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Synthesis and fluorescent properties of novel biotinylated labels Prospects for application in bioanalytical detections

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Abstract

Two novel biotinylated fluorophores, (2-biotinyloxyethyl) acridine-9-carboxylate (**4a**) and (2-biotinylamidoethyl) acridine-9-carboxamide (**4b**) have been synthesized and their fluorescent properties examined, in the presence and absence of avidin. In aqueous solutions the biotinylated conjugates **4**, as well as their precursors **3**, exhibit intense fluorescence and can be detected down to nanomolar concentrations. A four-atom spacer, ethylene glycol, in the case of **4a** and ethylene diamine, in the case of **4b** was chosen in order to minimize steric repulsion between two biotin-acridine conjugates adjacently bound on avidin. In the presence of avidin, they show fast and stoichiometric binding to the tetrameric protein. In contrast to most known biotin-fluorophore conjugates, the novel fluorescent labels **4a** and **4b** not only retain their fluorescence after binding to avidin but fluorescence is also enhanced by 37 and 16.5%, respectively, even when four ligands are bound per avidin tetramer. Consequently, these novel biotin-fluorophore conjugates are prospective fluorescent labels in bioanalytical applications.

Keywords: Acridine; Avidin; Biotin; Fluorescence

1. Introduction

Specific detection of immobilized biomolecules is a standard procedure in modern biochemistry [1]. Biotinylated biomolecules such as DNA, peptides and proteins can be recognized by strept(avidin) conjugated with a marker such as fluorescent label. Usually, a biotinylated probe molecule allows its indirect labelling by non-covalent binding of fluorescent (strept)avidin or (strept)avidin and a fluorescent biotin via biotin–(strept)avidin–biotin bridges. The latter seems to be attractive but fluorescent biotin derivatives lose most of their fluorescence when binding to (strept)avidin. However, there are two strategies to overcome this problem: (i) use of long spacers such as poly(ethylene glycol) between biotin and the fluorophore, but these have the disadvantage of relatively fast dissociation [2,3] or (ii) search for a fluorophore where quenching does not happen or even enhancement is observed, for specific reasons. Successful examples for the latter strategy have recently been published [4–9].

Generally, in order to be applicable in bioanalytical detections, the fluorescent labels must fulfil some criteria. They must have: (a) high values of fluorescence efficiency in aqueous solutions; (b) good emission in the bound state [10–12]; and (c) the spacer moiety between the fluorescent molecule and biotin must be of such length, that undesirable steric interactions or quenching effects would be avoided [2,3,9,11,13].

In the present study, the synthesis and fluorescent properties of two novel biotinylated fluorescent compounds, (2-biotinyloxyethyl)-acridine-9-carboxylate (**4a**) and (2-biotinylamidoethyl)-acridine-9-carboxamide (**4b**) are presented, which fulfil some of the above-mentioned

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criteria. Their fluorescent properties are compared to those of the non-biotinylated precursors **3a** and **3b** to show the effect of the biotin group on the fluorescence efficiency of the initial fluorophore. The novel fluorescent labels **4** form 4:1 complexes, like simple biotin, with the protein avidin and, in contrast to most other fluorescent biotins, they exhibit enhanced fluorescence intensity after binding to avidin.

2. Experimental

2.1. Equipment

^{1}H and ^{13}C NMR spectra were measured on a Brucker AC 250 spectrometer with tetramethylsilane as an internal standard. ESI mass spectra were recorded on a Finnigan spectrometer, AQA navigator. Infrared spectra were obtained using a Perkin-Elmer 283 FT-IR spectrometer. Elemental analyses were run on a Perkin-Elmer CHN 2004 instrument. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. Absorption spectra were run on a JASCO V-560 spectrophotometer and fluorescence spectra on a JASCO FP-777 spectrofluorimeter (scan speed 200 nm min^{-1}, emission bandwidth 5 nm).

