Fluorescent Probes

Negatively Charged Yellow-Emitting 1-Aminopyrene Dyes for Reductive Amination and Fluorescence Detection of Glycans

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Abstract: 1-Aminopyrenes with three α-hydroxylated N-alkylsulfonamido or alkylsulfonyl residues in positions 3, 6, and 8 were prepared, O-phosphorylated, and applied for reductive amination of oligosaccharides. The dyes (ε ≈ 20 000 M⁻¹ cm⁻¹) with six negative charges (pH ≥ 8) and low m/z ratios enable labeling and fluorescence detection of reducing sugars (glycans) related to the most structurally and functionally diverse class of natural products. Under excitation with a 488 nm laser, the new glycoconjugates emit yellow light of about 560 nm, outperforming (with respect to brightness and faster electrophoretic mobilities) the corresponding APTS derivatives (benchmark dye with green emission in conjugates).

Glycosylation is an enzymatically driven and highly diverse transformation of proteins, lipids, and other noncarbohydrates. The products of glycosylation (glycoconjugates) have a new chemical bond formed between a carbohydrate (glycan; donor) and another molecule (acceptor). Glycoconjugates represent one of the most structurally and functionally diverse class of natural products involved in fundamental biochemical processes in living matter. Only few specific functions of these complex and carbohydrate-rich molecules have been well understood so far.[2] Further progress in glycomics and glycobiology depends on the advances in analytic techniques applicable to complex carbohydrates.

Carbohydrates do not absorb visible light, and for the sensitive detection by emission they need to be labeled with a fluorescent tag.[3] Capillary gel electrophoresis (CGE) is an important method for analyzing glycoconjugates including glycoproteins, glycopeptides, and “released” (enzymatically cleaved from the acceptors) N- or O-glycans.[4] The net electrical charge is required for separation of the analytes by CGE. The native carbohydrates, except sialic or glucuronic acids, sulfated or phosphorylated derivatives, are uncharged and cannot be separated by their mass to charge ratio (electrophoresis). Importantly, the features of an “ideal” fluorescent label required for CGE—the electrical charge, emissive properties, and the reactive group—can be incorporated in one fluorescence dye with (multiple) charges and the amino group reacting with aldehyde residues in reducing sugars (Scheme 1). Combined with laser-induced fluores-

Scheme 1. Reductive amination of mono- and oligosaccharides.
bright fluorescent dyes with an aromatic amino group, multiple negative charges and yellow emission.

The high-performing fluorescent tags applicable in the reductive amination and CGE-LIF of glycans must have an amino group for which the $pK_a$ of the conjugated acid is in the range of 3–4 for the efficient reaction (Scheme 1) at $pH \approx 3$. The glycan conjugates should have a net charge of $-3, -6$ at $pH 8$ (pH of the buffer solution in CGE) to provide high electrophoretic mobility, should be soluble in aqueous buffers, and should be stable against reduction with boranes or borohydrides over a wide pH range (3–8). The absorption at 488 nm ($\epsilon_{488}$) or 505 nm ($\epsilon_{505}$) determined the excitation efficiency with an argon ion laser or solid-state laser; high brightness and the minimal cross-talk with the “APTS channel” in the detector (low emission of conjugates at 520 nm) are also required. These features are set by the reaction conditions in Scheme 1, and the properties of the standard DNA-sequencing equipment used for the separation and detection of the fluorescent glycan derivatives.

APTS is a reference dye with green emission in conjugates with oligosaccharides (see Figure 2 and the Supporting Information). We designed new dyes with yellow emission in conjugates, trying to minimize the interference with an APTS detection window.

To achieve this goal, we converted the sulfonic acid residues in APTS (Scheme 2) into more powerful electron acceptors—sulfonamides. Sulfonamides (represented by dyes 6–9 in Scheme 2) have higher values of the Hammett $s$-constants ($s_m = 0.53, s_p = 0.60$ for SO$_2$NH$_2$) than ionized sulfonic acid residues in APTS ($s_m = 0.05, s_p = 0.09$). The presence of an electron-donating (N-alkyl)amino group and the acceptor groups in “active” positions (3, 6, and 8) of the pyrene system leads to the “push–pull” dyes$^{[10]}$ emitting blue-green (APTS), green (6-R$^1$, 7-H), or yellow (7-Me, 9) light. The spectral properties of the dyes are given in Table 1 (see also the Supporting Information).

