Steady state and time-resolved fluorescence of 2-aminoacridone sugar derivatives

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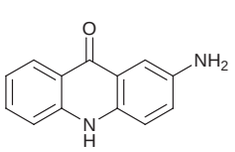
The fluorescence properties of 2-aminoacridone (2-AMAC) and some polysaccharide derivatives have been studied in the steady state and with time-correlated single-photon counting. 2-AMAC in methanol has an absorption maximum at 425 nm and a fluorescence emission maximum at 530 nm. The absorption spectrum shows a sufficiently broad tail for satisfactory excitation by the light from an argon ion laser (488 nm). Lifetime decay analysis shows that the fluorescence lifetime of the aminoacridone moiety is not changed significantly by the addition of a sugar chain, or by the length or structure of that chain. 2-AMAC exhibits a fluorescence decay that may be fitted to a single exponential in both methanol ($t \approx 12$ ns) and water ($t \approx 10$ ns); in an equal mixture of the solvents the behaviour is best described by a sum of two exponential terms. We have also shown that the absorbance of a complex mixture of 2-AMAC tagged glycans is directly proportional to their fluorescence emission at 525 nm. These results have confirmed our previous studies that 2-AMAC is a useful and non-selective fluorescent tag in the analysis of polysaccharide chains.

A large number of experimental and therapeutic proteins contain carbohydrate covalently linked either to asparagine (N-linked) or to serine and threonine (O-linked) residues. This carbohydrate not only plays an important role in modulating the biological properties of glycoproteins, but it can also affect physicochemical properties such as solubility, interaction with other proteins and protection against protease hydrolysis.[1] Analysis of oligosaccharides released from glycoproteins has become an essential tool in glycobiology, especially when studies are carried out that relate their structural properties to the biological function of the glycoprotein.

A number of techniques have been used in the analysis of carbohydrates. Mass spectrometry has been used effectively to give a qualitative assay of the complexity of glycan structures in a mixture.[2] Unfortunately, the majority of carbohydrates of interest do not contain a chromophore or fluorophore so that direct quantitative analysis is not possible. A number of authors have used several aromatic amines, which react easily and efficiently with reducing carbohydrates *via* reductive amination.[3,4]

We have used 2-aminoacridone (2-AMAC) extensively in the derivatisation of a variety of mono- and poly-saccharides.[5–8] 2-AMAC is favoured by us as it is neutral over a wide pH range (~2 to 9). This property, combined with its hydrophobicity and its UV–visible and fluorescence properties, allows 2-AMAC carbohydrate derivatives to be separated by a number of chromatographic modes (reverse- and normal-phase high-pressure liquid chromatography, and micellar electrokinetic capillary chromatography), and detected by several spectroscopic and spectrometric techniques.



2-AMAC

In order to quantify the relative proportions of fluorophore derivatised glycans in a mixture, it is important to establish that fluorescence properties are not affected by the nature of the carbohydrate. We now report some simple steady state and time-resolved fluorescence studies of 2-AMAC and some sugar derivatives. We have also directly compared signals of individual glycan components from their UV–visible absorbance to their fluorescence emission to assess the suitability of this dye for glycan quantification.

Experimental

Materials

2-AMAC was prepared as detailed by us previously.[7] Sodium cyanoborohydride, glacial acetic acid and dimethyl sulfoxide (used in the derivatisation of carbohydrates with 2-AMAC), were purchased from Aldrich (Gillingham, UK). HPLC grade methanol (BDH, Poole, UK) was used in fluorescence studies without further purification. A hydrolysed dextran ladder (for the preparation of the 2-AMAC derivatives of glucose, penta-glucose and deca-glucose), 6′-sialyllactose (6′-SL), lacto-*N*-neohexaose (LNnH) and the glycan mixture released from IgG were supplied by Oxford GlycoSciences (Abingdon, UK). 2-AMAC derivatives were stored below −20 °C, when not in use. Methanol solutions were prepared shortly before spectroscopic measurements were carried out.

