Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Photochemistry and Photobiology A: Chemistry 181 (2006) 126–131
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

10-(2-Biotinyloxyethyl)-9-acridone A novel fluorescent label for (strept)avidin–biotin based assays

K. Agiamarnioti [a], T. Triantis [a], K. Papadopoulos [a,*], A. Scorilas [b]

[a] *Institute of Physical Chemistry, NCSR "Demokritos", 15310 Athens, Greece*
[b] *Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, 157 01 Athens, Greece*

Received 1 September 2005; received in revised form 25 October 2005; accepted 13 November 2005
Available online 19 December 2005

Abstract

A novel biotinylated fluorophore, 10-(2-biotinyloxyethyl)-9-acridone **3** has been synthesized and its fluorescent properties were examined in the presence and absence of avidin or streptavidin. In aqueous solutions the novel fluorophore **3**, as well as its precursor 10-(2-hydroxyethyl)-9-acridone **2** exhibit intense fluorescence and can be detected down to 8.62×10^{-10} and 1.90×10^{-10} M, respectively. A short spacer was chosen in order to minimize steric repulsion between the adjacently bound to avidin or streptavidin residues of acridone. The novel biotinylated fluorophore **3** exhibits rapid, specific and stoichiometric binding to the four biotin binding sites in avidin or streptavidin tetramers, even at low concentrations (20 nM). Preliminary measurements showed that the new conjugate can be applied for the fluorimetric determination of solid-phase immobilized mouse IgG with detection limits down to 1.3 ng per assay.

Keywords: Acridone; Biotin; Avidin; Streptavidin; Fluorescence

1. Introduction

Biotin-fluorophores have often been used for the detection of biotinylated biomolecules, such as proteins, peptides or DNA via biotin–(strept)avidin–biotin bridges but most of them lose part of their fluorescence when binding to (strept)avidin. Preservation of high fluorescence upon binding to (strept)avidin is a necessary but insufficient criterion for biotinylated fluorophores if they are to be used as labels. Most important is that they must show (a) high values of fluorescence efficiency in aqueous solutions; (b) absence of non-specific binding and (c) high affinity to avidin or streptavidin.

In this paper, we present a novel biotin-fluorophore based on acridone which fulfil all the above mentioned criteria and retains about 60% (or 25%) of its fluorescence after binding to avidin (or streptavidin). A short three-atom spacer was chosen between the fluorescent molecule and biotin in order to minimize steric repulsion between the adjacently bound to avidin or streptavidin residues of acridone which comes in agreement with corresponding studies reported in literature [1–3]. Further, encouraged by our very promising published results in which acridinium–biotin conjugates comprising short spacer moieties can be applied for the detection of biotinylated proteins [4], we also decided to test the novel conjugate **3** for the detection of immobilized biotinylated mouse IgG.

2. Experimental

2.1. Equipment/reagents

^{1}H- and ^{13}C nmr spectra were measured on a Brucker AC 250 spectrometer. ESI Mass spectra were recorded on a Finnigan spectrometer, AQA Navigator at a flow rate of 0.1 ml min^{-1} using a Harvant Syringe pump. Hot nitrogen gas was used for desolvation (Dominic-Hunter UHPLC MS-10). Infrared spectra were obtained using a Perkin-Elmer 283 FT-IR spectrometer. Elemental analyses were obtained with a Perkin-Elmer CHN 2004 instrument. The melting point was recorded on a Gallenkamp apparatus and is uncorrected. Absorption spectra were run on a JASCO V-560 spectrophotometer while fluorescence spectra run on a JASCO FP-777 spectrofluorimeter (Scan speed 200 nm min^{-1}, emission band 5 nm). The solid-phase fluores-

* Corresponding author. Tel.: +30 210 6503647.
E-mail address: kyriakos@chem.demokritos.gr (K. Papadopoulos).

1010-6030/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2005.11.014

cence measurements were performed on a Perkin-Elmer LS-50B luminescence spectrometer.

2.2. Reagents

All solvents used in the present work [*N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), chloroform] were dried and distilled prior to their use. Affinity purified avidin, streptavidin and biotin were purchased from Sigma. Acridone, 10-methyl-9-acridone, ethylene carbonate and *N*,*N*-carbonyldiimidazole were purchashed from Aldrich and used without any further purification. Phosphate buffer, stock solution (PBS) was prepared from Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (140 mM), 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. 10-(2-Hydroxyethyl)-9-acridone, **2**