2.2. Reagents

Most of the reagents used in the present work were purified prior to their use. *N*,*N*-dimethylformamide, ethylenediamine, ethyleneglycol, isobutyl chloroformate and thionylchloride were used after drying and distillation, d-biotin and 1,1′-carbonyldiimidazole (CDI) were dried under reduced pressure over P_2O_5 for 24 h before use, while dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and affinity purified avidin were used without further purification. All the above reagents were purchased from Aldrich. Affinity purified and lyophilised streptavidin was purchased from Sigma. Phosphate buffer stock solution (PBS) was prepared from Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (140 mM) and KCl (2.7 mM), and maintained in refrigerator at 4 °C after filtering over 0.45 μm Whatman paper. Working solutions of PBS were daily prepared by 1:10 dilution of the stock solution and adjusted to pH 7.4.

2.3. Syntheses

2.3.1. Acridine-9-carbonyl chloride (2)

This compound was prepared following known procedure [12]. Specifically, a sample of acridine-9-carboxylic acid hydrate (**1**) (5.19 g, 30 mmol) was dissolved in 50 ml of thionylchloride and the solution was heated under reflux for 6 h. The excess of thionylchloride was evaporated under reduced pressure and the residue was collected and used without any further purification.

2.3.2. (2-Hydroxyethyl)-acridine-9-carboxylate (3a)

Acridine-9-carbonyl chloride (**2**) (920 mg, 3.3 mmol) was dissolved in a large excess of ethylene glycol (20 ml) containing 4 ml triethylamine. The mixture was first stirred for 12 h at room temperature and then heated at 100 °C for 4 h. After cooling, the residue was dissolved in 100 ml chloroform and 100 ml saturated $NaHCO_3$, the organic phase was separated and dried over $MgSO_4$. The solvents were evaporated under reduced pressure and the product was purified by column chromatography (silica gel, chloroform–methanol, 15:1, R_f = 0.57). Chemical yield (565 mg, 94%); mp: 184–186 °C; UV–vis (H_2O): λ_{max}, 350, 363, 384 nm; fluorescence (H_2O): λ_{max}, 440 nm, λ_{exc}, 363 nm; IR (KBr): ν_{max}, 3190, 3044, 2978, 2951, 2867, 2848, 1728 (CO), 1611, 1519, 1461, 1450, 1294, 1176, 1022, 762 cm^{-1}; ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.24 (d, 2H, J = 8.6 Hz), 8.13 (d, 2H, J = 8.2 Hz), 7.93 (m, 2H), 7.73 (m, 2H), 5.08 (m, 1H), 4.68 (m, 2H), 3.82 (m, 2H); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 166.98 (C=O), 147.74, 136.91, 130.84, 129.43, 127.58, 125.13, 121.39, 67.87, 58.95; MS (*m*/*z*, %): 268 (M^++1, 15), 267 (M^+, 92), 223 (77), 206 (59), 178 (100), 167 (23), 151 (42), 75 (30); Anal. Calc. for $C_{16}H_{13}NO_3$ (267.284): C 71.91 H 4.87, N 5.24; found C 71.81, H 4.66, N 5.44.

2.3.3. (2-Aminoethyl)-acridine-9-carboxamide (3b)

Acridine-9-carbonyl chloride (**2**) (1.18 g, 4.24 mmol) was added slowly, in small portions and under ice bath cooling, to a mixture of ethylenediamine (20 ml) and 4 ml triethylamine. The mixture was stirred for 24 h at room temperature. A saturated ammonium chloride solution (50 ml) was added and the mixture was extracted with chloroform (3 × 30 ml). The organic extracts were collected, dried over magnesium sulphate and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, chloroform–methanol, 10:1, R_f = 0.17). Chemical yield (450 mg, 40%); mp: 161–164 °C; UV–vis (H_2O): λ_{max}, 350, 361, 385 nm; fluorescence (H_2O): λ_{max}, 440 nm, λ_{exc}, 363 nm; IR (KBr): ν_{max} 3406, 3246, 3053, 2930, 2870, 1638 (CO), 1547, 1518, 1260, 856, 752, 645 cm^{-1}; ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.69 (t, 1H, J = 4.95 Hz), 8.13 (d, 2H, J = 8.66 Hz), 8.02 (d, 2H, J = 8.66), 7.75 (m, 2H), 7.53 (m, 2H), 3.69 (m, 2H), 3.02 (t, 2H, J = 5.77 Hz, CH_2NH_2); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 166.01 (C=O), 148.16, 142.55, 130.57, 129.20, 126.66, 125.13, 121.75, 42.96, 41.39; MS (*m*/*z*): 266.2 (M^+ + 1); Anal. Calc. for $C_{16}H_{15}N_3O$ (265.316): C 72.43, H 5.70, N 15.84; found C 72.25, H 5.63, N 15.60.