10 μM fluorescein disodium salt (ICN Biomedicals, Ohio, USA) in distilled water was used as a calibration standard for fluorescence lifetime measurements, that is, a good fit to a single exponential decay, with a typical lifetime of 4.65 ns.[9]

Preparative HPLC of 2-AMAC carbohydrate derivatives

Hydrolysed dextran ladder. The derivatisation of the hydrolysed dextran ladder with 2-AMAC by the reductive amination reaction has been previously described by us.[7] Separation of the

derivatised dextran mixture was performed using a Waters Alliance 2690 instrument, fitted with an Oxford GlycoSystems GlycoSepN column (3.9 mm id × 250 mm). About 60 μg of the 2-AMAC derivatives were injected on to the column and detected with a Waters 474 Scanning Fluorescence Detector (volume, 5 μl; pathlength, 1 mm). The excitation and emission wavelengths of the fluorescence detector were set at 428 nm and 525 nm, respectively.

The mobile phase was a gradient with acetonitrile (eluent A) and 50 mM ammonium formate, pH 4.40 (eluent B), such that step 1 was a linear gradient from 35 to 53% B for 72 mins (flow rate 0.4 ml min^{-1}); step 2, linear gradient from 53 to 100% B for 3 mins (flow rate 0.4 ml min^{-1}); step 3, isocratic at 100% B for 2 mins (flow increased to 1.0 ml min^{-1}); step 4, isocratic at 100% B for 15 mins (flow rate 1.0 ml min^{-1}); step 5, equilibration of the column at 35% B for 5 mins (flow rate 1.0 ml min^{-1}); step 6, equilibration of the column at 35% B for 5 mins (flow rate 0.4 ml min^{-1}).

Three glucose derivatives were collected as they emerged from the fluorescence detector. These were glucose (Glu-1), penta-glucose (Glu-5) and deca-glucose (Glu-10). The collected samples were frozen in solid carbon dioxide at −60 °C, freeze dried and stored dry at −20 °C until required.

Oligosaccharides from human milk. The experimental procedure described above was also used for these oligosaccharides, 6′-SL and LNnH. About 80 μg of each derivatised sugar was injected and the eluting samples were collected and processed as above.

Steady state fluorescence. Spectra were acquired either on a single-photon counting fluorimeter (Instruments SA Fluoromax-2, Stanmore, UK) (2-aminoacridone solutions) with 1 nm bandpass and 1 s integration time, or on the argon ion laser system described below (glucose derivatives).

Standard 10 mm pathlength cuvettes (Starna, from Optiglass, Hainault, UK) were quartz (bulk measurements) or optical glass (small volumes). They were new for these experiments, and thoroughly cleaned with methanol between each sample.

Fluorescence lifetime measurements. These were made with a conventional time-correlated single-photon counting system.[10] The excitation source was 488 nm light from a cavity dumped argon ion laser (Lexel 95). The detector was a dedicated single-photon counting photomultiplier tube (Hamamatsu R6358P). Single-photon counting electronics comprised a constant fraction discriminator (on board a Stanford Research Systems SR400 gated photon counter (Sunnyvale, California, USA)), delay unit and time-amplitude converter operated in reverse start-stop mode (EG&G Ortec 425A and 437A, Canada), and an Oxford Instruments data card (PCA-3) running on a Pentium PC. A monochromator (SPEX 1681, Instruments SA, Stanmore, UK) positioned before the detector enabled discrimination between excitation and emission wavelengths. 10 μM fluorescein disodium salt (FDSS) in water was used as the calibration standard for time channel determination. Instrument response functions were recorded with scattered light at 488 nm.

HPLC analysis of IgG glycans derivatised with 2-AMAC. Separation of the derivatised IgG glycan pool was carried out as previously described above. About 50 μg of the 2-AMAC derivative was injected on to the column and detected using a Waters 474 Scanning Fluorescence Detector (volume, 5 μl; pathlength, 1 mm) connected in series to a Kratos Spectroflow 757 UV absorbance detector (volume, 12 μl; pathlength, 8 mm). The excitation and emission wavelengths of the fluorescence detector were set at 428 and 525 nm, respectively, and the absorbance wavelength at 305 nm.