To provide multiple negative charges and high electrophoretic mobility at $pH$ 8, primary phosphates (R-OPO$_2$H$_2$) are preferred over phosphonates$^{[11a]}$ because their first and second $pK_a$ values are in the range of 1.5–1.9 and 6.3–6.8, respectively$^{[11b]}$. In the electrophoresis buffer solution, one primary phosphate group introduces two negative charges (phosphonates are less acidic and not fully ionized at $pH$ 8). APTS (Scheme 2) was converted into the relatively stable 1,3,6-tris(chlorosulfonyl)pyrene-8-amine (4) and then to sulfonamides (6-7Bu, 7-H) by reaction with the corresponding amine (5 or CH$_3$NHCH$_2$CH$_2$OH).

Direct phosphorylation of three hydroxy groups in 7-H or 8 (reagent $h$ in Scheme 2) followed by hydrolysis$^{[12]}$ afforded dyes 6-H (16 %) or 9 bearing six negative charges at $pH$ 8. To improve the yield, we prepared O-phosphorylated N-(methylamino)ethanol 5, let it react with compound 4, isolated the intermediate 6-7Bu, and then converted it into dye 6-H (83%).

To improve the polarity of the aminopyrene chromophore even further, we introduced alkyl sulfonyl groups into positions 3, 6, and 8 of 1-aminopyrene and prepared dyes 13, 15, and 16 (Scheme 3). Alkyl sulfonyl substituents have higher values of the Hammett $s$-constants ($s_m = 0.56–0.66, s_p = 0.68–0.77$ for SO$_2$Alkyl$^{[8]}$) than sulfonamides ($s_m = 0.53, s_p = 0.60$ for SO$_2$NH$_2$)$^{[9]}$. This indicates that they are more powerful acceptors than sulfonamides (compounds 6-R$^1$, 7-R$^2$, and 9 in Scheme 2).

We found that the trifluoroacetoyl residue is a better protecting group for 1-aminopyrene than acetyl$^{[13]}$ because it is acid-stable but can be easily cleaved under mild basic conditions. Bromination of 1-(trifluoroacetamino)pyrene led to tribromide 10 (Scheme 3), an important precursor to

Table 1: Spectral properties of the dyes and their $m/z$ ratios.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Absorption $\lambda_{max}$ [nm] $((\epsilon \cdot M^{-1} \cdot cm^{-1})$</th>
<th>Emission $\lambda_{max}$ [nm] ($\Phi_l$)$^{[6]}$</th>
<th>Solvent</th>
<th>Fluor. lifetime t [ns]</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTS$^{[9]}$ (151)</td>
<td>424 (20600)</td>
<td>500 (0.95)</td>
<td>aq. PBS</td>
<td>–</td>
</tr>
<tr>
<td>APTS-G$_{6b}$</td>
<td>455 (17000)</td>
<td>511</td>
<td>H$_2$O</td>
<td>–</td>
</tr>
<tr>
<td>6-H (144)</td>
<td>471</td>
<td>544 (0.88)</td>
<td>H$_2$O</td>
<td>5.9</td>
</tr>
<tr>
<td>7-H</td>
<td>477 (22400)</td>
<td>535 (0.96)</td>
<td>MeOH</td>
<td>5.6</td>
</tr>
<tr>
<td>7-Me</td>
<td>493 (23000)</td>
<td>549 (0.97)</td>
<td>MeOH</td>
<td>5.9</td>
</tr>
<tr>
<td>9</td>
<td>502</td>
<td>563 (0.85)</td>
<td>H$_2$O</td>
<td>3.6</td>
</tr>
<tr>
<td>13</td>
<td>502 (23400)</td>
<td>550 (0.88)</td>
<td>MeOH</td>
<td>6.3</td>
</tr>
<tr>
<td>15</td>
<td>509 (19500)</td>
<td>563 (0.67)</td>
<td>H$_2$O</td>
<td>6.4</td>
</tr>
<tr>
<td>15 (137)</td>
<td>486 (21000)</td>
<td>534 (0.80)$^{[6a]}$</td>
<td>MeOH</td>
<td>4.9</td>
</tr>
</tbody>
</table>