2.3.4. (2-Biotinyloxyethyl)-acridine-9-carboxylate (4a)

d-Biotin (367 mg, 1.5 mmol) was added under argon to 10 ml DMF, containing 50 mg molecular sieves (4 Å) and was heated at 80 °C until biotin was dissolved. Carbonyldiimidazole (243 mg, 1.5 mmol) was added and the mixture was heated at 80 °C for another 15 min until CO_2 evolution ceased. The mixture was then stirred for 2 h at room temperature during which time the intermediate biotinylimidazolide

came out of solution as a flocculent white precipitate. Then, a DMF solution containing 1.5 mmol (400 mg) of the acridine derivative **3a** was added and the mixture was stirred first at room temperature and then at 80 °C for 4 h. The solvents were evaporated under reduced pressure and the product was purified by column chromatography (silica gel, chloroform–methanol, 30:1, $R_f = 0.14$). Chemical yield (330 mg, 45%); UV–vis (H_2O): λ_{max} 350, 363, 384 nm; fluorescence (H_2O): λ_{max}, 445 nm, λ_{exc}, 363 nm; IR (KBr): ν_{max} 3190, 3076, 2926, 2856, 1730, 1701 (CO), 1519, 1461, 1440, 1371, 1290, 1263, 1166, 1209, 1028, 758, 645 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$): δ, 8.25 (d, 2H, $J = 8.93$ Hz), 8.04 (d, 2H, $J = 8.56$ Hz), 7.80 (m, 2H), 7.59 (m, 2H), 5.80 (s, NH), 5.32 (s, NH), 4.84 (m, 2H), 4.51 (m, 2H), 4.33 (m, 1H), 4.10 (m, 1H), 2.98–2.59 (complex area, 3H), 2.37 (t, 2H, $J = 7.44$ Hz), 1.69–1.32 (complex area, 6H); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 173.26 (C=O), 167.23 (C=O), 163.53 (C=O), 148.57, 136.37, 130.44, 129.90, 127.27, 125.01, 122.33, 63.75, 61.92, 61.78, 59.99, 55.24, 40.47, 33.70, 28.60, 28.13, 27.94; MS (*m*/*z*, %): 495.2 ($M^+ + 2$, 13), 494.2 ($M^+ + 2$, 37); Anal. Calc. for $C_{26}H_{27}N_3O_5S$ (493.584): C 63.26 H 5.51, N 8.51, found: C 63.50, H 5.62, N 8.12.

2.3.5. (2-Biotinylamidoethyl) acridine-9-carboxamide (4b)

d-Biotin (367 mg, 1.5 mmol) was added under argon to 10 ml DMF, containing 50 mg molecular sieves (4 Å) and was heated at 80 °C until biotin was dissolved. Carbonyl diimidazole (243 mg, 1.5 mmol) was added and the mixture was heated at 80 °C for another 15 min until CO_2 evolution ceased. The mixture was then stirred for 2 h at room temperature during which time the intermediate biotinylimidazolide came out of solution as a flocculent white precipitate. Then, a DMF solution containing 1.51 mmol (400 mg) of the acridine derivative **3b** was added and the mixture was stirred first at room temperature and then at 80 °C for 4 h. The solvents were evaporated under reduced pressure and the product was purified by column chromatography (silica gel, chloroform–methanol, 3:1, $R_f = 0.59$). Chemical yield (305 mg, 40%); UV–vis (H_2O): λ_{max} 350, 361, 385 nm; fluorescence (H_2O), λ_{max}, 445 nm, λ_{exc}, 361 nm; IR (KBr): ν_{max} 3281, 3065, 2930, 2860, 1705 (CO), 1650 (C=O), 1540, 1535, 1462, 1440, 1340, 1320, 1242, 1200, 1163, 1028, 752, 670 cm^{-1}; ^1H NMR (250 MHz, DMSO): δ 9.08 (m, 1H), 8.19 (d, 2H, $J = 8.66$ Hz), 8.02 (d, 2H, $J = 7.84$ Hz), 7.89 (m, 2H), 7.67 (m, 2H), 6.45 (s, 1H, NH), 6.38 (s, 1H, NH), 4.29 (m, 1H), 4.12 (m, 1H), 3.55 (m, 4H), 3.09–2.80 (complex area, 4H), 2.70–2.09 (complex area, 4H), 1.69–1.24 (complex area, 6H); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 172.42 (C=O), 166.21 (C=O), 162.75 (C=O) 148.18, 142.26, 130.70, 129.21, 126.75, 125.84, 121.73, 61.07, 61.01, 60.7, 59.19, 55.43, 55.41, 40.47, 35.35, 28.27, 28.14; MS (*m*/*z*, %): 494.2 ($M^+ + 3$, 13), 493.2 ($M^+ + 2$, 42), 492.3 ($M^+ + 1$, 93); Anal. Calc. for $C_{26}H_{29}N_5O_3S$ (491.613): C 63.52, H 5.94, N 9.76, found C 62.95, H 5.82, N 9.32.