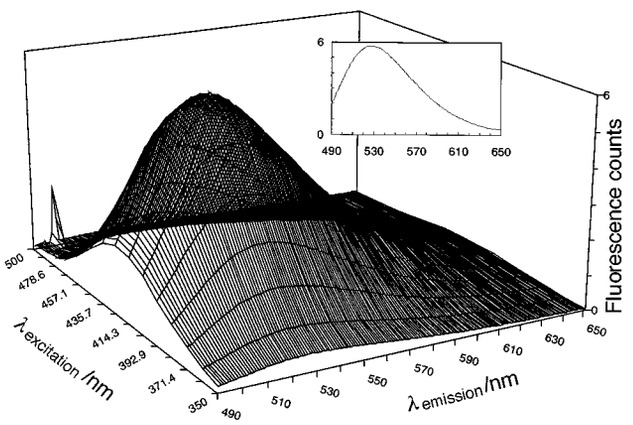


Fig. 1 Three-dimensional fluorescence excitation/emission plot for 2-AMAC in methanol: 1 nm bandpass, 1 s acquisition time per point. Inset shows emission profile after excitation at λ_{max}, 425 nm.

The mobile phase used was a gradient of acetonitrile (eluent A) to 250 mM ammonium formate, pH 4.40 (eluent B) in the following ratios. Step 1, linear gradient from 35 to 39% B for 35 mins (flow rate 0.4 ml min^{-1}); step 2, linear gradient from 39 to 53% B for 37 mins (flow rate 0.4 ml min^{-1}); step 3, linear gradient from 53 to 100% B for 3 mins (flow rate 0.4 ml min^{-1}); step 4, isocratic at 100% B for 2 mins (flow increased to 1.0 ml min^{-1}); step 5, isocratic at 100% B for 15 mins (flow rate 1.0 ml min^{-1}); step 6, equilibration of the column at 35% B for 5 mins (flow rate 1.0 ml min^{-1}); step 7, equilibration of the column at 35% B for 5 mins (flow rate 0.4 ml min^{-1}).

Results and discussion

Steady state fluorescence of 2-AMAC

The three-dimensional excitation/emission plot for 2-AMAC in methanol is shown in Fig. 1. The inset shows the fluorescence emission profile for excitation at the absorption maximum, 425 nm. Emission profiles corresponding to excitation at other wavelengths are essentially reduced-scale versions of this curve. Maximum emission is near 530 nm; significantly shifted from excitation wavelengths so that good spectral separation is possible. It is interesting that, after excitation of 2-AMAC at 425 nm, emission extends to 600 nm. This is well within the visible region and makes 2-AMAC a very selective derivatising agent with spectroscopic properties which do not interfere with common natural fluorophores such as tyrosine and tryptophan. Absorption at 488 nm is also adequate for time-resolved studies using argon ion laser excitation.

Time-resolved fluorescence

The technique of time-correlated single-photon counting was used to record the fluorescence decays of 2-AMAC in different solvents (Fig. 2). The solutions had visually similar optical densities; fluorescence decays were recorded at 530 nm and instrument responses at 488 nm. Monochromator slits corresponded to a 15 nm bandpass.

Analyses of the data using a sum of exponentials (SOE) iterative reconvolution routine[11] show that, in methanol or water, the fluorescence kinetics are described adequately by single exponential decay laws with lifetimes of approximately 12 and 10 ns respectively. In a 50 : 50 methanol–water mixture, the observed behaviour is more complex, and a fit to a sum of two exponential terms is necessary to describe the decay (Table 1). Independent analyses of the decays using the alternative Maximum Entropy Method (MEM)[12] support these results.