$^{[a]}$ Absolute values of the fluorescence quantum yields (if not stated otherwise).
$^{[b]}$ Data from Ref. [6b], abs. measured in H$_2$O, emission measured in aq. phosphate buffer at $pH$ 7.4.
$^{[c]}$ Conjugate with maltolhexose (G$_2$), data from Ref. [5b].
$^{[d]}$ Excitation at 375 nm. [e] Rhodamine 6G as a reference dye with $\Phi_l = 0.9$. [f] Aq. Et$_3$N$+$/H$_2$CO$_3$, pH 8–8.5. [g] Fluorescein as a reference dye with $\Phi_l = 0.9$ in 0.1 M NaOH.
the functionally substituted aminopyrenes. Palladium-catalyzed cross-coupling of tribromide 10 with 3-mercapto-1-propanol afforded triol 11 with three alkyl aryl sulfide residues. Oxidation of 11 with hydrogen peroxide in acetic acid in the presence of sodium tungstate led to trisulfonyl derivative 12. Deprotection of the amino group in compound 12 by heating with aq. NaOH in methanol gave amine 15 (model compound). Another model dye with an N-methyl group (13) was prepared from intermediate 11 by N-methylation of the trifluoracetylamino group and mild hydrolysis of the amide group. Phosphorylation of 12 followed by hydrolysis led to aminopyrene 16 with three primary phosphate groups attached to alkyl sulfonyl residues. The phosphate groups in dyes 6-H, 9, and 16 are hydrolytically stable over a broad pH range, from pH 3 (reductive amination conditions) to pH 8.3 (electrophoresis) and beyond.

The model pyrenes with N-methylamino groups (7-Me, 9 in Scheme 2, and 13 in Scheme 3) are structurally similar to the final products formed in Scheme 1 upon reductive amination of carbohydrates; they make it possible to measure the red-shifts in the absorption and emission spectra and extinction coefficients (7-Me, 13 in Table 1). Importantly, dyes 7-Me, 9, and 13 did not participate in the reductive amination of glucose (even under harsh conditions). This result may be explained by the additional steric hindrance associated with the planar iminium ion (Scheme 3), in which two aromatic hydrogen atoms adjacent to the reaction center are expected to “repulse” the N-methyl group.

The photophysical properties of APTS, its conjugate with maltohexaose (APTS-G6), and the new pyrenes, are given in Table 1. The dyes in Table 1 form two groups: compounds with a primary amino group (APTS, 6-H, 7-H, 15, and 16) and dyes with a secondary amino group (APTS-G6, 7-Me, 9, and 13). Pyrenes in the first group absorb at 424 nm (APTS) to 486 nm (15). Compounds in the second group are related to the products formed in the course of reductive amination (Scheme 1); their absorption maxima are red-shifted and found in the range from 455 nm (APTS-G6) to 509 nm (13) in aqueous solutions. For example, N-methylation (6-H—9) shifted the absorption maximum to the red by 31 nm, while the emission underwent bathofluorochromic shift of “only” 19 nm. Thus, the Stokes shift was reduced from 73 nm (6-H) to 61 nm (9). Importantly, within each group the spectrum of APTS or APTS-G6 conjugate is separated from the other spectra of the same group by 42–69 nm (absorption maxima) and by 38–54 nm (emission maxima): new dyes absorb and emit at longer wavelengths. This feature is important, as the glycan conjugates of dyes 6-H and 16 are intended to have minimal emission in the APTS detection window. Comparing sulfonamides (6-H, 7-Me) with structurally related alkyl sulfones (13, 16) we observed the red shift of only 6–9 nm in absorption, while the positions and shapes of emission bands remained the same (Table 1 and Figure 1).