2.4. Fluorescence measurements

2.4.1. Fluorescence quantum yields and determination of detection limits

For the determination of fluorescence quantum yields, compounds **3a**, **3b** and **4a**, **4b** were dissolved in anhydrous DMF at concentrations 10^{-3} M (stock solutions) and diluted in various solvents so that the absorbance at 362 nm was less than 0.05. All diluted samples were deoxygenated with argon for 5 min before measuring their fluorescence. The measured areas between 380 and 600 nm of the fluorescence spectra of compounds **3a**, **3b** and **4a**, **4b** as well as this of the reference compound were used for the estimation of fluorescence quantum yields. Methyl acridine-9-carboxylate was employed as reference compound. Its fluorescence quantum yield in ethanol is equal to 0.030 [14] and its fluorescence spectra are similar to those of the compounds **3a**, **4b** and **4a**, **4b**. The fluorescence quantum yields were calculated by the equation: $\Phi_u = (F_u A_s n_u^2 / F_s A_u n_s^2) \times \Phi_s$ [15] where Φ_u and Φ_s are the fluorescence quantum yields of unknown and reference compound; F_u and F_s the integrated emission area of the unknown and reference compound between 380 nm and 600 nm; A_u and A_s the absorbance of the unknown and standard compound at the excitation wavelength; absorbances of the sample and reference were adjusted to the same value to avoid errors due to inner filter effects [16]; n_u and n_s the refractive indexes of the solvents containing the unknown and reference compound. Following the procedure described above, we were able to determine the fluorescence quantum yield of methyl 9-acridine carboxylate in water.

For the determination of the detection limit of the compounds **3a**, **3b** and **4a**, **4b** a solution of 10^{-4} M was prepared by diluting 1 ml aliquot of the stock solution (10^{-3} M) in anhydrous DMF to 10 ml with distilled water. By sequential dilutions with distilled water, solutions of 10^{-5} to 10^{-11} M of the compounds **3** and **4** were prepared. The fluorescence measurements were performed with $\lambda_{ex} = 362$ nm, $\lambda_{em} = 444$ nm and excitation and emission slits equal to 5 nm.

2.4.2. Determination of avidin functional concentration

The primary standard solution of 10 μM d-biotin was prepared by dissolving 99% pure d-biotin in DMSO (10^{-3} M), followed by dilution of 0.1 ml aliquots in 10 ml PBS buffer. The concentration of functional avidin was determined by titration with d-biotin using a known procedure [17]. In a typical experiment, avidin was dissolved in PBS buffer at 4 μM (stock solution, nominal concentration by weight). 3 ml of avidin solution in PBS (100 nM, nominal concentration) was pipetted into a 4 ml fluorimeter cell and titrated with a 10 μM biotin standard solution by successive additions of 10 μl increments at 1 min intervals, using a Hamilton syringe. The decrease in tryptophan fluorescence was monitored at 350 nm with excitation at 290 nm. Additions were made until no further decrease in fluorescence was observed. The breakpoint between the progressive quenching and the