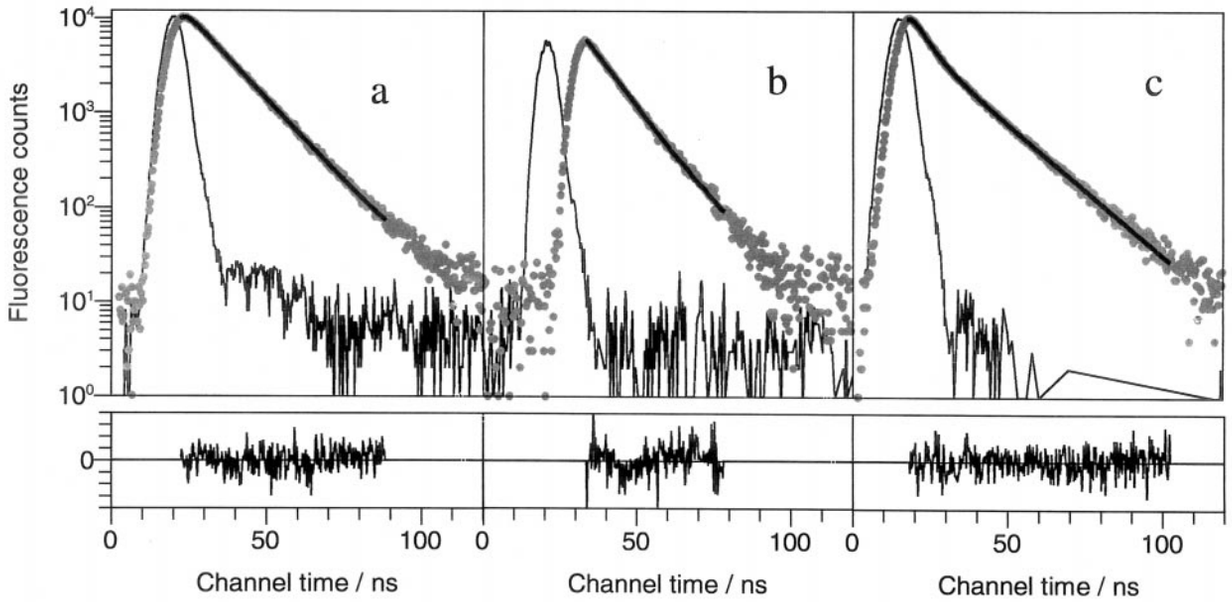


Fig. 2 Fluorescence decays and sum of exponential (SOE) fits for 2-AMAC in a) methanol, b) water, c) 50 : 50 methanol–water. Corresponding instrument response functions are also shown.

Table 1 Results of sum of exponentials (SOE) fitting for the decays shown in Fig. 2

	τ/ns[a]	A (%)[b]	χ^2[c]	DW[d]
a) 2-AMAC/methanol	12.1	100	1.02	1.90
b) 2-AMAC/water (saturated)	10.1	100	1.74[e]	1.68
c) 2-AMAC/50 : 50 methanol–water	15.7	65	1.19	1.93
	4.4	35		

[a] Fitted lifetime. [b] Pre-exponential factor. [c] Quality of fit. [d] Durbin–Watson parameter. [e] The statistics for this sample are poor, but are not improved by fitting to 2 or 3 exponential terms. The single-exponential model is the best choice, supported by MEM analysis.

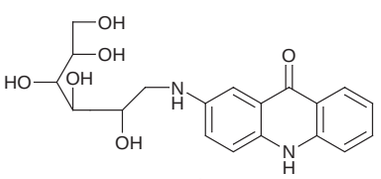
Table 2 Results of SOE fitting for the decays given in Fig. 4 and 5

	τ/ns[a]	χ^2[b]	DW[c]
2-AMAC	12.1	1.02	1.90
19 μM Glu-1	12.3	1.79[d]	1.0
19 μM Glu-5	12.9	1.97[d]	1.1
5.4 μM Glu-10	12.8	[d]	
30 μM 6′SL	12.2	1.24	1.6
20 μM neohexose	13.3	1.38	1.5

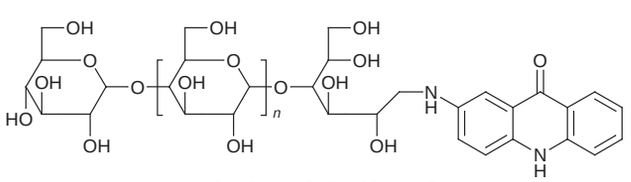
[a] Fitted lifetime. [b] Quality of fit. [c] Durbin–Watson parameter. [d] The statistics for these samples are poor, but are not improved by fitting to 2 or 3 exponential terms. The single-exponential model is the best choice, supported by MEM analysis.

Steady state fluorescence of sugar derivatives

The three glucose derivatives were chromatographically prepared using an analytical column, before fluorescence studies were carried out on solutions of these compounds. This procedure ensured that no excess 2-AMAC was in these samples. The three 2-AMAC derivatives of glucose itself (Glu-1), penta-glucose (Glu-5) and deca-glucose (Glu-10) can be represented by the structures shown.



Glu-1



Glu-5, n = 3; Glu-10, n = 8

Fluorescence emission profiles of the free dye and glucose chain derivatives were recorded under equivalent conditions for direct comparison. These are reproduced together in Fig. 3: the 2-AMAC fluorescence spectrum is given along with the spectra of 19 μM Glu-1, 19 μM Glu-5 and 5.4 μM Glu-10 in methanol. The monochromator slits selected gave a 9 nm bandpass, and excitation was at 488 nm.

