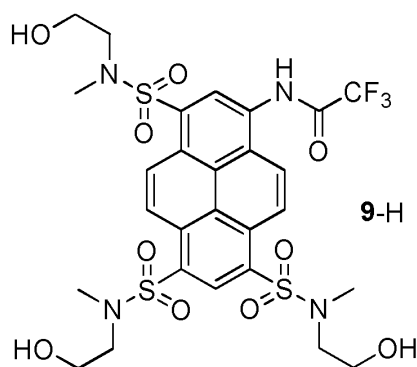


Compound **7-H** (40 mg, 64 μ mol) was dissolved in $(\text{MeO})_3\text{PO}$ (5 mL) and this solution was added to freshly distilled POCl_3 (0.2 mL) with stirring at room temperature. A weak exothermic reaction was observed, and the solution turned orange-brown. The reaction mixture was stirred several hours at room temperature. All volatile materials (excess of POCl_3 and most of trimethyl phosphate) were removed in vacuum (first using rotary evaporator and then - oil pump; 0.5 mbar, 60 $^{\circ}\text{C}$, cold trap cooled with dry ice for collecting trimethyl phosphate and POCl_3). The residue was treated and stirred with 1 M $\text{Et}_3\text{N}^+\text{H}_2\text{CO}_3^-$ buffer (TEAB; initial pH = 8), and the pH was controlled. Phosphorylation of the amino group also takes place, but this phosphate group is readily cleaved. Fresh portions of the TEAB buffer were added when the solution became acidic, until the pH stabilized at about 5-7. TLC control on regular SiO_2 was possible in $i\text{PrOH}/\text{H}_2\text{O}/25\%$ aq. NH_3 (10/5/1); $R_f \sim 0.3$ (yellow spot with green fluorescence). The reaction mixture was lyophilized, and the title compound was isolated by chromatography on regular SiO_2 using $i\text{PrOH}/\text{H}_2\text{O}/25\%$ aq. NH_3 (10/5/1) mixture as an eluent. HPLC control indicated homogeneous fractions containing the substance with $t_R = 10.2$

min (ACN/H₂O (+0.1% TFA): 20/80 – 50/50 in 25 min, 1.2 mL/min, 254 nm). Lyophilization afforded 60 mg of the red-orange foam. C₂₅H₃₅N₄O₁₈P₃S₃ (exact mass 868.0321) * × NH₃ ESI-MS, negative mode: *m/z* (rel. int., %) = 867 (100) [*M*-H]⁻, 889 (60) [*M*-2H+Na]⁻.

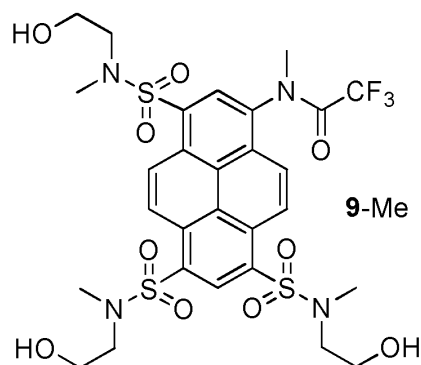
Final purification was achieved by preparative HPLC using a 0.1 M TEAB buffer and a preparative Kintex column (e.g., Kinetex, 5 μm C18 100, 250 × 4.6 mm, ACN/H₂O: 10/90 – 30/70 in 20 min, 1.2 mL/min; *t_R* = 10.8 min). Freeze-drying of the eluate gave the title compound as yellow foam. ¹H NMR (400 MHz, CD₃OD) δ = 9.14 (d, *J* = 9.7 Hz, 1 H), 9.03 (s, 1 H), 8.96 (d, *J* = 9.7 Hz, 1 H), 8.77 (d, *J* = 9.8 Hz, 1 H), 8.67 (d, *J* = 9.7 Hz, 1 H), 8.17 (s, 1 H), 3.94–4.06 (m, 6 H, CH₂), 3.49–3.59 (m, 6 H, CH₂), 3.08 (s, 3 H, NCH₃), 3.04 (q, *J* = 7.3 Hz, 18 H, CH₂N in Et₃N), 3.02 (s, 3 H, NCH₃), 1.22 (t, *J* = 7.3 Hz, 27 H, CH₃CH₂ in Et₃N) ppm; λ_{max} (absorption) = 205, 237, 305 and 471 nm (0.05 M aq Et₃N*H₂CO₃ buffer, pH 8), λ_{max} (emission) = 544 (0.05 M aq Et₃N*H₂CO₃ buffer, pH 8); fluorescence lifetime 5.9 ns (in H₂O; excitation at 470 nm); fluorescence quantum yield: 0.77 (H₂O, standard: Coumarin 153 with emission efficiency of 0.54 in ethanol, λ_{excit} = 400 nm), 0.88 (absolute value in 0.05 M aq Et₃N*H₂CO₃ buffer, pH 8; excitation at 460 nm).

3-(Trifluoroacetyl)amino-*N,N',N''*-tris(2-hydroxyethyl)-*N,N',N''*-trimethylpyrene-1,6,8-trisulfonamide (9-H)



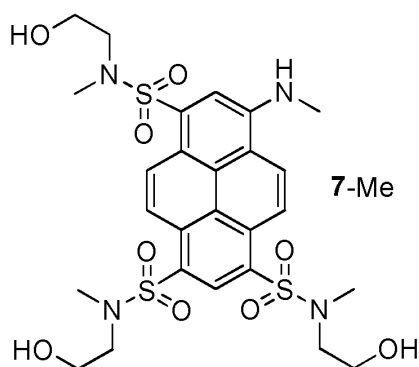
Compound **7-H** (179 mg, 0.28 mmol) was suspended in 10 mL of DCM, and a 10% solution of trifluoroacetic anhydride in DCM ($d = 1.33$ g/mL, 0.9 mL, ~ 0.57 mmol) followed by Et_3N (126 μL , $d = 0.73$ g/mL, 0.91 mmol) was added at room temperature. The reaction mixture was stirred for 30 min. All volatile materials were evaporated under reduced pressure, the residue was dissolved in methanol (50 mL) and NaHCO_3 (50 mg) was added. After stirring at room temperature for 30 min, the reaction mixture was neutralized with acetic acid, and all volatile materials were removed *in vacuo*. These operations remove trifluoroacetate groups from hydroxyl groups. The title compound was isolated by chromatography regular silica gel (50 g) using $\text{CH}_2\text{Cl}_2/\text{aceton}$ (2:1) as an eluent. Yield - 165 mg (82%) of a yellow solid. HPLC: $t_R = 10.8$ min, ACN/ H_2O : 20/80 - 100/0 in 25 min, 254 nm. $\text{C}_{27}\text{H}_{31}\text{F}_3\text{N}_4\text{O}_{10}\text{S}_3$, (exact mass 724.1154). ESI-MS, negative mode: m/z (rel. int., %) = 723 (100) $[\text{M}-\text{H}]^-$. ^1H NMR (400 MHz, acetone- d_6) $\delta = 11.2$ (br. s, 1 H, NH), 9.49 (d, $J = 10.0$ Hz, 1 H), 9.38 ("dd", $J = 9.7$ and 1.9 Hz, 2 H), 9.26 (s, 1 H), 9.02 (s, 1 H), 8.76 (d, $J = 9.7$ Hz, 1 H), 3.83-3.90 (m, 3 H, OH), 3.71 (m, 6 H, OCH_2), 3.43 (m, 6 H, NCH_2), 3.08 / 3.06 / 3.06 (3xs, Σ 9 H, NMe) ppm. ^{19}F NMR (376 MHz, acetone- d_6) $\delta = -75.7$ ppm

3-[N-methyl-N-(trifluoroacetyl)]amino-N,N',N''-tris(2-hydroxyethyl)-N,N',N''-trimethylpyrene-1,6,8-trisulfonamide (9-Me)



To a solution of compound **7-H** (120 mg, 0.17 mmol) in DMF (2 mL), CsCO₃ (42 mg, 0.13 mmol) and CH₃I (960 mg, 6.7 mmol) were added under argon. The reaction mixture was stirred at 70°C for 40 min, and the solvent was evaporated under reduced pressure. The title compound was isolated by chromatography on regular SiO₂ (50 g) using a 15:1 mixture of DCM and methanol as an eluent; yield - 110 mg (88%) of a yellow solid. Due to the presence of the secondary amide group, two rotamers (two sets of signals) were detected in ¹H NMR spectrum of this substance, and therefore it was difficult to interpret. HPLC: *t*_R = 12.2 min, ACN/H₂O: 20/80 - 100/0 in 25 min, 254 nm. C₂₈H₃₃F₃N₄O₁₀S₃ (exact mass 738.1311). ESI-MS, positive mode: *m/z* (rel. int., %) = 739 (100) [M+H]⁺, 761 (35) [M+Na]⁺.

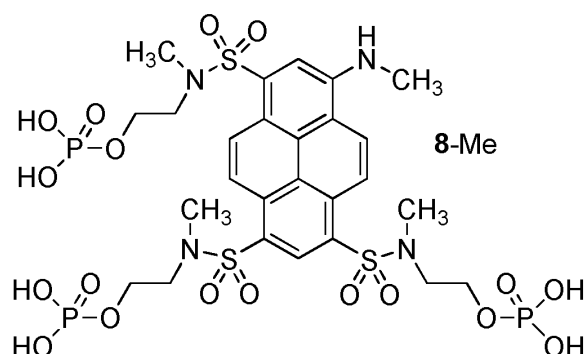
3-Methylamino-*N,N',N''*-tris(2-hydroxyethyl)-*N,N',N''*-trimethylpyrene-1,6,8-trisulfonamide (7-Me)



The title compound was obtained from the corresponding *N*-methyl-*N*-trifluoroacetamide (**9-Me**; see above) upon treatment with Na₂CO₃, which was taken in excess as a saturated solution, in aqueous methanol (ca. 1:1) so that reaction mixture remained homogeneous. The product was isolated as an orange solid (20 mg) by chromatography on SiO₂ using a 15:1 mixture of DCM and methanol as an eluent.

^1H NMR (400 MHz, CD_3OD) δ = 9.15 (d, J = 9.8 Hz, 1 H), 9.06 (s, 1 H), 8.98 (d, J = 9.6 Hz, 1 H), 8.77 (d, J = 9.8 Hz, 1 H), 8.63 (d, J = 9.7 Hz, 1 H), 7.93 (s, 1 H), 3.77–3.63 (m, 6 H, CH_2), 3.40 (m, 6 H, CH_2), 3.21 (s, 3 H, NCH_3), 3.00 (s, 3 H, NCH_3), 2.99 (s, 6 H, $2\times\text{NCH}_3$) ppm; $\text{C}_{26}\text{H}_{34}\text{N}_4\text{O}_9\text{S}_3$ (exact mass 642.1488). HRMS (ESI): 665.1353 (found $[\text{M}+\text{Na}]^+$), calculated: 665.1380. λ_{max} (absorption) = 493 nm (ϵ = 23000 $\text{M}^{-1}\text{cm}^{-1}$, MeOH), λ_{max} (emission) = 549 nm (MeOH); fluorescence lifetime 5.9 ns (MeOH), fluorescence quantum yield: 0.97 (absolute value in MeOH); 0.83 (relative value obtained in MeOH using Rhodamine 6G as a standard (QY = 0.94 in ethanol), excitation at 480 nm).