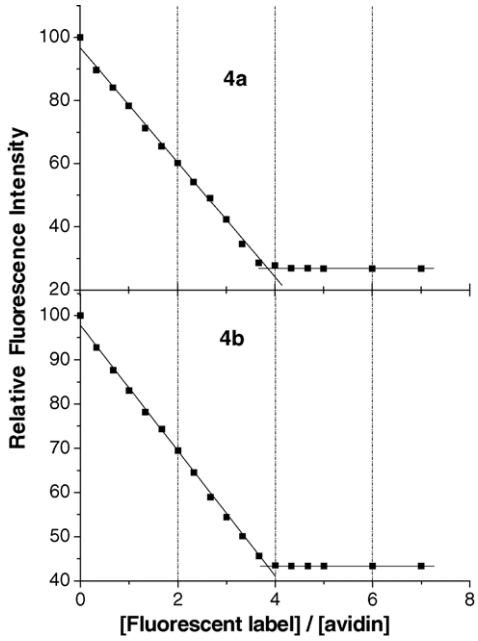


Fig. 1. Dependence of the relative fluorescence intensity of avidin at various concentrations of biotinylated labels, **4a** and **4b**.

subsequent plateau indicates the amount of d-biotin needed for saturation of all biotin-binding sites.

The functional concentration of the fluorescent conjugates **4a** and **4b** was determined with the same method, using the standardized avidin solution. Typically, 3 ml of the standardized avidin solution in PBS (100 nM) was titrated by successive additions of 10 μl aliquots of conjugates **4a** or **4b** in PBS (10 μM). The fluorescence intensity was monitored at 350 nm with excitation at 290 nm. The decrease in tryptophan fluorescence at 350 nm continued until the ligand/avidin concentration ratio attained the value of nearly 4/1 (Fig. 1).

*2.4.3. Fluorescence measurements of the biotinylated conjugates **4a** and **4b** in the presence and absence of avidin*

The effect of avidin on the fluorescence of the novel biotinylated conjugates **4a** or **4b**, was determined following fluorescence measurements. In a typical "forward titration", 3 ml of a PBS standardized avidin solution (100 nM) (solid squares, Fig. 2) or 3 ml of a PBS buffer solution (solid triangles, Fig. 2) was titrated with a 10 μM standardized biotinylated conjugate **4a** or **4b** solution in PBS buffer by successive additions of 10 μl increments. Further, in a parallel control experiment, 3 ml pre-saturated avidin (100 nM) with a 80-fold excess of d-biotin was titrated by successive additions of 10 μl aliquots of a 10 μM biotin fluorescent derivatives **4a** or **4b** solution in PBS buffer (white circles, Fig. 2). Previously, optimization fluorescence experiments were made to determine the time required for equilibration at room temperature between the biotinylated conjugate and avidin. Five minute was the time required for the equilibrium to be reached, so this was the time interval between successive additions of

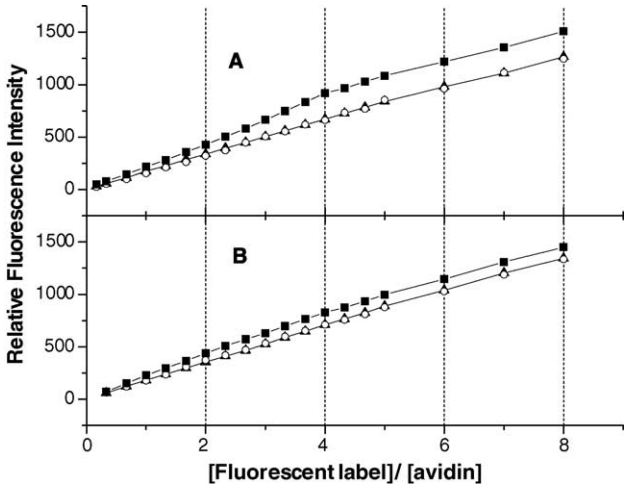


Fig. 2. Relative fluorescense intensities of biotinylated labels **4a** and **4b**, in the presence (■) and absence (▲) of avidin. In parallel runs, avidin had been blocked with 80 μM biotin before titration with fluorescent ligand (◯). 100 nM avidin were titrated with 10 μM **4a** and with 10 μM **4b**.