The emission spectra of all four samples were very similar, whether the derivative included only an alditol moiety or was a relatively much larger derivative containing an alditol group and nine glucose units. This result indicates that the steady state fluorescence properties of 2-AMAC are not significantly modified when derivatised with carbohydrate.

Time-resolved fluorescence

The fluorescence decays and single exponential fits for the derivatised polysaccharide samples Glu-1, Glu-5 and Glu-10 are given in Fig. 4. We also extended (Fig. 5) these studies to the 2-AMAC derivatives of a trisaccharide containing a sialic acid, a galactose and a glucose residue (6′SL), and a branched oligosaccharide containing six sugar residues (LNnH), comprising one glucose, two *N*-acetylgalactosamine and three galactose residues.

A single exponential term may be found to describe the decay of each sample. The results of reconvolution analysis are given in Table 2. The fitted lifetimes, τ, of each decay do not differ significantly from each other; the values quoted lie within the limits of experimental error. The quality of the fit (indicated by the reduced chi-squared value, χ^2, and the Durbin–Watson parameter, DW) does vary between samples; but complementary analyses with the Maximum Entropy Method support the single exponential model for each one.

Fluorescence lifetimes are particularly sensitive to changes in environment or structure. However, it is clear from the present

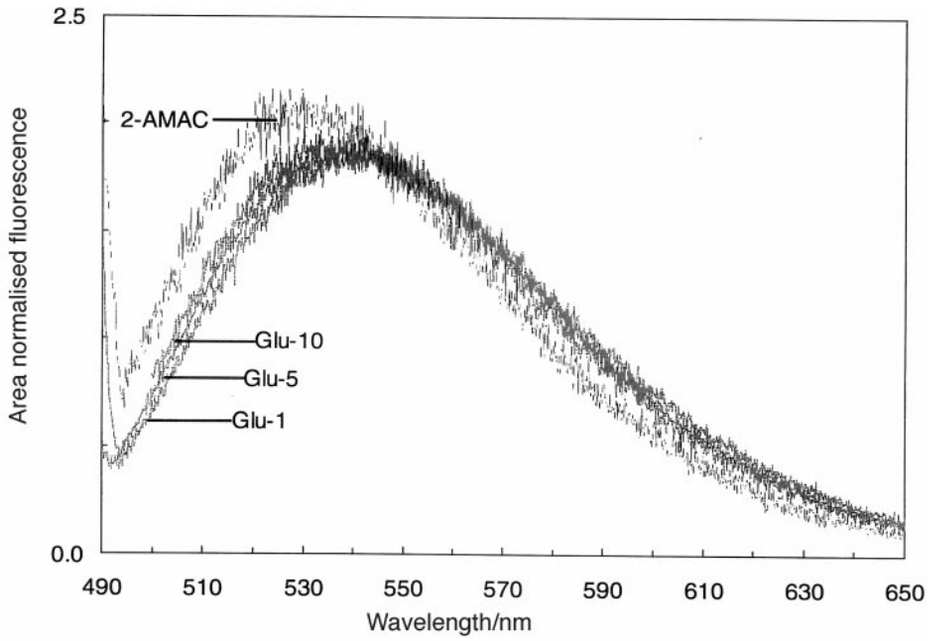


Fig. 3 Comparison of area normalised fluorescence emission profiles; excitation wavelength, 488 nm; 9 nm bandpass; 0.8 s acquisition time per point.