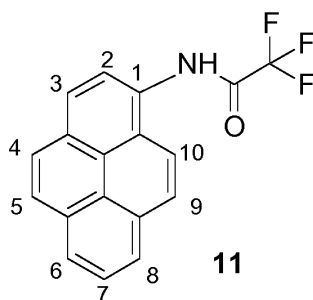
3-Methylamino-*N,N',N''*-tris(2-hydroxyethyl)-*N,N',N''*-trimethylpyrene-1,6,8-trisulfonamide *O,O',O''*-triphosphate (8-Me) as tris(triethylammonium salt)



To a solution of POCl_3 (74 mg, 480 μmol) in 0.1 mL of $(\text{MeO})_3\text{PO}$, the solution of compound **7-Me** (40 mg, 54 μmol) in 0.5 mL of $(\text{MeO})_3\text{PO}$ was added dropwise at 0°C . Then the reaction mixture was stirred for 1.5 h at room temperature. All volatile materials (excess of POCl_3 and most of trimethyl phosphate) were removed in vacuum (first, using rotary evaporator and then an oil pump; 0.5 mbar, 60°C , cold trap cooled with dry ice for collecting trimethyl phosphate). The residue was treated and stirred with

1 M $\text{Et}_3\text{N}\cdot\text{H}_2\text{CO}_3$ buffer (TEAB; initial pH = 8) and pH-value was controlled. Fresh portions of the TEAB buffer were added when the solution became acidic, until the pH-value stabilized at about 5-7. Then the reaction mixture was loaded on RP-18 (ca. 30 g) and the title compound was eluted using 1:4 mixture of MeCN and aqueous 0.1 M $\text{Et}_3\text{N}\cdot\text{H}_2\text{CO}_3$ buffer (pH 7.5). Yield - 63%, a yellow solid. HPLC: t_R = 6.9 min $\text{H}_2\text{O}/\text{ACN}$ (+0.1% TFA): 80/20 $\rightarrow$ 0/100 in 25 min, 254 nm. $\text{C}_{26}\text{H}_{37}\text{N}_4\text{O}_{18}\text{P}_3\text{S}_3$ (exact mass 882,0478). ESI-MS, negative mode: m/z (rel. int., %) = 881 (100) $[\text{M}-\text{H}]^-$, 440 (15) $[\text{M}-2\text{H}]^{2-}$. λ_{max} (absorption) = 502 nm (H_2O), λ_{max} (emission) = 563 nm (H_2O); fluorescence quantum yield 0.85 (H_2O , standard: Rhodamine 6G with emission efficiency of 0.94 in ethanol, λ_{excit} = 500 nm). This substance was found to be unreactive in the reductive amination of sugars.

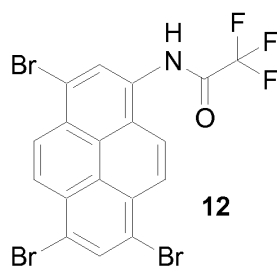
2,2,2-Trifluoro-N-(pyren-1-yl) acetamide (11)



1-Aminopyrene (**10**) (1.00 g, 4.60 mmol) was dissolved in dry DCM (30 mL) under stirring and by using an ultrasonic bath. Trifluoroacetic anhydride ($d=1.51$, 772 μL , 1.17 g, 5.55 mmol, 1.21 eq.) was added dropwise over a period of 10 min. The obtained suspension was stirred for 30 min at r.t. The precipitate was removed by filtration and washed with cyclohexane (3 x 15 mL) and dried *in vacuo*. A white precipitate observed in the filtrate and was also filtered off. The combined precipitates (a light grey powder) represented the title compound (1.13 g, 3.61 mmol, 79%). ^1H NMR (400 MHz, $\text{acetone}-d_6$): δ = 8.11 (t, $^3J_{\text{H,H}}$ = 7.6 Hz, 1 H, 7-H), 8.20 (d, $^3J_{\text{H,H}}$ = 9.1 Hz, 1

H), 8.22 (d, $^3J_{\text{H,H}} = 8.4$ Hz, 1 H, 2-H), 8.23 (d, $^3J_{\text{H,H}} = 9.3$ Hz, 1 H), 8.23 (d, $^3J_{\text{H,H}} = 9.1$ Hz, 1 H), 8.34 (d, $^3J_{\text{H,H}} = 7.6$ Hz, 2 H, 6-H, 8-H), 8.35 (d, $^3J_{\text{H,H}} = 8.4$ Hz, 1 H, 3-H), 8.82 (d, $^3J_{\text{H,H}} = 9.3$ Hz, 1 H), 10.78 (br. s, 1 H, NH) ppm. ^{13}C NMR (101 MHz, acetone- d_6): δ (ppm) = 122.5 (CH), 125.2 (C_q), 125.4 (CH), 125.9 (C_q), 126.0 (CH), 126.7 (CH), 126.9 (CH), 127.0 (C_q), 127.6 (CH), 128.2 (CH), 128.8 (CH), 129.1 (C_q), 129.2 (CH), 131.7 (C_q), 131.8 (C_q), 132.2 (C_q) ppm. CF_3 and CO signals were not detected due to low intensities. ^{19}F NMR (376 MHz, acetone- d_6): δ (ppm) = -75.7 (s, CF_3) ppm. $\text{C}_{18}\text{H}_{10}\text{F}_3\text{NO}$ (313.0714) HR-MS (ESI): found 314.0783 $[\text{M}+\text{H}]^+$; calcd. 314.0787.

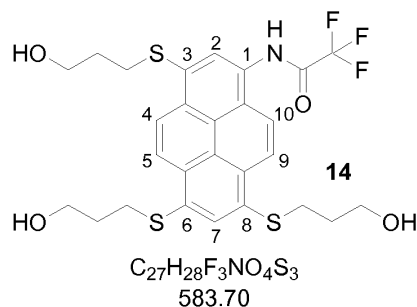
2,2,2-Trifluoro-N-[3,6,8-tris(bromo)pyren-1-yl]- acetamide
(12)



N-Trifluoroacetyl-1-aminopyrene **11** (870 mg, 2.76 mmol) was suspended in nitrobenzene (30 mL) and stirred at 50 °C for 30 min. Bromine (500 μL , 19.5 mmol, 7.07 eq.), dissolved in nitrobenzene (5 mL), was added to the solution of pyrene. The reaction mixture was stirred in a closed vessel at 80 °C for 30 min. Afterwards, the mixture was allowed to reach rt. After diluting with cyclohexane (20 mL), the precipitate was removed by filtration, washed with cyclohexane (2 x 25 mL) and dried *in vacuo*. The product was obtained as a light yellow powder (1224 mg, 2.26 mmol, 81%). ^1H NMR (400 MHz, DMSO- d_6): δ = 8.39 (d, $^3J_{\text{H,H}} = 9.7$ Hz, 1 H, 10-H), 8.47 (d, $^3J_{\text{H,H}} = 9.7$ Hz, 1 H, 9-H), 8.51 (d, $^3J_{\text{H,H}} = 9.3$ Hz, 1 H, 5-H), 8.59 (d, $^3J_{\text{H,H}} = 9.3$ Hz, 1 H, 4-H), 8.66 (s, 1 H, 2-H), 8.80 (s, 1 H, 7-H) ppm. ^{19}F NMR (376 MHz, DMSO- d_6): δ = -73.57 (s, CF_3). MS (ESI): m/z (negative mode, %) = 548 (100)

[M]⁻. HR-MS (ESI): calcd. for C₁₈H₇NOF₃⁷⁹Br₂⁸⁰Br ([M-H]⁻) 547.7937, found 547.7930.

2,2,2-Trifluoro-N-[3,6,8-tris[(3-hydroxypropyl)sulfanyl]pyrene-1-yl]-acetamide (14)

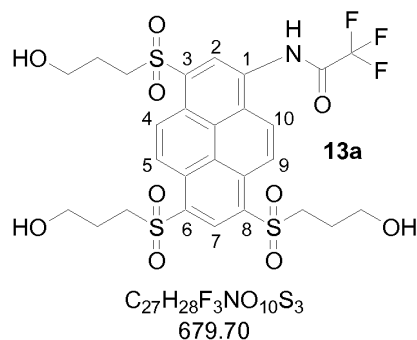


K₂CO₃ (221.2 mg, 400 μmol, 0.53 eq.) and NEt₃ (40 μL) were suspended in dry DMF (4 mL) and flushed with argon for 15 min. 3-Mercapto-1-propanol (d = 1.067 mg/mL, 272 μL, 290 mg, 3160 μmol, 4.35 eq.), brominated pyrene derivative **12** (400 mg, 757 μmol) and dry DMF (40 mL) were added, and a gentle argon stream was bubbled through the solution for 20 min. Afterwards, Pd₂(dba)₃ (197.6 mg, 216 μmol, 0.30 eq.) and Xantphos (140.4 mg, 242.8 μmol, 0.33 eq.) were added. The mixture was stirred at 85 °C under argon for 18 h. The solvents were removed *in vacuo*, the residue was dissolved in MeOH, applied to Celite® and submitted to flash chromatography (SNAP Ultra 100 g cartridge, DCM/MeOH with 2-18% MeOH-gradient over 15 CV) to provide the title compound **14** (298 mg, 511 μmol, 70%) as a pale-yellow solid. This procedure has been repeated several times on various scales and reliably gave 50-70% yields of the title product. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.80 (m, 6 H, CH₂CH₂CH₂), 3.28 (m, 6 H, SCH₂CH₂), 3.55 (m, 6 H, HOCH₂CH₂), 4.63 (m, 3 H, OH), 8.06 (d, ³J_{H,H} = 9.47 Hz, 1 H, 9-H), 8.17 (br. s, 2 H, 2-H, 7-H), 8.50 (d, ³J_{H,H} = 9.47 Hz, 1 H, 9-H), 8.53 (d, ³J_{H,H} = 9.50 Hz, 1 H, 5-H), 8.53 (d, ³J_{H,H} = 9.50 Hz, 1 H, 4-H), 11.85 (s, NH, 1H) ppm. ¹³C NMR (101 MHz, DMSO-*d*₆): δ = 29.9 (CH₂), 30.0 (CH₂), 31.7 (CH₂), 31.9 (CH₂),

32.0 (CH₂), 59.2 (CH₂), 59.2 (CH₂), 59.2 (CH₂), 116.2 (d, ¹J_{F,C} = 289.3 Hz), 121.8 (CH), 123.2 (CH), 123.7 (CH), 124.2 (CH), 124.4 (C_q), 124.6 (C_q), 124.7 (C_q), 125.5 (CH), 127.2 (CH), 127.4 (C_q), 127.6 (C_q), 128.6 (2 x C_q), 132.3 (C_q), 132.8 (C_q), 132.9 (C_q), 156.2 (d, ²J_{C,F} = 36.7 Hz) ppm.

¹⁹F NMR (376 MHz, DMSO-*d*₆): δ = -73.15 (s, CF₃) ppm. **HPLC**: *t*_R = 8.3 min (MeCN/H₂O 50:50 → 100:0 + 0.1% TFA in 25 min detected at 254 nm). **HR-MS** (ESI): Calcd. for C₂₇H₂₈NOF₃S₃ ([M-H]⁻) 582.1060, found 582.1049.