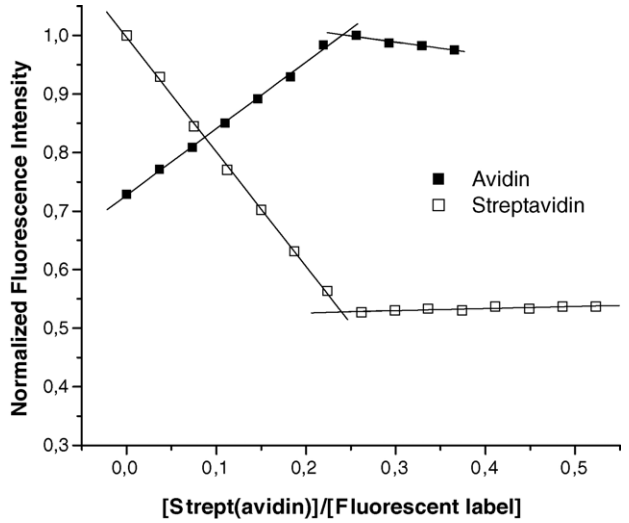


Fig. 3. Inverse titration of 400 nM **4a** with 2.2 μM avidin or 3.4 μM streptavidin stock solutions; (similar results observed for **4b** using avidin).

the biotinylated conjugates. The excitation wavelength on the monochromator was set at 362 nm while the fluorescence intensity was monitored at 444 nm. In both cases, the bandwidth was set at 5 nm. In the "reverse titration" mode, 3 ml of standardized fluorescent label **4a** or **4b** (400 nM) in PBS was titrated with a stock solution of standardized avidin by successive additions of 20 μl increments at 5 min intervals Fig. 3.

3. Results and discussion

The primary aim of this study was to determine all the fluorescence properties of the novel biotinylated labels **4a** and **4b** as well as these of their non-biotinylated precursors **3a**

and **3b** in pure solvents and to compare them. Further, it was of interest to determine the effect that proteins have on the fluorescence of the labels **4a** and **4b** after binding to them. As mentioned in the introduction, fluorescent labels must have high fluorescence quantum yields in aqueous solutions, in order to be applicable in bioanalytical assays. The acridine-9-carboxylic acid was chosen as the light emitting group of the label, due to the relative high fluorescence efficiency of acridine derivatives in aqueous solutions [18]. Because of the wide range of applications that the avidin–biotin system has in modern bioscience and the advantages that this presents over conventional detection methods [1], biotin was chosen to be the reactive group of the fluorescent labels. Furthermore, having in mind that in many cases derivatization of the initial fluorescent group affects its fluorescence, we have chosen two different kinds of reagents as spacer moieties, a diol (ethylene glycol) and a diamine (ethylene diamine) to compare their effect on the fluorescence. These four-atom spacers were used in order to minimize steric repulsion between adjacently bound acridine residues, achieving specific, strong binding to avidin and conservation of the initial fluorescence of label.

3.1. Synthesis of biotinylated acridines *4a* and *4b*

The synthesis of the fluorescent biotinylated acridine labels **4** is carried out in three steps (Scheme 1). In the first step, acridine-9-carboxylic acid (**1**) is converted to its acridine-9-carbonyl chloride (**2**) by its reaction with thionylchloride by a known method [19]. After removing the excess of thionylchloride the crude product was allowed to react with ethylene glycol or ethylene diamine giving the ester **3a** and the amide **3b** in good yields. For the biotinylation of acridine derivatives **3**, different methods were tested ranging from the DCC [20], chloroformate [21], NHS activated biotin-ester [22] to the CDI-method [23]. The highest chemical yields were obtained by the last method which was, therefore, chosen for the preparation of the derivatives **4**.

Table 1
Fluorescence quantum yields of biotinylated acridines **4a** and **4b** and their precursors **3a** and **3b** in protic and non-protic solvents

Compound	Fluorescent quantum yield[a]			
	Ethanol	Chloroform	DMF	Water
3a	0.024	0.031	0.0086	0.456
3b	0.022	0.012	0.0066	0.304
4a	0.020	0.028	0.0074	0.184
4b	0.014	0.013	0.0077	0.118