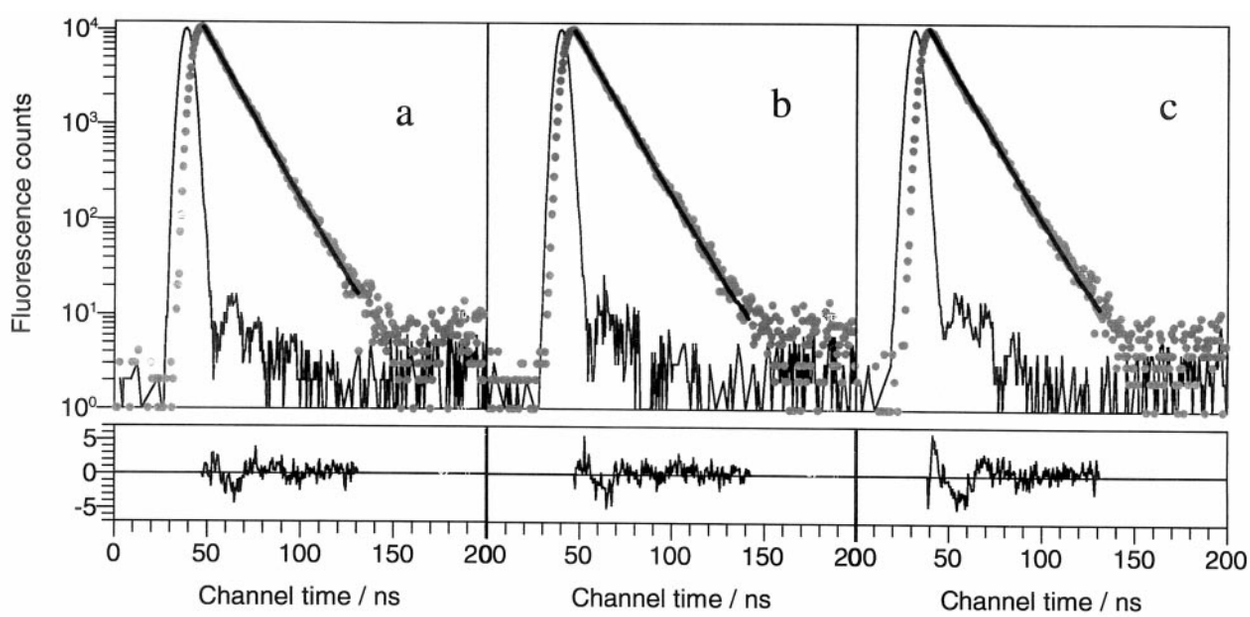
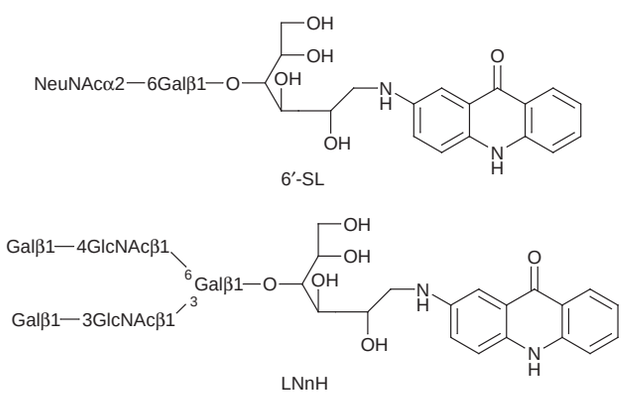


Fig. 4 Fluorescence decays and SOE fits for glucose chain derivatives. a) 19 μM Glu-1, b) 19 μM Glu-5, c) 5.4 μM Glu-10. Instrument response functions are also shown.



studies that the emission profiles and fluorescence lifetime decay of 2-AMAC do not change significantly when it is covalently attached to a saccharide chain *via* the amine group. The sugar units are linked to 2-AMAC through an sp^3 hybridised carbon atom. *Ab initio* molecular modelling calculations could show the influence of this linkage, but qualitatively we expect minimal interaction with the π-electron system of the acridone moiety and therefore little alteration in the fluorescence properties. This is borne out in our findings.

As a final test of the suitability of 2-AMAC as a derivatising agent for carbohydrates we derivatised a complex mixture of glycans released from human IgG. This glycoprotein contains a number of biantennary complex glycans, some of which have one or two sialic acid residues at the non-reducing end.[13] Derivatisation with 2-AMAC gives derivatives linked to the fluorophore *via* an acetylglucosamine residue. We separated this mixture of 2-AMAC derivatives chromatographically, and recorded both the UV–visible absorbance and fluorescence emission, using two detectors connected in series. The two chromatograms are shown in Fig. 6(a). Despite the longer pathlength used for detection by absorbance, fluorescence detection