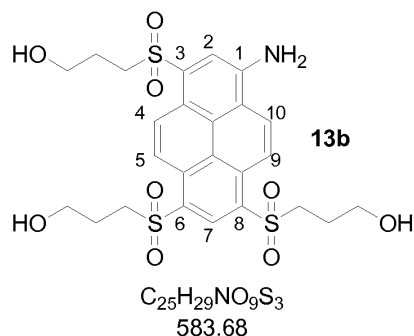
2,2,2-Trifluoro-N-[3,6,8-tris[(3-hydroxypropyl)sulfonyl]pyren-1-yl]-acetamide (13a)



Triol **14** (135 mg, 232 μmol) was suspended in a mixture of AcOH (20 mL) and H₂O (1 mL). Afterwards, sodium tungstate dihydrate (19.1 mg, 57.9 μmol, 0.25 eq.) was added, and the solution was cooled with an ice-bath until the mixture became viscous. Then aq. H₂O₂ solution (85-90%, d = 1.45, 5.00 mL, 189 mmol, 82 eq.) was added over a period of 10 min. The solution was stirred for 30 min in the ice-bath. After removing the ice-bath, the mixture was stirred for 2 at rt, until the reaction was complete (TLC). The solvents were removed by freeze-drying, and the residue was dissolved in MeCN/H₂O. *Celite*® was added and, after removing all solvents *in vacuo*, the sample was submitted to flash chromatography (SNAP Ultra 25 g cartridge, ACN/MeOH + 5% DCM with MeOH 1-10%-gradient over 20 CV). The isolated compound **13a** was obtained as a yellow solid (113 mg, 116 μmol, 50%).

^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 1.78 (m, 6 H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 3.41 (m, 6 H, HOCH_2CH_2), 3.73 (m, 6 H, SCH_2CH_2), 4.59 (m, 3 H, OH), 8.83 (d, $^3J_{\text{H,H}}$ = 9.46 Hz, 1 H, 10-H), 9.02 (s, 1 H, 2-H), 9.26 (s, 1 H, 7-H), 9.35 (m, 2 H, 4-H, 4-H), 9.47 (d, $^3J_{\text{H,H}}$ = 9.46 Hz, 1 H, 9-H), 12.35 (s, NH , 1H)ppm. ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$): δ = 25.7 (CH_2), 25.9 (CH_2), 53.2 (CH_2), 53.3 (CH_2), 53.5 (CH_2), 58.4 (CH_2), 58.4 (CH_2), 115.91 (d, $^1J_{\text{F,C}}$ = 289.0 Hz), 124.3 (CH), 124.8 (CH), 125.0, 125.3, 127.3, 127.9, 128.0, 128.1, 129.5 (CH), 131.1 (CH), 132.1 (CH), 132.2, 132.3, 134.5 (CH), 156.5 (t, $^2J_{\text{C,F}}$ = 38.2 Hz)ppm. ^{19}F NMR (376 MHz, $\text{DMSO}-d_6$): δ = -73.46 (s, CF_3)ppm. TLC (SiO_2): R_f = 0.68 (ACN:DCM:H₂O = 10:1:1). HR-MS (ESI): Calcd. for $\text{C}_{27}\text{H}_{28}\text{NO}_{10}\text{F}_3\text{S}_3$ ($[\text{M}-\text{H}]^-$) 678.0755, found 678.0756.

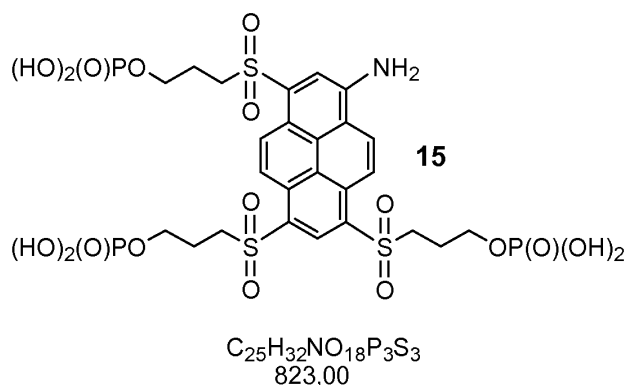
3,6,8-Tris[(3-hydroxypropyl)sulfonyl]-pyrene-1-amine (13b)



Protected pyrene derivative **13a** (20 mg, 29 μmol) was suspended in MeOH (6 mL). Diluted aq. Na_2CO_3 (750 μL of the sat. aq. Na_2CO_3 -solution mixed with 5 mL H_2O) was added, and the solution was stirred for 30 min at rt, followed by 30 min at 50 °C and 90 min at rt. Celite® and MeOH were added and the solvents were removed under reduced pressure. The crude product was purified by fcc (SNAP Ultra 10 g cartridge, DCM/MeOH with 2-20% MeOH-gradient over 12 CV), and the title product **13b** was isolated as an orange solid (14 mg, 24 μmol , 81%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ (ppm) = 1.73 (m, 6 H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 3.45 (m, 6 H, OCH_2CH_2), 3.51 (m, 6 H, SCH_2CH_2), 4.60 (m, 3 H, OH), 8.25 (s, 1 H, 2-H), 7.83 (br. s, 2 H, NH_2), 8.77 (d, $^3J_{\text{H,H}}$ = 9.56 Hz, 1 H), 8.87 (d, $^3J_{\text{H,H}}$ = 9.66

Hz, 1 H), 8.93 (d, $^3J_{\text{H,H}} = 9.66$ Hz, 1 H), 9.02 (s, 1 H), 9.15 (d, $^3J_{\text{H,H}} = 9.56$ Hz, 1 H) ppm. ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$): $\delta = 25.9, 26.0, 26.1, 52.3, 52.5, 53.4, 58.5, 58.5, 58.6, 115.8, 115.9, 116.9, 118.5, 121.0, 126.1, 126.4, 127.3, 127.9, 128.0, 128.8, 130.0, 132.8, 134.1, 136.9, 148.5$ ppm. HPLC: $t_R = 13.1$ min ($\text{MeCN}/\text{H}_2\text{O}$ 10:90 $\rightarrow$ 100:0 + 0.1% TFA in 25 min detected at 225 nm). TLC (SiO_2): $R_F = 0.38$ ($\text{DCM}:\text{MeOH} = 9:2$). HR-MS (ESI): Calcd. for $\text{C}_{25}\text{H}_{29}\text{NO}_9\text{S}_3$ ($[\text{M}-\text{H}]^-$) 582.0932, found 582.0927. UV-VIS (In MeOH): $\epsilon = 21000 \text{ M}^{-1} \text{ cm}^{-1}$, $\lambda_{\text{max}}(\text{absorption}) = 486 \text{ nm}$, $\lambda_{\text{max}}(\text{florescence}) = 534 \text{ nm}$, QY = 0.80.

Tri-O-phosphorylated 3,6,8-tris[(3-hydroxypropyl)sulfonyl]-pyrene-1-amine (15)



A solution of the deprotected pyrene derivative **13b** (2.00 mg, 3.43 μmol) in trimethylphosphate (0.2 mL) was added dropwise to freshly distilled and ice-cooled POCl_3 (0.25 mL, 2.69 mmol) under argon atmosphere. The mixture was stirred at 0 °C for 30 min and 4 h at room temperature. All volatile components were distilled *in vacuo* (0.7 mbar) into a flask cooled in a dry ice/acetone bath (first at room temperature, later heating until 60 °C) and the residue was further dried by lyophilization (0.02 mbar). An aqueous $\text{Et}_3\text{N}-\text{H}_2\text{CO}_3$ buffer (1 M, ca. 6-8 mL) was added to the

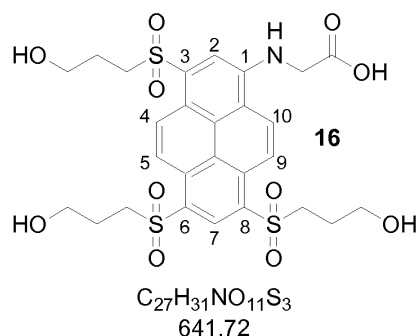
residue until the pH is adjusted to 8 (gas evolution). The sample was concentrated to a volume of ca 1 mL by freeze-drying and purified by preparative HPLC (Kinetex 5 μ m EVO C18 100A 250x21 mm column, MeCN/water + 0.05 M TEAB, with MeCN-gradient 10-30% over 20 min, peak at 9.1 min).

^1H NMR (400 MHz, CD_3OD): δ = 1.24 (t, 36 H, J = 7 Hz, 12 CH_3 , 3 Et_3N) 2.04 (m, 6 H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 3.58 – 3.72 (m, 6 H, $\text{Ar-SO}_2\text{CH}_2\text{CH}_2$), 3.07 (q, J = 7 Hz, 24 H, CH_2CH_3 , 3 Et_3N), 3.88 – 3.93 (m, 6 H, $\text{CH}_2\text{CH}_2\text{SO}_3$), 8.17 (s, 1 H), 8.73 (d, J = 9.5 Hz, 1 H), 8.73 (d, J = 9.5 Hz, 1 H), 8.86 (d, J = 9.5 Hz, 1 H), 9.18 (s, 1 H) 9.21 (d, J = 9.5 Hz, 1 H), ppm.

^{13}C NMR (101 MHz, CD_3OD): δ = 9.2 (CH_3), 21.6 (CH_2), 22.1 (CH_2), 25.8 (CH_2), 47.4 (CH_2), 54.1 (CH_2), 55.3 (CH_2), 56.5 (CH_2), 63.8 (CH_2), 71.0 (CH_2), 71.6 (CH_2), 117.8 (C), 118.3 (CH), 120.2 (CH), 122.9 (CH), 127.7 (C), 128.2 (C), 128.7 (CH), 129.3 (C), 129.8 (C), 130.2 (CH), 131.8 (CH), 134.8 (C), 136.0 (C), 138.3 (C), 149.8 (C), ppm. **^{31}P NMR** (161.9 MHz, CD_3OD): δ = 0.97 ppm (s, OP(O)(OH)_2).

HPLC: t_R = 5.48 min (MeCN/ H_2O 10:90 $\rightarrow$ 100:0 + 0.05 M TEAB in 20 min detected at 254 nm). TLC (RP-SiO₂ C18): R_f = 0.7 – 0.8 (MeCN: H_2O + 0.05 M TEAB = 9:2). MS (ESI): Calcd. for $\text{C}_{25}\text{H}_{32}\text{NO}_{18}\text{P}_3\text{S}_3$ (822.9994): $[\text{M-H}]^-$ 821.9922. UV-VIS (0.05 M aq. $\text{Et}_3\text{N}\cdot\text{H}_2\text{CO}_3$ (TEAB) buffer, pH 8 – 8.5): λ_{max} (absorption) = 203, 236, 305, 477 nm; λ_{max} (emission) = 542 nm, ϵ = 19600 $\text{M}^{-1}\text{cm}^{-1}$, $\text{QY}_{\text{fl.}}$ = 92% (absolute value in a TEAB buffer, pH 8 – 8.5, as measured by Hamamatsu apparatus C11347-12 with an integration sphere) and 74-76%, respectively, as measured with a Fluorolog-3 spectrometer with an R2658 PMT, relative to an alkaline solution of fluorescein as a standard, whose $\text{QY}_{\text{fl.}}$ = 90% in a 0.1M NaOH under excitation at 496 nm).