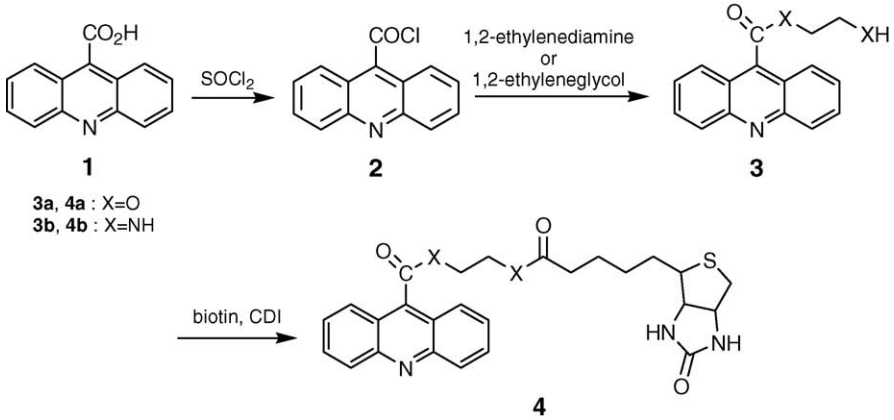
[a] Calculated by the equation $\Phi_u = (F_u A_s n_u^2 / F_s A_u n_s^2) \times \Phi_s$ [15].

The conventional synthetic route to fluorescent conjugates usually starts with the preparation of the biotinylated spacer arm and finishes with the attachment of the fluorophore to it [2,3,13].

In our case, we preferred to follow the opposite synthetic route because firstly, acridine-9-carboxylic acid is much less expensive than biotin and secondly, in that way we could synthesize novel fluorescent molecules **3a**, **3b**. These compounds having a free reactive group can easily be activated and transformed into fluorescent probes that could possibly be used in analytical methods. For instance, the hydroxyl group of **3a** can be converted to an activated carbamate moiety after reaction with 1,1′-carbonyldiimidazole (CDI). Likewise, **3b** can be converted to the corresponding urea. Both of these fluorescent derivatives could be applied in the detection of free amine groups in a sample.

3.2. Fluorescent properties

The fluorescence efficiency of compounds **3** and **4** is strongly affected by the solvent used. Generally, the fluorescence quantum yields of all derivatives in water are up to 20-fold higher than in ethanol or chloroform and about 100-fold higher than in DMF (Table 1). The highest efficiency is observed in aqueous solutions (Φ_{FL} = 0.118–0.456) and the lowest in polar aprotic solvents, such as *N*,*N*-dimethylformamide (Φ_{FL} = 0.0066–0.0086). This observation is very important if these molecules are to be used in analytical applications in



Scheme 1. Synthetic route of the heterobifunctional fluorescent labels **4a** and **4b**.

Table 2
Analytical characteristics of the biotinylated acridines **4a** and **4b** and their non-biotinylated precursors **3a** and **3b** associated with fluorescence measurements

Compound	Fluorescence[a] (arbitrary units)	Linear range[b] (M)	Detection limit[c] (M)	*A*	*B*	S.D.	*r*
3a	1513	10^{-7}–10^{-9}	7.10×10^{-10}	8.039	0.698	0.029	0.9990
3b	671	10^{-7}–10^{-9}	4.55×10^{-10}	6.268	0.494	0.016	0.9994
4a	276	10^{-6}–10^{-8}	2.51×10^{-9}	5.979	0.503	0.038	0.9967
4b	286	10^{-6}–10^{-8}	7.16×10^{-9}	7.355	0.700	0.035	0.9981

[a] Referred to sample concentration of 10^{-7} M in water; λ_{em} = 444 nm, λ_{ex} = 362 nm.
[b] Calculated from the equation: $\log I_{FL} = A + B\log C$.
[c] As detection limit was considered the analyte concentration which gives signals three times greater than blank sample (pure solvent).

aqueous solutions. As it is further shown in Table 1, it is not only the solvent that has an impressive effect on the fluorescence, but also the spacer molecule and the biotin group. The acridine ester **3a** has higher fluorescence quantum yields in all solvents than the acridine amide **3b**, but these values are lower than those of methyl acridine-9-carboxylate. A comparison of the fluorescence quantum yields of derivatives **3** and **4** in water to that of methyl acridine-9-carboxylate (Φ_{FL} = 0.589) shows that the fluorescence of biotinylated labels **4** has been reduced by up to 80%. Despite the negative effect that the spacer molecules and biotin have on the initial fluorescence of the acridine fluorophore in aqueous solutions, the biotinylated fluorescent labels **4** as well as their precursors **3** can be detected down to nanomolar concentrations in aqueous solutions (Table 2).