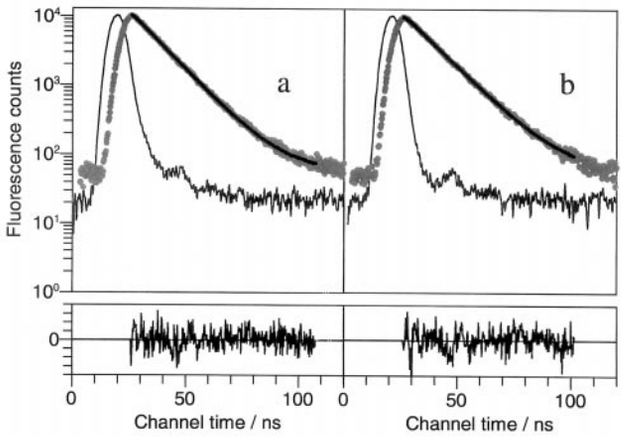


Fig. 5 Fluorescence decays and associated SOE fits for mixed chain derivatives. a) 25 mg dm^{-3} 6′SL (straight chain), b) 25 mg dm^{-3} neohexose (branched chain). Instrument response functions are shown.

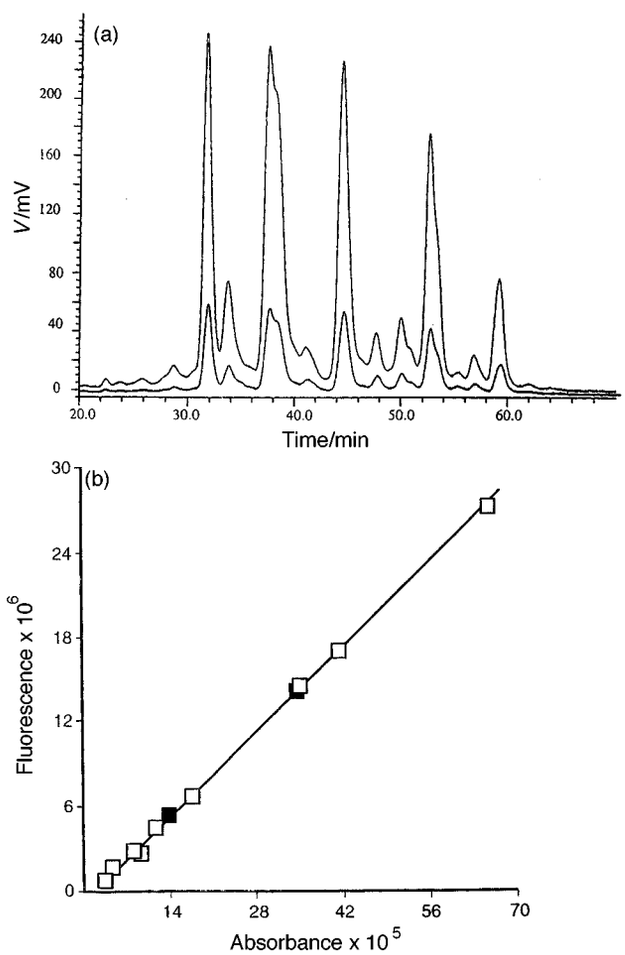


Fig. 6 (a) Chromatograms of the 2-AMAC derivatised glycan mixture released from human IgG, detected by fluorescence (stronger signal) and absorbance (weaker signal); (b) linear plot of the fluorescence and absorbance intensities for individual glycans, ■ sialylated, □ neutral. On the *y*-axis $\lambda_{ex} = 428$ nm, $\lambda_{em} = 525$ nm, pathlength = 1 mm; on the *x*-axis $\lambda = 305$ nm, pathlength = 8 mm.

was about five times more sensitive. Peak areas obtained from both chromatograms were plotted as shown in Fig. 6(b). The straight line that passes through the origin confirms that the two measurements are directly proportional.

In conclusion, we have shown that 2-AMAC is a good choice as a fluorescent tag for a variety of polysaccharides, since its fluorescence does not seem to be sensitive to its molecular environment. It absorbs light at experimentally convenient wavelengths, and fluorescence emission is well separated from suitable excitation frequencies; this separation simplifies discrimination and aids detection of fluorescence. Moreover, we have shown recently [14] that 2-AMAC is suitable for the derivatisation of glycan pools of a total weight as low as 50 ng. Using this fluorophore reliable information can be obtained about the relative proportions of glycans in a mixture, where the lowest level components are normally present at the lower (1 to 5) picomole levels.

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