For the synthesis of this compound a known procedure was followed [5]. In a 100 ml round flask containing 4 ml DMF, acridone (1.0 g, 5.12 mmol), ethylene carbonate (0.8 g, 9.1 mmol) and KOH (11 mg, 0.196 mmol) were added successively and the mixture was stirred under reflux for 6 h. Then, the solvents were evaporated under reduced pressure chloroform was added to the residue and then extracted with water. The product precipitated as a yellow solid in the aqueous layer and was filtered. Chemical yield (400 mg, 33%); m.p.: 192–193 °C; UV–vis (H_2O): λ = 393, 410 nm; fluorescence (H_2O): λ_{em} = 427, 450 nm, λ_{exc} = 393 nm; IR (KBr): ν_{max} 3321, 2972, 2922, 2905, 2874, 1610 (C=O), 1596, 1589, 1498, 1460, 1379, 1350, 1290, 1269, 1182, 1177, 1088, 756, 673 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ 8.34 (dd, 2H, *J* = 7.79, 1.38 Hz), 7.88 (brd, 2H, *J* = 8.71 Hz), 7.80 (ddd, 2H, *J* = 8.71, 7.33, 1.38 Hz), 7.32 (ddd, 2H, *J* = 7.79, 7.33, 0.46 Hz), 5.09 (t, 1H, *J* = 5.5 Hz), 4.59 (t, 2H, *J* = 5.96 Hz), 3.89 (dt, 2H, *J* = 5.96, 5.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 176.45 (C=O), 142.08, 133.87, 126.50, 121.54, 121.15, 116.34, 58.27, 47.54; MS (*m*/*z*, %): 240.2 (M^++1, 67), 196.2 (M^+-43, 100), 167.2 (14); Anal. Calc. for $C_{15}H_{13}NO_2$ (239.26): C 75.30, H 5.47, N 5.85; found C 74.89, H 5.71, N 5.68.

2.3.2. 10-(2-Biotinyloxyethyl)-9-acridone, **3**

Biotin (244 mg, 1 mmol) was added under argon to 5 ml DMF containing 50 mg molecular sieves (4 Å) and was heated at 80 °C until biotin was dissolved. *N*,*N*′-Carbonyldiimidazole (162 mg, 1 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 of 10-(2-hydroxyethyl)-9-acridone (240 mg, 1 mmol) was added at room temperature and stirred at 110 °C for 7 h. The solvents were evaporated under reduced pressure and the product was purified by flash chromatography (silica gel, firstly with chloroform/diethylether/acetone 9:3:1 in order to remove the residual reactant **2** R_f = 0.41, and then chloroform/methanol 3:1 in order to obtain the product). Chemical yield: 230 mg (50%); UV–vis (H_2O): λ = 391, 408 nm; Fluorescence (H_2O): λ_{em} = 425, 448 nm, λ_{exc} = 393 nm; IR (KBr): ν_{max} 3391, 3271, 3074, 2926, 2856, 1732, 1701, 1630, 1607, 1597, 1493, 1462, 1375, 1292, 1265, 1180, 756, 675 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.42 (dd, 2H, *J* = 7.79, 1.38 Hz), 7.62 (ddd, 2H, *J* = 8.71, 7.33, 1.38 Hz), 7.51 (brd, 2H, *J* = 8.71 Hz), 7.18 (ddd, 2H, *J* = 7.79, 7.33, 0.46 Hz), 6.30 (s, NH), 5.96 (s, NH), 4.53 (t, 2H, *J* = 6.87 Hz), 4.43 (t, 2H, *J* = 6.87 Hz), 4.40 (m, 1H), 4.18 (m, 1H), 3.03–2.63 (complex area, 3H), 2.20 (t, 2H, *J* = 7.33 Hz), 1.62–1.32 (complex area, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.61 (C=O), 173.29 (C=O), 163.89 (C=O), 141.68, 133.90, 127.50, 122.17, 121.41, 114.51, 61.81, 60.02, 59.99, 55.32, 43.71, 40.35, 33.48, 28.14, 28.03, 24.34; MS (*m*/*z*, %): 467.2 (M^+ +2, 31), 466.2 (M^+ + 1, 100), 227 (26); Anal. Calc. for $C_{25}H_{27}N_3O_4S$ (465.57): C 64.50 H 5.84, N 9.03, found: C 64.82 H 6.03, N 8.87.

2.4. Fluorescence measurements

2.4.1. Fluorescence quantum yields and detection limits

The fluorescence quantum yields of the compounds **2** and **3** were determined according to a known procedure [6]. 10-Methyl-9-acridone was employed as reference compound considering that its fluorescence spectra are similar to those of the compounds **2** and **3**. Its fluorescence quantum yield in water is equal to 0.82 [7]. The fluorescence quantum yields were calculated by the equation: $\Phi_u = (F_u A_s n_u^2 / F_s A_u n_s^2)\Phi_s$ 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 400 nm and 550 nm; A_u and A_s the absorbance of the unknown and standard compound at the excitation wavelength; n_u and n_s the refractive indexes of the solvents containing the unknown and reference compound. For the determination of the detection limits of the compounds **2** and **3** aqueous solutions of 10^{-4} to 10^{-11} M were prepared by sequential dilutions of a stock solution (10^{-3} M) in anhydrous DMSO with distilled water. The fluorescence measurements were performed at λ_{exc} = 393 nm, λ_{em} = 425 nm and excitation/emission slits equal to 5 nm.

2.4.2. Determination of functional concentrations of avidin/streptavidin and biotinylated compound **3**

The functional concentration of (strept)avidin was determined by titration with D-biotin by monitoring its fluorescence quenching at 350 nm (excitation at 290 nm) [8]. In a typical experiment, avidin or streptavidin was dissolved in PBS buffer at ∼3 μM (stock solution, nominal concentration by weight). Three millilitres of (strept)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 3-min intervals. The breakpoint between the progressive quenching and the subsequent plateau indicates the amount of D-biotin needed for satura-