3.3. *Fluorescence properties of biotinylated conjugates **4a** and **4b** before/after binding to avidin*

As mentioned above, most biotin-fluorophore conjugates lose fluorescence when binding to avidin. Therefore, it was of interest to know if this is also true for the novel synthesized biotinylated fluorescenct labels **4a** and **4b** in the presence of avidin. As shown in Fig. 2 (solid squares), the fluorescence of both biotinylated conjugates **4a** and **4b** is not only retained in the presence of avidin, but on the contrary, a steadily increasing enhancement of up to 4:1 of label to avidin was observed. In the case of the conjugate **4a**, the fluorescence is enhanced up to 37% and the fluorescence of **4b** is enhanced up to 16.5%. The highest enhancement is observed at a molar ratio of 4, indicating stoichiometric binding of the biotinylated labels **4a** and **4b** to the tetrameric binding sites of avidin. The stoichiometric binding was our goal when using a four-atom spacer, hoping that the adjacently bound acridine residues would not contact or interact. As it is shown in Fig. 2, the enhancement of the fluorescence remains constant at molar ratios of label to avidin > 4:1, indicating that no further interaction exists between the labels and the tetrameric binding sites of avidin. The absence of non-specific binding is evidenced by the strictly parallel nature of the line that corresponds to the fluorescence intensity at molar ratios of label to avidin > 4:1 to the line that occurs from the corresponding control experiments (white circles, Fig. 2).

It is also important to note that before the above cumulative titrations, optimization experiments were performed of the incubation time required for the equilibration between the biotinylated conjugates **4a** or **4b** and avidin at room temperature. We observed that after 5 min incubation, the fluorescence intensity remained stable even after 90 min of incubation. This indicates the rapid binding of the biotinylated conjugates **4a**, **4b** to avidin (<5 min).

The fluorescence enhancement of the novel biotinylated fluorophores is more obvious in a "reverse titration" mode (Fig. 3). As shown in Fig. 3, in case of derivative **4a**, the highest enhancement is observed at a molar ratio of 0.25 indicating stoichiometric binding of the biotinylated label **4a** to the tetrameric binding sites of avidin. Interestingly, when similar experiment was performed using streptavidin instead of avidin, the addition of 4 equivalent of biotin **4a** to tetrameric binding sites of streptavidin was also observed, but the fluorescence intensity was quenched by 47.3%. This result cannot be explicitly explained. It can only be assumed that the interaction of the fluorophore moiety of the biotinylated derivatives with streptavidin is different from the one with avidin due to the unlike structure and size of the biotin binding sites of these proteins [24].

4. Conclusions

In this paper, we presented the synthesis of two novel fluorescent biotinylated acridine conjugates, containing ethylene glycol or ethylene diamine as spacer moieties and examined their fluorescent properties in the presence and absence of avidin. Despite the reduction of the fluorescence quantum yield that is caused firstly by the derivatization of the acridine-9-carboxylic acid with the diol or the diamine and then by the attachment of the biotin moiety, the novel biotinylated fluorescent conjugates **4** as well as their precursors **3** could be detected down to few nanomolar concentrations in aqueous solutions. This fact makes the fluorescent molecules **3** prospective analytical reagents in aqueous solutions.

In contrast to many known fluorescent conjugates in which quenching of fluorescence is observed in the presence of proteins, the novel biotinylated compounds **4a** and **4b** not only retain their fluorescence in the presence of avidin, a tetrameric protein specific for biotin, but an enhancement up to 37% for

4a and 16.5% for **4b** is also observed. Enhancement of the fluorescence of a biotinylated label after binding to avidin is a phenomenon that has been reported in the literature, so far, only few times [4–9]. These novel fluorescent conjugates present some other advantages: they show stoichiometric ligand binding, as four biotinylated molecules bind to avidin, non-specific binding is absent and the biotinylated conjugate–avidin complex is formed in less than 5 min.

In conclusion, the novel biotinylated fluorescent conjugates **4a** and **4b** combine all these properties that make them prospective fluorescent labels in bioanalytical methods where the avidin–biotin system is used.

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