**N-[3,6,8-Tris[(3-hydroxypropyl)sulfonyl]pyren-1-yl]glycine
(16)**



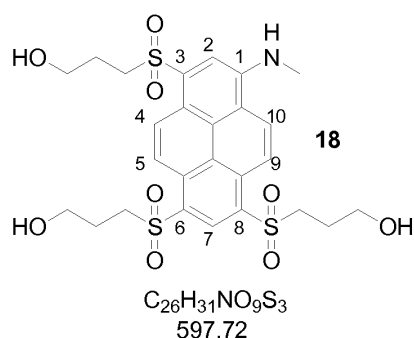
The deprotected amino pyrene **13b** (19 mg, 34 μ mol) was suspended in MeOH (11 mL). Glyoxylic acid monohydrate (12.6 mg, 136 μ mol, 4.0 eq.) was added, and the mixture was acidified by addition of conc. HCl (12 μ L, 140 μ mol, 4.3 eq.). After 1 h stirring at rt, NaBH₃CN (17 mg, 270 μ mol, 8.0 eq.) was added. After 3 h, another portion of glyoxylic acid monohydrate (11 mg, 120 μ mol, 3.5 eq.) was added. After stirring overnight, a second portion of NaBH₃CN (29 mg, 460 μ mol, 14 eq.) and conc. HCl (20 μ L, 240 μ mol, 7.1 eq.) were added. After stirring for one more day at rt, a third portion of glyoxylic acid monohydrate (91 mg, 990 μ mol, 29 eq.), NaBH₃CN (27 mg, 430 μ mol, 12.6 eq.) and conc. HCl (20 μ L, 240 μ mol, 7.1 eq.) were added, and the reaction was stirred for one night. The solvent was removed *in vacuo*, and the crude product was purified by RP-column chromatography with H₂O/ACN + 0.2% TFA with 9-25%-ACN gradient. The product was isolated as a red solid (8.3 mg, 13 μ mol, 38%).

¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.74 (m, 6 H, CH₂CH₂CH₂), 3.38 (m, 6 H, SCH₂CH₂), 3.63 (m, 6 H, HOCH₂CH₂), 4.26 (br. s, 2 H, CH₂COOH), 4.59 (m, 3 H, OH), 7.89 (br. s, 1 H, NH), 8.45 (m, 2 H, 4-H, 5-H), 8.81 (d, ³J_{H,H} = 9.46 Hz, 1 H, 10-H), 8.89 (s, 1 H, 2-H), 9.06 (s, 1 H, 7-H), 9.21 (d, ³J_{H,H} = 9.46 Hz, 1 H, 9-H) ppm. TLC (SiO₂): R_F = 0.22 (ACN:H₂O:DCM + formic acid = 20:2:1).

HPLC: *t*_R = 4.4 min (MeCN/H₂O 30:70 → 100:0 + 0.1% TFA in 25 min detected at 254 nm). HPLC: *t*_R = 9.5 min (MeCN/H₂O 10:90 → 100:0 +

0.1% TFA in 25 min detected at 465 nm). MS (ESI): m/z (negative mode, %) = 641 (100) $[M]^-$. HR-MS (ESI): Calcd. for $C_{27}H_{31}NO_{11}S_3$ ($[M+Na]^+$) 664.0951, found 664.0925. UV-VIS (In MeOH): ϵ = 18000 $M^{-1} cm^{-1}$, λ_{max} . (absorption) = 499 nm, λ_{max} . (fluorescence) = 553 nm, QY = 0.71.

3,6,8-Tris[(3-hydroxypropyl)sulfonyl]pyrene-1-(methylanine)
(18)

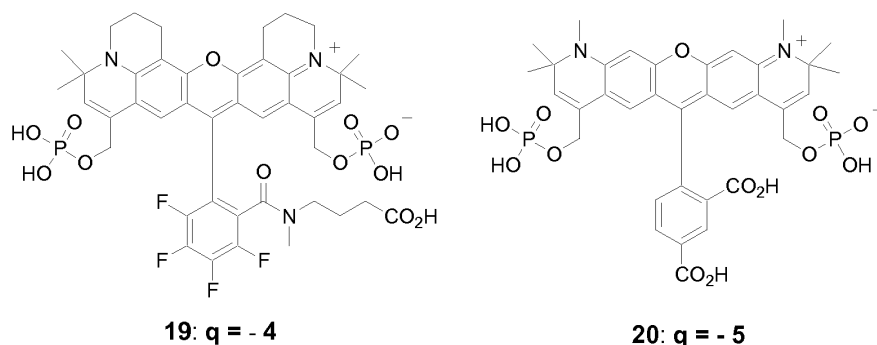


Compound **14** (75 mg, 0.13 mmol) was suspended in dry DMF (0.1 mL) under argon, Cs_2CO_3 (55 mg, 0.17 mmol) was added followed by MeI (0.15 mL). The reaction mixture was stirred for 1 h in a screw-cap tube at 50 °C. HPLC indicated that the reaction was complete. HPLC: starting material t_R = 13.7 min (MeCN/ H_2O 30:70 $\rightarrow$ 100:0 + 0.1% TFA in 25 min detected at 254 nm). HPLC: product t_R = 15.8 min (MeCN/ H_2O 30:70 $\rightarrow$ 100:0 + 0.1% TFA in 25 min detected at 254 nm). DMF was removed under vacuum, and the residue was taken up in dichloromethane - water mixture. The organic layer was separated, washed with brine, dried over sodium sulfate, filtered and evaporated in vacuo. Compound **17** (56 mg, 78%, the *N*-methylated product, see the general scheme of the synthesis for structure) was isolated by chromatography on SiO_2 (25 g) in the course of elution with 0 - 5% MeOH in ethyl acetate. To oxidize it into the sulfone, compound 17 was dissolved in acetic acid (5 mL), water was added (3 mL), followed by $Na_2WO_4 \cdot 2H_2O$ (12 mg, catalyst), and the mixture was cooled to 0 °C. Hydrogen peroxide (1.5 mL of a ca. 80% solution) was added at 0 °C. The

reaction mixture turned dark, and then the dark color disappeared. HPLC indicated complete conversion to a new substance with $t_R = 8.48$ min (MeCN/H₂O 30:70 → 100:0 + 0.1% TFA in 25 min detected at 254 nm). All volatile materials were removed *in vacuo*, and the dry residue was dissolved in aqueous acetonitrile. The cleavage of the CF₃CO group (deprotection) in the intermediate compound was performed as follows: a saturated aqueous solution of Na₂CO₃ (ca. 20 wt.-%, 1.5 mL) was added, and the solution was stirred at room temperature. The ratio acetonitrile - water has to be chosen in such a way, that the addition of 1.5 mL of saturated aqueous Na₂CO₃ solution will provide a homogeneous reaction mixture. The reaction mixture turns to be bright orange in several minutes; it was stirred overnight at room temperature. HPLC indicated complete conversion to a new substance (title compound) with $t_R = 6.4$ min (MeCN/H₂O 30:70 → 100:0 + 0.1% TFA in 25 min detected at 254 nm). Sodium carbonate was neutralized by addition of glacial AcOH, and the frozen reaction mixture was lyophilized. The solid residue was dissolved in hot aqueous acetonitrile and applied onto a column with RP-SiO₂ (C18, 50 g). Elution with acetonitrile - water mixture (1/3 - 1/2, + 1% AcOH) resulted first in a green-yellow "band" of impurity, followed by an orange zone of the title compound (**17**). Lyophilization of the orange solution afforded 48 mg (80%) of the title compound as an orange - red powder (slightly soluble in methanol and water). HPLC: $t_R = 6.0$ min (MeCN/H₂O 30:70 → 100:0 + 0.1% TFA in 20 min detected at 254 nm; Kinetex column). ¹H NMR (400 MHz, CD₃OD + DMSO-*d*₆, ref. 3.30 ppm for CHD₂OD): $\delta = 1.92$ (m, 6 H, CH₂CH₂CH₂), 3.28 (s, 3 H, MeNH), 3.57 (q, 6 H, ³J_{H,H} = 6.1 Hz, OCH₂CH₂), 3.68 (m, 6 H, SO₂CH₂CH₂), 8.12 (s, 1 H, 2-H), 8.89 (d, ³J_{H,H} = 9.6 Hz, 1 H), 8.93 (d, ³J_{H,H} = 9.6 Hz, 1 H), 9.10 (d, ³J_{H,H} = 9.6 Hz, 1 H), 9.22 (s, 1 H), 9.32 (d, ³J_{H,H} = 9.7 Hz, 1 H) ppm. HR-MS (ESI): C₂₆H₃₁NO₉S₃ (597.1116); [M-H]⁻ found 596,1078, calc. 596.1088. UV-VIS (MeOH): $\epsilon = 23400$ M⁻¹ cm⁻¹, $\lambda_{\max}$.(absorption) = 502 nm, $\lambda_{\max}$.(fluorescence)

= 550 nm, QY = 0.88. UV-VIS (H₂O): ϵ = 19500 M⁻¹ cm⁻¹, λ_{max} . (absorption) = 509 nm, λ_{max} . (fluorescence) = 563 nm, QY = 0.88.

Some additional selected fluorescent dyes with large negative net charges and favorable spectral properties (see also Scheme 12 and Table 2).



The red-emitting rhodamine dye with multiple ionizable groups of structure **20** was obtained by phosphorylation of the corresponding hydroxyl-substituted rhodamine precursor and isolated analogously to compound **19** (another phosphorylated rhodamine dye, see Schemes 6 and 11 above) previously described by K. Kolmakov, C. A. Wurm, R. Hennig, E. Rapp, S. Jakobs, V. N. Belov and S. W. Hell in *Chem. Eur. J.* **2012**, *18*, 12986-12998 (see compound 7-H therein for the properties and the phosphorylation details). The hydroxyl-substituted precursor for compound **20** was synthesized according to K. Kolmakov, C. A. Wurm, D. N. H. Meineke, F. Göttfert, V. P. Boyarskiy, V. N. Belov and S. W. Hell (*Chem. Eur. Journal*, **2013**, *20*, 146-157; see compound 14-Et therein). The phosphorylation was followed by saponification of the ethyl ester group via a routine procedure, as described.

Purity and identity of compound **20** was confirmed by the following analytical data: ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.23 (s, 6 H, CH₃), 1.28 (s, 6 H, CH₃), 2.62 (s, 6 H, NCH₃), 4.21 (m, 4 H, 2CH₂), 5.70 (s, 2 H), 6.76 (s, 2 H), 7.16 - 7.30 (br. m, 4 H),

8.55 (m, 1H), 8.36 (m, 1 H) ppm. ^{13}C NMR (101 MHz, DMSO- d_6): δ = 29.1 (CH₃), 34.2 (CH₃), 95.8 (CH₂), 118.2 (CH), 121.7 (C), 122.6 (C), 125.5 (CH), 127.3 (CH), 127.4 (CH), 128.0 (CH), 129.8 (CH), 133.9 (C), 136.8 (C), 155.0 (CO), 157.0 (CO) ppm.

^1H NMR (400 MHz, CD₃OD, **20** as a Et₃N-salt): δ = 1.12 (t, J = 7 Hz, 9H, CH₃CH₂), 1.25 (t, J = 7 Hz, 27 H, CH₃CH₂), 1.52 (s, 6 H, CH₃), 1.53 (s, 6 H, CH₃), 3.11, 3.31 (m, 24 H, CH₃CH₂), 3.18 (s, 6 H, NCH₃), 3.61 (m, 2 H, CH₂), 4.45 (m, 2 H, CH₂), 6.03 (s, 2 H), 6.8 (s, 2 H), 6.9 (s, 2 H), 7.28 (d, J = 8 Hz, 1 H), 8.16 (d, J = 8 Hz, 1 H), 8.66 (m, 1 H) ppm. ^{31}P NMR (161.9 MHz): δ = -0.2 (DMSO- d_6) and 0.63 (CD₃OD) ppm (s, OP(O)(OH)₂).

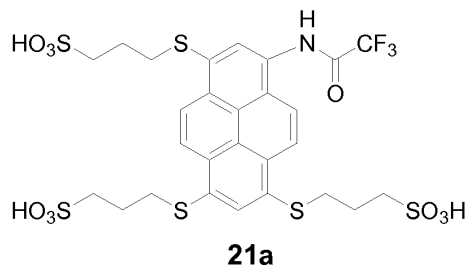
HPLC: t_R = 3.9 min (Kinetex EVO C-18 column, with 0.02 M aq. Et₃N (A) and 3% MeCN (B), isocratic flow 0.5 mL/min, detection at 254 nm). **TLC**: R_f = 0.25 (silica gel plates, MeCN/H₂O 5:1 + 0.2% Et₃N).