The brightness of a glycan label is termed as a product of the extinction coefficient (at 488 nm, as an excitation wavelength) and the fluorescence quantum yield. The fluorescence quantum yields of dyes 6-H and 16 (Table 1) are 0.88 and 0.92, respectively. For APTS conjugates, the extinction coefficient at the maximum (455 nm) is 17000 μm⁻¹ cm⁻¹, and the absorption at 488 nm is ca. 35 % of the maximal value. For all new N-alkylated pyrenes (7-Me, 9, 13, 6-H + G, 16 + G) we can assume the extinction coefficient at 488 nm to be about 18000 μm⁻¹ cm⁻¹. Therefore, the conjugates of the new dyes are ca. three times brighter than APTS derivatives (under excitation with the 488 nm laser).

We used APTS as a reference dye and compounds 6-H and 16 as new reagents, and obtained their conjugates with glucose (G) and oligomers (maltohexaose (G6), and maltoheptaose (G7)), mannose (M), and oligomers [2-O-, 3-O- and 4-O-(a-D-mannopyranosyl)-α-D-mannoses (M2-2O, M2-3O and M2-4O), mannitol (M1), mannotetraose (M4)], as well as 3’- and 6’-sialylglycoses. Due to the presence of the strong acceptors—sulfonamido and alkylsulfonyl groups—dyes 6-H...
and 16 undergo reductive alkylation more reluctantly than APTS. The detailed procedures for reductive amination and yields of the individual conjugates are given in Supporting Information. Figure 1 shows absorption and emission spectra of glucose conjugates prepared from dyes 6-H and 16. The absorption spectra are very similar, and the emission spectra are practically identical. Therefore, we preferentially used dye 16 in reductive amination. Figure 2 shows the gel electrophoresis results obtained with APTS and its conjugates (lanes 1, 3, 5), as well as compound 16 and its conjugates with reducing sugars (lanes 2, 4, 6). The conjugates of dye 16 move faster than the corresponding conjugates of APTS. Due to higher net charge of dyes 6-H and 16 with six negative charges may reveal “heavy” glycans undetectable with APTS due to very long retention times caused by the relatively low charge (−3) and the limited brightness. Access to the DNA sequencer with a CGE-LIF unit will make it possible to evaluate the crosstalk between the emission signals of APTS, on one hand, and the dyes 6-H and 16, on the other hand (also in conjugates), and their applicability for calibration of the retention times in CGE.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 2. Gel electrophoresis (migration from “north” to “south”, pH 8.3); detection by emission (excitation at 365 nm). From bottom to top. Lane 1: APTS (lowest; blue), APTS + G, APTS + G3, APTS + G7, (green). Lane 2: dye 16 (green), 16 + G, 16 + G3, 16 + G7 (yellow). Lane 3: APTS + M, APTS + M2-2O/APTS + M2-3O (unresolved), APTS + M4-4O, APTS + M3, and APTS + M. Lane 4: dye 16, 16 + M, 16 + M2-3O, 16 + M2-2O/16 + M2-4O (unresolved), 16 + M4, and 16 + M3. Lane 5: APTS, APTS-labeled 3'- and 6'-sialyllactoses. Lane 6: dye 16 and its conjugates with 3'- and 6'-sialyllactoses.


[9] H. Zollinger, W. Buchler, C. Wittwer, *Helv. Chim. Acta* 1953, 36, 1711–1722; larger values for $\alpha_{\text{SO}_3^-} = 0.30$ and $\alpha_{\text{sp}} = 0.35$ are mentioned in ref. [8a] as a private communication of Viktor Palm.


[12] Hydrolysis with aq. Et$_3$N/H$_2$CO$_3$ buffer (after removal of POCl$_3$ and trimethyl phosphate) transforms $O$-alkyldichlorophosphates to primary alkyl phosphates and cleaves the N–P bond formed upon phosphorylation of the weakly nucleophilic amine.


[17] Yields were determined by integration of HPLC peak areas for the free dyes and their conjugates at isosbestic points; see Table S1 in the Supporting Information.

[18] 6-H$^+$ G moves slower than APTS$^+$ G (Figure S5).