HR-MS (ESI): calc. for C₃₅H₃₅N₂O₁₃P₂⁻ ([M-H]⁻) 753.1614, found 753.1672. A red-violet highly water-soluble dye with a very intense red fluorescence: **UV-VIS** (PBS buffer, pH = 7.4) $\lambda_{\text{max. abs.}}$ = 582 nm, $\lambda_{\text{max. fl.}}$ = 609 nm, ϵ = 120000 M⁻¹ cm⁻¹, QY fl. = 0.76% (abs. value in TEAB buffer, excitation at 540 nm).

3,6,8-Tris[(3-sulfopropyl)sulfonyl]-pyrene-1-amine (23a)

The synthesis of a sulfoalkylsulfonyl-substituted 1-aminopyrene derivative **23a** and of its homolog **23b** involved halogen exchange in the tribromo-substituted precursor **12** (see Scheme 11 above) with synthon containing SH and SO₃H functions and was followed by oxidation to the sulfone.

2,2,2-Trifluoro-*N*-[3,6,8-tris[(3-sulfopropyl) sulfanyl]pyrene-1-yl]-acetamide (21a)



2,2,2-trifluoro-*N*-[3,6,8-tris(bromo)pyren-1-yl]-acetamide (tribromo-substituted precursor **12**, 180 mg, 0,33 mmol; for preparation and properties see above) and sodium 3-mercapto-1-propanesulfonate (600 mg, 3,37 mmol, 10 equiv) was reacted in dry DMF (20 mL) at 90°C upon stirring under an argon atmosphere in presence of triethylamine (0,6 mL) as a base with Pd₂(dba)₃ (20 mg, 0,02 mmol) and xantphos reagent (30 mg, 0.05 mmol) as catalysts. As the reaction completed (3-5 h), the crude product (Na salt of **21a**) was first purified over reversed-phase silica gel (C-18) and H₂O - MeCN (gradient 0-70%) as mobile phase. The homogeneous fractions were combined, filtered (syringe filters 0.45 μm), concentrated and freeze-dried. The additional purification of the crude product (340 mg, containing inorganic salts) was performed by repeated preparative HPLC using Kinetex Gemini NX C-18 solid phase (5 μm) with 50 mmol TEAB buffer (A) and MeCN (B) under gradient (0-20%B) conditions. The pure fractions were combined, concentrated at t ≤ 40°C and freeze-dried to furnish 70-80% of the yellow crystalline water-soluble solid as a triethylammonium salt of compound **21a**.

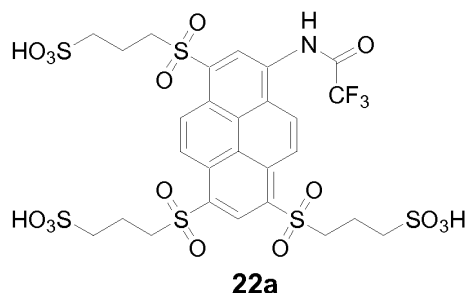
Analytical data: ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.08 (m, 27H, CH₃CH₂) 1.98 (m, 6 H, CH₂CH₂CH₂), 2.66 (m, 6 H, SCH₂CH₂), 2,88 (m, 18H, CH₂CH₃), 3.39 (m, 6 H, O₃SCH₂CH₂), 8.13 (d, *J* = 9.5 Hz, 1 H), 8.15 (d, *J* = 9.5 Hz, 1 H), 8.26 (br. s, 1 H), 8.45 (d, *J* = 9.5 Hz, 1 H), 8.48 (d, *J* = 9.5 Hz, 1 H), 8.52 (s, 2 H) ppm. ¹H NMR

(400 MHz, CD₃OD): δ = 1.21 (t, J = 7 Hz, 27H, CH₃CH₂) 2.15 (m, 6 H, CH₂CH₂CH₂), 2.99 (m, 6 H, SCH₂CH₂), 3.10 (q, J = 7 Hz, 18H, CH₂CH₃), 3.38 (m, 6 H, O₃SCH₂CH₂), 8.06 (d, J = 9.5 Hz, 1 H), 8.22 (s, 1 H), 8.31 (s, 1 H), 8.43 (d, J = 9.5 Hz, 1 H), 8.51 (br. s, 1 H, NH), 8.63 – 8.70 (m, 2 H) ppm.

¹³C NMR (101 MHz, DMSO-*d*₆): δ = 9.4 (CH₃), 22.5 (CH₂), 25.0 (CH₂), 25.1 (CH₂), 32.3 (CH₂), 32.4 (CH₂), 45.7 (CH₂), 50.0 (CH₂), 115.3 (CH), 118.2 (CH), 122.3 (CH), 123.1 (CH), 123.4 (CH), 124.3 (C), 124.8 (CH), 124.9 (C), 125.5 (CH), 127.2 (CH), 127.3 (C), 127.7 (C), 127.9 (C), 132.0 (C), 132.1 (C), 156.3 (C), 156.6 (C), 171.0 (CO) ppm. **¹⁹F NMR** (376 MHz, DMSO-*d*₆): δ = -73.20 (s, CF₃) ppm.

HPLC: t_R = 8.2 min (Kinetex column EVO C-18 100, 5 μ m, 4.6 x 250 mm), 0.05 M aq. TEAB (A) and 25% MeCN (B), isocratic flow 0.5 mL/min, detection at 254 nm). **TLC**: R_f = 0.6 (silica gel plates, MeCN/H₂O 7:1 + 0.2% Et₃N, also seen by its intense blue fluorescence under illumination with UV-light at 365 nm). **HR-MS** (ESI): calc. for C₂₇H₂₇F₃NO₁₀S₆⁻ ([M-H]⁻) 773.9911, found 773.9845. **UV-VIS** (PBS buffer, pH = 7.4) λ_{max} . (absorption) = 428 nm. The synthesis is based on the general recipe for palladium-catalyzed cross-coupling of thiols and aryl bromides described by C. Mispeleare-Canivet and co-workers, *Tetrahedron* **2005**, 61, 5253-5259.

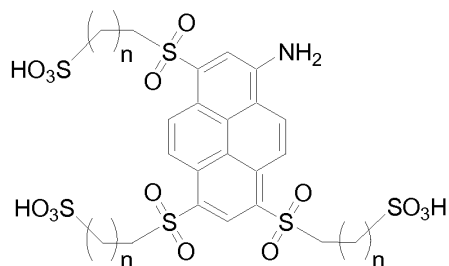
2,2,2-Trifluoro-*N*-[3,6,8-tris[(3-sulfopropyl)sulfonyl]pyrene-1-yl]-acetamide (22a)



The thioether **21a** (as a triethylammonium salt) was oxidized to the sulfone as follows: 50 mg (0,046 mmol, as a Et₃N salt with M = 1077) of the substrate was dissolved in a mixture of HOAc (4 mL) and water (0,4 mL). Upon cooling to +5°C 1 ml of 50 wt.% H₂O₂ and 0,5 mL of 1 wt.% aq. solution of Na₂WO₄ x 2H₂O (a catalyst) was added. The reaction mixture was kept for 30 min at this temperature, left overnight at RT, diluted with water (20 mL) and freeze-dried. The product was purified by means of preparative HPLC using Kinetex Gemini NX C-18 solid phase (5 µm) with 0,5 vol. % aqueous HOAc (A) and MeCN (B) under gradient (0-50%B) conditions. The pure fractions were pooled, concentrated at t ≤ 40°C and freeze-dried to furnish 28 mg (52%) of a red crystalline solid as a triethylammonium salt (M.W. = 1173), which has an intense yellow-orange fluorescence in water.

Analytical data for compound **22a**: ¹H NMR (400 MHz, CD₃OD): δ = 1.27 (m, 27H, CH₃CH₂) 2.24 (m, 6 H, CH₂CH₂CH₂), 2.97 (m, 6 H, SO₂CH₂CH₂), 3,19 (m, 18H, CH₂CH₃), 3.84 (m, 6 H, CH₂CH₂SO₃), 8.73 (d, J = 9 Hz, 1 H), 9.03 (s, 1 H), 9.39 - 9.41 (m, 2 H), 9.02 (s, 1 H), 9.52 (d, J = 9 Hz, 1 H) ppm. ¹³C NMR (101 MHz, CD₃OD): δ = 8.9 (CH₃), 20.1 (CH₂), 47.9 (CH₂), 50.2 (CH₂), 55.7 (CH₂), 56.2 (CH₂), 56.6 (CH₂), 126.4 (C), 126.6 (CH), 127.0 (C), 128,0 (CH), 129,6 (CH), 130,0 (C), 131.7 (C), 133.5 (C), 134.1 (C), 134.4 (C), 134.9 (C), 136.4 (C), 158.8 (C=O), ppm. ¹⁹F NMR (376 MHz, CD₃OD): δ = -76.50 (s, CF₃) ppm.

HPLC: t_R = 5.2 min (Kinetex column EVO C-18 100, with 0,05 M aq. TEAB (A) and 25% MeCN (B), isocratic flow 0.5 mL/min, detection at 254 nm). **TLC**: R_f = 0,5 (silica gel plates, MeCN/H₂O 7:1 + 0,2% Et₃N, seen by its intense orange fluorescence under illumination with UV-light at 365 nm). **HR-MS** (ESI): calc. for C₂₇H₂₇F₃NO₁₆S₆⁻ ([M-H]⁻) 869.9606, found 869.9536. **UV-VIS** (PBS buffer, pH = 7.4) λ_{max}. abs. = 476 nm.

3,6,8-Tris[(3-sulfopropyl)sulfonyl]-pyrene-1-amine (23a)**23a** (n = 2)**23b** (n = 1)

The protective COCF_3 group in compound **22a** was cleaved by a conventional method under basic conditions at RT (see T. W Greene, P. G. M. Wuts "Protective groups in organic synthesis" Third Edition 1999, p. 557). The reaction progress was monitored by TLC, the reaction mixture neutralized to pH 7 - 8 with HOAc and concentrated at $t \leq 40^\circ\text{C}$. The residue was subjected to preparative HPLC using Gemini NX C-18 solid phase (5 μm) with 0,3 vol. % aqueous Et_3N (A) and 10% MeCN (B) under isocratic mode. The pure fractions were concentrated and freeze-dried to furnish 22 mg (79%) of compound **23a** as a triethylammonium salt. Properties: red crystalline solid, well-soluble in water to give solutions with a very intense yellow-greenish fluorescence.

Analytical data: ^1H NMR (400 MHz, CD_3OD): δ = 1.27 (t, 27 H, J = 7 Hz, CH_3CH_2 , 3 Et_3N), 2.13 - 2.29 (m, 6 H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 2.88 - 3.17 (m, 6 H, Ar- $\text{SO}_2\text{CH}_2\text{CH}_2$), 3,16 (q, J = 7 Hz, 18 H, CH_2CH_3 , 3 Et_3N), 3.69 - 3.84 (m, 6 H, $\text{CH}_2\text{CH}_2\text{SO}_3$), 8.13 (s, 1 H), 8.66 (d, J = 9 Hz, 1 H), 8.83 (d, J = 9 Hz, 1 H), 8.93 (d, J = 9 Hz, 1 H), 9.17 (d, J = 9 Hz, 1 H), 9.20 (s, 1 H) ppm. ^{13}C NMR (101 MHz, CD_3OD): δ = 9.2 (CH_3), 20.0 (CH_2), 20.1 (CH_2), 20.2 (CH_2), 47.9 (CH_2), 50.4 (CH_2), 55.4 (CH_2), 55.7 (CH_2), 56.5 (CH_2), 60.1 (CH_2), 117.7 (C), 118.3 (CH), 120,3 (CH), 123,0 (CH), 127,7 (C), 128.1 (C), 128.6 (CH), 129.4 (C), 129.9 (C), 130.2 (CH), 131.7 (CH), 134.9 (C), 136.0 (C), 138.1 (C), 149.6 (C), ppm. HPLC: t_R = 4.7 min (Kinetex column EVO C-18 100, with 0,05 M aq. TEAB (A) and 25%

MeCN (B), isocratic, 0.5 mL/min, detection at 254 nm). **TLC:** R_f = 0,4 (silica gel plates, MeCN/H₂O 7:1 + 0,2% Et₃N, seen by its intense green fluorescence under illumination with UV-light at 365 nm). **HR-MS** (ESI) negative mode: calc. for C₂₅H₂₈NO₁₅S₆⁻ ([M-H]⁻) 774.9873, found 774.9804; positive mode: calc. for C₂₅H₃₃N₂O₁₅S₆⁺ ([M+NH₄]⁺) 793,0205, found 793.0146. **UV-VIS** (TEAB buffer, pH = 8.5): ϵ = 21 000 M⁻¹ cm⁻¹, $\lambda_{\max}$. abs. = 486 nm, $\lambda_{\max}$. fl. = 542 nm., Φ_{fl} = 86% (in TEAB buffer, relative to fluorescein as a reference dye with Φ_{fl} = 0.9 in 0.1 M NaOH under excitation at 496 nm).

Using the commercially available sodium 2-mercaptoethanesulfonate as a thiol-containing reagent with a shorter alkyl chain, the dye **23b** (a homolog of dye **23a** depicted above, which also belongs to the general Formula C with each X = SO₃H and n = 1) was obtained in the exactly same fashion and with a high yield from bromide **12**. Its spectral and photophysical properties (ϵ , $\lambda_{\max}$ and Φ_{fl} , see also Table 2) proved to be fully identical to those of the dye **23a**.

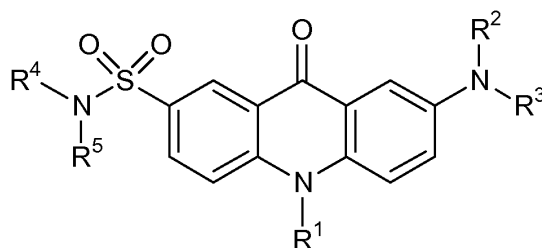
EXAMPLE 3

Reductive amination of glycans

For reductive amination of glycans using the compounds of the present invention, the prior art protocol for fluorescent labeling of N-glycans with 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) and a borane-based reducing agent (Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB, *Analytical Biochemistry* **1995**, 230, 229-238; Ruhaak LR, Hennig R, Huhn C, Borowiak M, Dolhain RJEM, Deelder AM, Rapp E, Wuhrer M, *Journal of Proteome Research* **2010**, 9, 6655-6664) or a modified version thereof may be followed.

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 A and B or of salts thereof:

**Formula A**

wherein

R^1 , R^2 , R^3 , R^4 , R^5 are independent from each other and may represent:

H, CH_3 , C_2H_5 , a straight or branched C_3 - C_{12} , preferably C_3 - C_6 , alkyl or perfluoroalkyl group, a phosphonylated alkyl group $(CH_2)_mP(O)(OH)_2$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain, $(CH_2)_nCOOH$, where $n = 1-12$, preferably 1-5, or $(CH_2)_nCOOR^6$, where $n = 1-12$, preferably 1-5, and R^6 may be 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 or other potentially nucleophile-reactive leaving groups, alkyl sulfonate $((CH_2)_nSO_3H)$ or alkyl sulfate $((CH_2)_nOSO_3H)$ where $n = 1-12$, preferably 1-5, and the alkyl chain in any $(CH_2)_n$ may be straight or branched;

a hydroxyalkyl group $(CH_2)_mOH$ or thioalkyl group $(CH_2)_mSH$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain, a phosphorylated hydroxyalkyl group $(CH_2)_mOP(O)(OH)_2$, 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 $(CH_2)_mOCOOR^7$ or $(CH_2)_mNHCOOR^7$, where $m = 1-12$ and $R^7 =$ methyl, ethyl, tert-butyl, benzyl, fluoren-9-yl, CH_2CN , *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, phenyl, substituted phenyl group, e.g., 2- or 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, 4-pyridyl, pyrimid-4-yl;

$(CH_2)_mNR^aR^b$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain; R^a , R^b are independent from each other and represent hydrogen and/or C_1-C_4 alkyl groups, a hydroxyalkyl group $(CH_2)_mOH$, where $m = 2-6$, with a straight or branched alkyl chain, a phosphorylated hydroxyalkyl group $(CH_2)_mOP(O)(OH)_2$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain;

an alkyl azide $(CH_2)_mN_3$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain;

R^1 , R^2 , R^3 , R^4 , R^5 may contain a terminal alkyloxyamino group $(CH_2)_mONH_2$, where $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain, that can include one or multiple alkylamino $(CH_2)_mNH$ or alkylamido $(CH_2)_mCONH$ groups in all possible combinations with $m = 0-12$;

$(CH_2)_nCONHR^8$, with $n = 1-12$, preferably 1-5; $R^8 = H$, C_1-C_6 alkyl, $(CH_2)_mN_3$, or $(CH_2)_m-N$ -maleimido, $(CH_2)_m-NH-COCH_2X$ ($X = Br$ or I), with $m = 1-12$, preferably 2-6, and with straight or branched alkyl chains in $(CH_2)_n$, $(CH_2)_m$ and R^8 ;

a primary amino group, preferably as R^1 , R^2 , or R^3 , which forms aryl hydrazines;

a hydroxy group, preferably as R^2 or R^3 , which forms aryl hydroxylamines;

an alkyloxyamino group $(CH_2)_nONH_2$ with $n = 1-12$;

further, one of the residues R^1 , R^2 , R^3 , R^4 , R^5 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

C₅H₃N being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl;

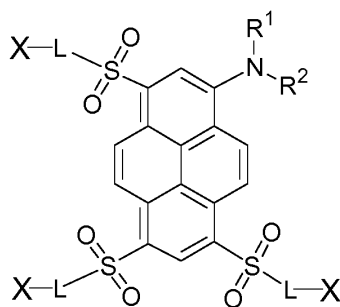
additionally, R²---R³ (R⁴---R⁵) may form a four-, five, six-, or seven-membered cycle, or a four-, five, six-, or seven-membered cycle with or without a primary amino group NH₂, secondary amino group NHR^a, where R^a = C₁-C₆ alkyl, a hydroxyl group OH, or a phosphorylated hydroxyl group -OP(O)(OH)₂ attached to one of the carbon atoms in this cycle;

optionally R²---R³ (R⁴---R⁵) may form a four-, five, six-, or seven-membered heterocycle with an additional 1-3 heteroatoms such as O, N or S included into this heterocycle;

further, R¹ may represent an unsubstituted phenyl group, a phenyl group with one or several electron-donor substituents chosen from the set of OH, SH, NH₂, NHR^a, NR^aR^b, R^aO, R^aS, where R^a and R^b are independent from each other and may be C₁-C₆ alkyl groups with straight or branched carbon chains, a phenyl group with one or several electron-acceptors chosen from the set of NO₂, CN, COH, COOH, CH=CHCN, CH=C(CN)₂, SO₂R^a, COR^a, COOR^a, CH=CHCOR^a, CH=CHCOOR^a, CONHR^a, SO₂NR^aR^b, CONR^aR^b, where R^a and R^b are independent from each other and may be H, or C₁-C₆ alkyl group(s) with straight or branched carbon chains;

or R¹ may represent a heteroaromatic group;

with the proviso that in all compounds of Formula A above at least two, preferably at least 3, 4, 5 or 6 negatively charged groups are present 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, a sulfonic acid residue SO₃H, a primary phosphate group OP(O)(OH)₂, a secondary phosphate group OP(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl, a primary phosphonate group P(O)(OH)₂, a secondary phosphonate group P(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl;

**Formula B**

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

H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3 , C_2H_5 , a linear or branched $\text{C}_3\text{-C}_{12}$ alkyl or perfluoroalkyl group, or a substituted $\text{C}_2\text{-C}_{12}$ alkyl group; in particular, $(\text{CH}_2)_n\text{COOR}^3$, where $n = 1\text{-}12$, preferably 1-5, R^3 may be H, alkyl, in particular $\text{C}_1\text{-C}_6$, 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, or other potentially nucleophile-reactive leaving groups, and the alkyl chain in $(\text{CH}_2)_n$ may be straight or branched; and

$R^1\text{---}R^2$ 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 $R^a = \text{C}_1\text{-C}_6$ alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally $R^1\text{---}R^2$ 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 $(\text{CH}_2)_m\text{OH}$, where $m = 1\text{-}12$, 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 $(\text{CH}_2)_m\text{OCOOR}^4$ or $(\text{CH}_2)_m\text{NHCOOR}^4$, where $m = 1\text{-}12$ and $R^4 = \text{methyl}$,

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;

$(\text{CH}_2)_m\text{NR}^a\text{R}^b$, 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 $\text{C}_1\text{-C}_4$ alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(\text{CH}_2)_m\text{N}_3$ with $m = 2-6$ and a straight or branched alkyl chain; one of R^1 or R^2 may be $(\text{CH}_2)_n\text{SO}_2\text{NR}^5\text{NH}_2$ with $n = 1-12$, while the substituent R^5 can be represented by H, alkyl, hydroxyalkyl or perfluoroalkyl groups $\text{C}_1\text{-C}_{12}$;

one of R^1 or R^2 groups may be a primary amino group to form aryl hydrazines $\text{Ar-NR}^6\text{NH}_2$ where Ar is the entire pyrene residue in Formula B and $\text{R}^6 = \text{H}$ or alkyl;

one of R^1 or R^2 groups may be a hydroxy group to form aryl hydroxylamines $\text{Ar-NR}^7\text{OH}$ where Ar is the entire pyrene residue in Formula B and $\text{R}^7 = \text{H}$ or alkyl;

one of R^1 or R^2 groups may be an alkyloxyamino group $(\text{CH}_2)_n\text{ONH}_2$ with $n = 1-12$;

one of R^1 or R^2 groups may be $\text{CO}(\text{CH}_2)_n\text{COOR}^8$, with $n = 1-5$ and a straight or branched alkyl chain $(\text{CH}_2)_n$ and with R^8 selected from H, straight or branched $\text{C}_1\text{-C}_6$ 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 $(\text{CH}_2)_n\text{CONHR}^9$, with $n = 1-5$ and $\text{R}^9 = \text{H}$, $\text{C}_1\text{-C}_6$ alkyl, $(\text{CH}_2)_m\text{N}_3$, $(\text{CH}_2)_m\text{-N-maleimido}$, $(\text{CH}_2)_m\text{-NHCOCH}_2\text{X}$ ($\text{X} = \text{Br}$ or I), where $m = 2-6$ and with straight or branched alkyl chains in $(\text{CH}_2)_n$ and R^9 ;

or one of R^1 or R^2 may represent $\text{CH}_2\text{-C}_6\text{H}_4\text{-NH}_2$, $\text{COC}_6\text{H}_4\text{-NH}_2$, $\text{CONHC}_6\text{H}_4\text{-NH}_2$ or $\text{CSNHC}_6\text{H}_4\text{-NH}_2$ with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, $\text{COC}_5\text{H}_3\text{N-NH}_2$ or $\text{CH}_2\text{-C}_5\text{H}_3\text{N-NH}_2$ with $\text{C}_5\text{H}_3\text{N}$ 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;

The linker L may be divalent or polyvalent and comprises at least one carbon atom and may comprise alkyl, heteroalkyl, perfluoroalkyl, alkyloxy, such as CH_2OCH_2 , CH_2CH_2O $CH_2CH_2OCH_2$, alkylamino or dialkylamino, particularly diethanolamine or *N*-methyl (alkyl) monoethanolamine moieties such as $N(CH_3)CH_2CH_2O-$ and $N(CH_2CH_2O-)_2$, one or multiple amide, 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} ;

The linker L may also include a carbonyl (CH_2CO , CF_2CO) moiety, also as a part of an amide group;

The linker L may also comprise or contain 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, pipercolines, 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;

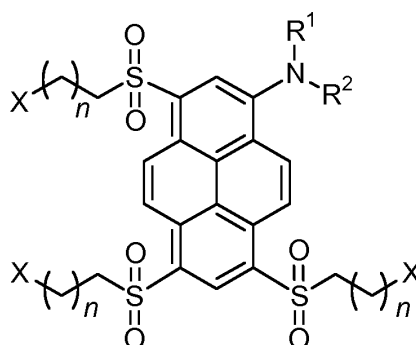
with the proviso that in all compounds represented by Formula B three or six negatively charged groups are present in the residues X of Formula B under basic conditions, i.e. $7 < pH < 14$, and these negatively charged groups represent at least

partially deprotonated residues of ionizable groups selected from the following: SH, COOH, SO₃H, OSO₃H, OP(O)(OH)₂, OP(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl, P(O)(OH)₂, P(O)(OH)R^a, where R^a = C₁-C₄ alkyl or substituted C₁-C₄ alkyl.

2. A fluorescent dye according to claim 1, wherein R¹ in Formula A represents an unsubstituted phenyl group, a phenyl group with one or several electron-donor substituents chosen from the set of OH, SH, NH₂, NHR^a, NR^aR^b, R^aO, R^aS, where R^a and R^b are independent from each other and may be C₁-C₆ alkyl groups with straight or branched carbon chains, a phenyl group with one or several electron-acceptors chosen from the set of NO₂, CN, CF₃, COH, COOH, CH=CHCN, CH=C(CN)₂, SO₂R^a, SO₃R, COR^a, COOR^a, CH=CHCOR^a, CH=CHCOOR^a, CONHR^a, SO₂NR^aR^b, CONR^aR^b, P(O)(OR^a)(OR^b) where R^a and R^b are independent from each other and may be H, or C₁-C₆ alkyl group(s) with straight or branched carbon chains; or wherein R¹ represents an aromatic heterocyclic group, in particular, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl, pyrimidin-4-yl, pyrimidin-2-yl, pyrimidin-5-yl, or other electron acceptor groups derived from aromatic heterocycles, such as 4-pyridyl-N-oxides, N-alkylpyridinium salts, or betaines, in particular, N-(ω-sulfoalkyl)-4-pyridinium, N-(ω-sulfoalkyl)-2-pyridinium, N-(1-hydroxy-4,4,5,5-tetrafluoro-cyclopent-1-en-3-on-2-yl)-4-pyridinium, N-(1-hydroxy-4,4,5,5-tetrafluorocyclopent-1-en-3-on-2-yl)-2-pyridinium.

3. A fluorescent dye according to claim 1 having Formula B wherein X at each occurrence may represent 1 to 4 groups SO₃H attached to the respective linker group L at one or more sites.

4. A fluorescent dye according to claim 1 having Formula C and at least one alkyl sulfonyl group

**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 deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. CH_3 , C_2H_5 , a straight or branched $\text{C}_3\text{-C}_{12}$, preferably $\text{C}_3\text{-C}_6$, alkyl group, or a substituted $\text{C}_2\text{-C}_{12}$, preferably $\text{C}_2\text{-C}_6$, alkyl group; in particular, $(\text{CH}_2)_n\text{COOR}^3$, 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 $(\text{CH}_2)_n$ may be straight or branched; and

$R^1\text{---}R^2$ 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 $R^a = \text{C}_1\text{-C}_6$ alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; optionally $R^1\text{---}R^2$ 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 $(\text{CH}_2)_m\text{OH}$, 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 $(\text{CH}_2)_m\text{OCOOR}^4$ or

$(\text{CH}_2)_m\text{NHCOOR}^4$, where $m = 1-12$ and $\text{R}^4 =$ 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;

$(\text{CH}_2)_m\text{NR}^a\text{R}^b$, 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 $\text{C}_1\text{-C}_4$ alkyl group(s), in particular, one of R^1 or R^2 groups may be an alkyl azide group $(\text{CH}_2)_m\text{N}_3$ with $m = 2-6$ and a straight or branched alkyl chain; one of R^1 or R^2 groups may be $(\text{CH}_2)_n\text{COOR}^5$, with $n = 1-5$ and a straight or branched alkyl chain $(\text{CH}_2)_n$ and with R^5 selected from H, straight or branched $\text{C}_1\text{-C}_6$ 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 $(\text{CH}_2)_n\text{ONH}_2$ with $n = 1-12$;

further, one of R^1 or R^2 may be $(\text{CH}_2)_n\text{CONHR}^6$, with $n = 1-12$, preferably 1-5, and $\text{R}^6 =$ H, $\text{C}_1\text{-C}_6$ alkyl, $(\text{CH}_2)_m\text{N}_3$, $(\text{CH}_2)_m\text{-N-maleimido}$, $(\text{CH}_2)_m\text{-NHCOCH}_2\text{X}$ ($\text{X} = \text{Br}$ or I), where $m = 2-6$ and with straight or branched alkyl chains in $(\text{CH}_2)_n$ and R^6 ; or one of R^1 or R^2 may represent $\text{CH}_2\text{-C}_6\text{H}_4\text{-NH}_2$, $\text{COC}_6\text{H}_4\text{-NH}_2$, $\text{CONHC}_6\text{H}_4\text{-NH}_2$ or $\text{CSNHC}_6\text{H}_4\text{-NH}_2$ with C_6H_4 being a 1,2-, 1,3- or 1,4-phenylene, $\text{COC}_5\text{H}_3\text{N-NH}_2$ or $\text{CH}_2\text{-C}_5\text{H}_3\text{N-NH}_2$ with $\text{C}_5\text{H}_3\text{N}$ being pyridin-2,4-diyl, pyridin-2,5-diyl, pyridin-2,6-diyl, or pyridin-3,5-diyl;

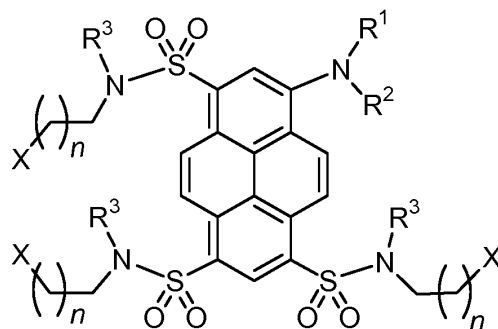
the $(\text{CH}_2)_n\text{-CH}_2$ linker, with $n = 1-12$, preferably 1-5, between the SO_2 fragment and the residue X in Formula B may represent a straight-chain, branched or cyclic group having 2-6 carbon atoms;

$X = \text{SH}, \text{COOH}, \text{SO}_3\text{H}, \text{OSO}_3\text{H}, \text{OP}(\text{O})(\text{OH})_2, \text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ optionally substituted $\text{C}_1\text{-C}_4$ alkyl, $\text{P}(\text{O})(\text{OH})_2, \text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a =$ optionally substituted $\text{C}_1\text{-C}_4$ 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. $7 < \text{pH} < 14$, and these negatively charged groups represent at least partially deprotonated residues of ionizable groups selected from the following: $\text{SH}, \text{COOH}, \text{SO}_3\text{H}, \text{OSO}_3\text{H}, \text{OP}(\text{O})(\text{OH})_2, \text{OP}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl, $\text{P}(\text{O})(\text{OH})_2, \text{P}(\text{O})(\text{OH})\text{R}^a$, where $\text{R}^a = \text{C}_1\text{-C}_4$ alkyl or substituted $\text{C}_1\text{-C}_4$ alkyl.

5. 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.

6. A fluorescent dye according to claim 1 having the following Formula D and at least one sulfonamide group



Formula D

or a salt thereof,

wherein

R^1 and/or R^2 are independent from each other and may represent H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-substituted alkyl with 1-12 C atoms, preferably 1-6 C atoms, e.g. $\text{CH}_3, \text{C}_2\text{H}_5$,

or a straight or branched, optionally substituted, C_3-C_{12} , preferably C_3-C_6 , alkyl group; in particular, $(CH_2)_nCOOR^4$, where $n = 1-12$, preferably 1-5,

R^4 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 = optionally substituted C_1-C_6 alkyl, or hydroxyl group OH attached to one of the carbon atoms in this cycle; or optionally R^1 --- R^2 may form a four-, five, six-, or seven-membered non-aromatic heterocycle with a heteroatom such as O, N or S included into this heterocycle;

R^1 and/or R^2 may further represent:

a hydroxyalkyl group $(CH_2)_mOH$, where $m = 1-12$, preferably 2-6, with a straight or branched, optionally substituted alkyl chain; one of R^1 or R^2 groups may be a carbonate or carbamate derivative $(CH_2)_mOCOOR^5$ or $(CH_2)_mNHCOOR^5$, where $m = 1-12$ and R^5 = methyl, ethyl, 2-chloroethyl, CH_2CN , *N*-succinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl, a phenyl group or substituted phenyl group, such as 2- and 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 2,3,5,6-tetrafluorophenyl, 2-pyridyl, 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 represent hydrogen and/or optionally substituted C_1-C_4 alkyl groups;

$(CH_2)_mN_3$, $m = 1-12$, preferably 2-6, with a straight or branched alkyl chain;

$CH_2)_nCONHR^6$, where $n = 1-12$, preferably 1-5 and R^6 = H, substituted or unsubstituted C_1-C_6 alkyl, $(CH_2)_mN_3$, $(CH_2)_m-N$ -maleimido, $(CH_2)_m-NHCOCH_2Y$ ($Y = Br, I$) where $m = 2-6$, with straight or branched alkyl chains in $(CH_2)_n$ and R^6 ;

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, 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;

R^3 may represent H, $(CH_2)_qCH_2X$, C_2H_5 , a straight or branched C_3-C_6 alkyl group, $C_mH_{2m}OR^7$, where $m = 2-6$, with a straight or branched alkan-diyl chain C_mH_{2m} , and $R^7 = H$, CH_3 , C_2H_5 , C_3H_7 , $CH_3(CH_2CH_2O)_kCH_2CH_2$; with $k = 1-12$; while the $(CH_2)_qCH_2$ linker may represent a straight-chain, branched or cyclic group having 2-6 carbon atoms;

in Formula D, the $(CH_2)_n-CH_2$ linker, with $n = 1-12$, preferably 1-5, between the sulfonamide fragment SO_2N and the residue X 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 $R^a =$ substituted or unsubstituted C_1-C_4 alkyl, $P(O)(OH)_2$, $P(O)(OH)R^a$, where $R^a =$ substituted or unsubstituted C_1-C_4 alkyl;

with the proviso that in all compounds represented by Formula D three, six, nine or twelve negatively charged groups are present in the residues X of Formula C 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.

7. A fluorescent dye according to claims 1-6 wherein R^1 and/or R^2 in Formula B, C or D represents H, deuterium, alkyl or deuterio-substituted alkyl, wherein one, several or all H atoms in the alkyl group may be replaced by deuterium atoms, in particular alkyl or deuterio-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 heterocycle 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.

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

9. A fluorescent dye according to claim 1 or 8, wherein the substituents R^1 , R^2 and R^3 in Formula A represent one or more carbonyl-reactive aryl hydrazine moieties, e.g. $R^2 = H$, R^1 or $R^3 = NH_2$ or $R^1 = NH_2$, $R^2, R^3 =$ alkyl, perfluoroalkyl or alkyl, and which are further conjugated or substituted with solubilizing and/or

anion-providing moieties, listed as possible candidates for R^4 and R^5 , particularly: hydroxyalkyl $(CH_2)_nOH$, thioalkyl $((CH_2)_nSH)$, carboxyalkyl $((CH_2)_nCO_2H)$, 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 or 1 to 12;

or, alternatively, the hydrazine derivatives might be represented by sulfonyl hydrazide moieties, where $R^4 = NH_2$, while R^5 are alkyl, perfluoroalkyl or alkyl groups conjugated or substituted with solubilizing and/or anion-providing groups of the types mentioned above.

10. The fluorescent dye according to claim 1, wherein the aryl amino groups NR^1 and NR^2R^3 in Formula A are connected to an analyte-reactive group, in particular selected from the group comprising an aromatic or heterocyclic amine, carboxylic acid, ester of the carboxylic acid, in particular *N*-hydroxysuccinimidyl, sulfo-*N*-succinimidyl, 1-oxybenzotriazolyl or another amino reactive ester, alkyl azide, alkyne, $(CH_2)_nONH_2$, maleimido, $COCH_2X$ with $X = Cl, Br$ and I , $NRCOCH_2X$, with $R = H, C_1-C_6$ -alkyl, $X = Cl, Br, I$, either directly or indirectly via a (poly)methylene, carbonyl, amido, nitrogen, oxygen or sulfur-containing linker, particularly $(CH_2)_mCON(R^9)$, $CO(O)NR'$, $CO(CH_2)_mN(R^9)$, $CO(CH_2)_mS(CH_2)_n$, $(CH_2)_mS(CH_2)_nCO$, $CO(CH_2)_mSO_2(CH_2)_n$, $(CH_2)_mSO_2(CH_2)_nCO$, or combinations thereof; with m and n being integers in the range from 0 or 1 to 12, and substituent R^9 may be a residue as defined for any of R^1, R^2, R^3, R^4 and R^5 in claim 1 above;

or, alternatively, wherein the aryl amino groups NR^1 and/or NR^2R^3 in Formula A are connected to an acyl hydrazine or alkyl hydrazine moiety indirectly, via linkers, in particular linkers as defined above, thus representing hydrazides ($ZCONHNH_2$) or hydrazines ($ZNHNH_2$), respectively;

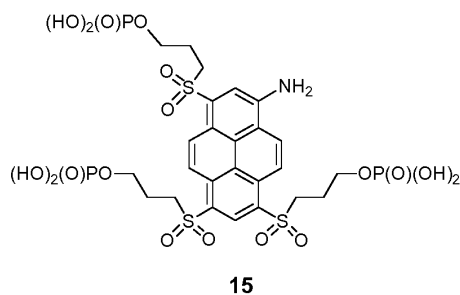
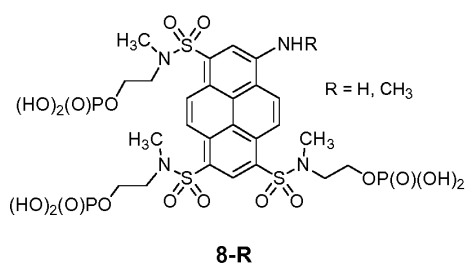
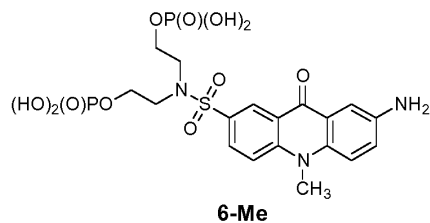
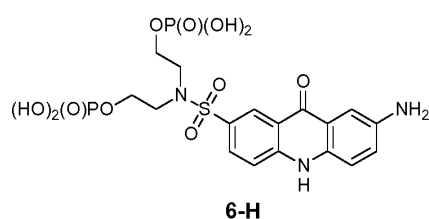
wherein Z denotes the dye residue of Formula A that includes aryl amino groups and linkers;

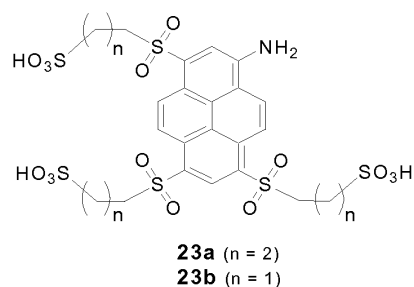
in particular, R^1 and R^2 may be represented by: $(CH_2)_mCON(R^{10})$, $CO(CH_2)_mN(R^{10})$, $CO(CH_2)_mS(CH_2)_n$, $(CH_2)_mS(CH_2)_nCO$, $CO(CH_2)_mSO_2(CH_2)_n$, $(CH_2)_mSO_2(CH_2)_nCO$ and their combinations; with m and n being integers ranging from 0 or 1 to 12;

R^{10} is defined as any of the functional groups R^1 , R^2 , R^3 , R^4 and R^5 in claim 1 above; in particular hydroxyalkyl $(CH_2)_nOH$, thioalkyl $((CH_2)_nSH)$, carboxyalkyl $((CH_2)_nCO_2H)$, 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 or 1 to 12;

linkers may also be represented by non-aromatic O, N and S-containing heterocycles, e.g., piperazines, pipercolines.

11. A compound according to one of the general formulae A-D of claims 1-6 having one of the following formulae:





or a salt thereof.

12. A compound according to Formula A above which has a net charge q of 0, -2, -4 or higher, preferably -4, or a compound of Formulae B, C and D above 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.

13. A fluorescent dye salt according to any one of claims 1-12, 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 A-D with a counterion selected from anions of a strong mineral, organic or a Lewis acid.

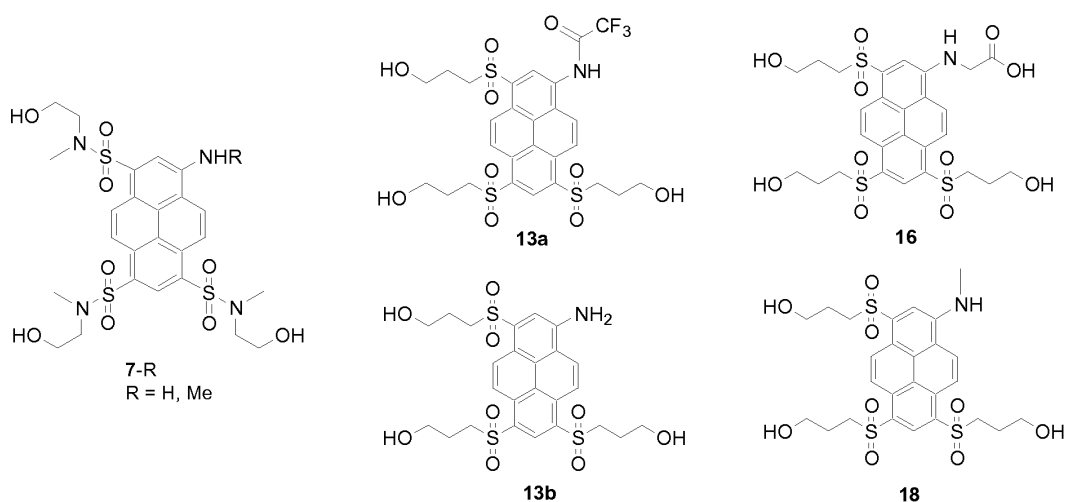
14. A fluorescent dye according to any one of claims 1-13, wherein carbohydrate-reactive groups, particularly hydrazine $N(R)NH_2$, hydroxylamine $N(R)OH$ or aminooxy ONH_2 groups with R being H, alkyl, heteroalkyl, alkenyl or perfluoroalkyl are connected to the nitrogen site $N(R^1)R^2$ in the dye of formulae A or B via all types of linkers listed for group L in formula B.

15. A compound according to any one of claims 1-14 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.

16. A compound according to any one of claims 1-14 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.

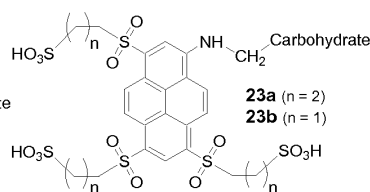
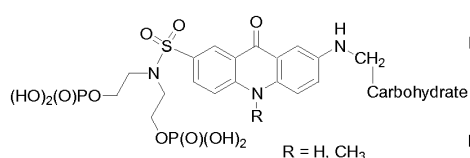
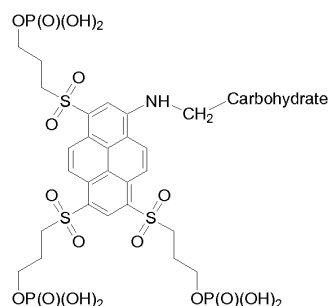
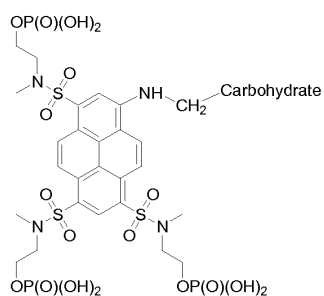
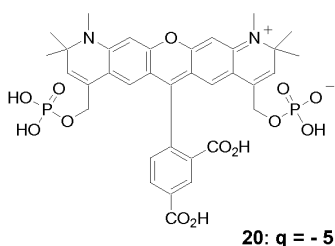
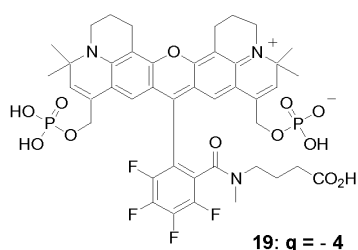
17. Compounds according to claim 16 which belong to the following compound set: **7-R** (R = H, Me), **13a**, **13b**, **16** and **18**.



18. Use of the compounds according to any one of claims 1-14 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.

19. Carbohydrate-dye conjugates comprising fluorescent dyes according to any one of claims 1-14.

20. The carbohydrate-dye conjugates according to claim 19 wherein the dye is selected from the compounds of the formulae **6-H**, **6-Me**, **8-H**, **15**, **23a** and **23b**, as shown in claim 11 and below



21. A kit or composition comprising one or more of the dyes of claims 1-14 or one or more of the carbohydrate conjugates of claims 19 and 20.

22. A method for synthesizing fluorescent aminopyrene dyes with absorbance maxima of 477 - 510 nm, net charge of 0 or down to - 6, emission maxima of 535 - 560 nm in water, having the general formulae B and C, particularly compounds **7-R**, **13a**, **13b**, **15**, **16**, **18**, **23a** and **23b**, which involves an exchange reaction of the aryl halogen to a substituted thioalkyl function, followed by oxidation to the sulfone and, optionally, by phosphorylation at the hydroxyl site.

23. A method for synthesizing phosphorylated fluorescent aminopyrene or aminoacridone dyes with net charges of -4, -6 and larger, that have the general formulae A and D, particularly compounds **6-R** and **8-R**, which involves sulfamidation of amino alcohols, followed by phosphorylation at the hydroxyl site.