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ORIGINAL ARTICLE

2-Oxo-2*H*-benzo[*h*]benzopyran as a new light sensitive protecting group for neurotransmitter amino acids

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Abstract Aiming at the development of new benzopyran-based photocleavable protecting groups, novel chloromethylated and hydroxymethylated 2-oxo-2*H*-benzo[*h*]benzopyran derivatives bearing a methoxy substituent were designed and used in the synthesis of a series of fluorescent bioconjugates, by linking through an ester or urethane bond to several model neurotransmitter amino acids (glycine, alanine, β-alanine and γ-aminobutyric acid, GABA). The resulting fluorescent bioconjugates with emission in the visible range and high fluorescent quantum yields, were subjected to photocleavage reaction in methanol/HEPES buffer (80:20) solution at different wavelengths of irradiation (250, 300, 350 and 419 nm) and photocleavage kinetic data were obtained.

Keywords Oxobenzobenzopyran · Coumarin · Neurotransmitters · Amino acids · Photocleavable protecting groups

Introduction

The control over the spatial and temporal sustained release of chemically and biologically relevant species, in connection with chemical, biological and medical research fields, has known a great development in recent years. The triggering agents usually employed can be enzymes (Anderson et al. 2004), pH changes (Lee and Chmielewski 2005), reducing or oxidising agents (Khurana and Arora 2009; Vaxelaire et al. 2009), temperature (Han et al. 2005) and light (Mayer and Heckel 2006); this later being an example of a green chemical process as the release of the relevant species occurs without the need for additional chemical reagents, and as an alternative to classical deprotection methods. Photoactivable molecules represent a recent and fast developing methodology that can find applications in drug delivery, enabling on demand release of a well-defined amount of drug in a predetermined time profile; in the elucidation of signalling processes involving neurotransmitters (amino acids, physiological amines or nucleotides) within cellular systems and in metabolic processes; in multistep organic synthesis, with polyfunctional reagents that require multiple protection/deprotection procedures; in combinatorial and analytical chemistry, among other examples.

2-Oxo-benzopyrans (trivially known as coumarins) have received considerable attention in applied photochemistry due to their photocleavage capabilities. Coumarin derivatives have been reported as suitable photocleavable protecting groups for alcohols (Gilbert et al. 2007), amines (Takaoka et al. 2004), phosphates (Hagen et al. 2005), carboxylic acids (Shembekar et al. 2005), aldehydes and ketones (Lu et al. 2003) in cell biology and biophysical studies. Benzopyrans possess additional features, from which fluorescence stands out, with a broad spectral range and high fluorescence quantum yields. This fact leads to the assembly of fluorescent conjugates whenever benzopyran derivatives are used as photocleavable protecting groups (Piloto et al. 2006a, b; Fonseca et al. 2007; Fernandes et al. 2008a, b).

Neurotransmitter amino acids are a class of compounds where the caging strategy has been largely applied, usually through an ester linkage of a suitable photoreleasable group (including coumarins) to their carboxylic terminal (Fernandes et al. 2007, 2008a, b). Certain amino acids play

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an important role in neuronal communication at the central nervous system (CNS), such as glycine (Gly) and γ-aminobutyric acid (GABA), which act as the main inhibitory transmitters within the mammalian CNS (Banerjee et al. 2003; Curten et al. 2005). β-Alanine (β-Ala) is the only naturally occurring beta amino acid and acts as a physiological transmitter, being the rate-limiting precursor of carnosine, which is a β-alanine-histidine dipeptide present in muscle and brain tissues (Hill et al. 2007). Alanine (Ala) is also an inhibitory neurotransmitter and an agonist of ionotropic glycinergic and GABA receptors (Treffort et al. 2006; Legendre 2001).

In pursuit of our current research interests, which involve the synthesis and application of novel oxygen and nitrogen polycyclic heterocycles, as novel fluorescent tags and photoreleasable protecting groups for the carboxylic acid and amine functions of amino acids (Piloto et al. 2005, 2006a, b; Fonseca et al. 2007; Fernandes et al. 2007, 2008a, b), we are interested in the evaluation and improvement of their photophysical properties (as possible fluorogenic derivatisation reagents) and also in the study of their behaviour towards photocleavage under UV irradiation at different wavelengths (254, 300, 350 and 419 nm), in simulated physiological environment in a methanol/HEPES buffer (80:20) solution. Recent results reported by us indicated that a chloromethylated benzo[*f*]benzopyran was an appropriate fluorescent tagging and photocleavable protecting group for carboxylic acids, with photocleavage at 350 nm occurring smoothly in short times (Fernandes et al. 2008a, b), which is preferable in studies involving biomolecules as it avoids cell damage due to short-wavelength light.

In view of these facts, we now report the synthesis of novel structurally related chloromethylated and hydroxymethylated 2-oxo-2*H*-benzo[*h*]benzopyrans and their application in the synthesis of a set of fluorescent ester and urethane conjugates of neurotransmitter amino acids, such as glycine, alanine, β-alanine and γ-aminobutyric acid. The resulting bioconjugates were irradiated in a photochemical reactor at the above-mentioned wavelengths of irradiation and photocleavage kinetic data were obtained.

Experimental

General

All melting points were measured on a Stuart SMP3 melting point apparatus and are uncorrected. TLC analyses were carried out on 0.25-mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230–240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer using KBr discs. UV–visible absorption spectra (200–800 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H NMR and 75.4 MHz for ^{13}C NMR or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H NMR and 100.6 MHz for ^{13}C NMR using the solvent peak as internal reference at 25°C. All chemical shifts are given in ppm using δ_H $Me_4Si = 0$ ppm as reference, and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities, and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear HMBC and HMQC correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. Photolyses were carried out using a Rayonet RPR-100 chamber reactor equipped with ten lamps of 254 (35 W), 300 (21 W), 350 (24 W) and 419 (14 W) nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 μm) column in a HPLC system composed by a Jasco PU-980 pump, a UV/Vis Shimadzu SPD-GAV detector and a Shimadzu C-RGA Chromatopac register. All reagents were used as received.

Synthesis of 4-(chloromethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**1**)

To a solution of 4-methoxy-1-naphthol (0.501 g, 2.88×10^{-3} mol) in 70% aqueous sulphuric acid (3 mL), ethyl chloroacetoacetate (0.6 mL, 4.36×10^{-3} mol) was added and stirred at room temperature for 4 h. The reaction mixture was poured into ice water and stirred for 2 h to give a fine greenish precipitate. The solid was collected by filtration, washed with cold water and dried in a vacuum oven. After purification by dry flash chromatography, using ethyl acetate/*n*-hexane, mixtures of increasing polarity as eluent, compound **1** was obtained as a green solid (0.358 g, 45%). mp = 180.5–181.9°C. TLC (ethyl acetate/*n*-hexane, 1:1): $R_f = 0.69$. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta_H = 4.07$ (s, 3 H, OCH_3), 4.73 (s, 2 H, CH_2Cl), 6.63 (t, $J = 0.8$ Hz, 1 H, H-3), 6.84 (s, 1 H, H-5), 7.64–7.72 (m, 2 H, H-8 and H-9), 8.25–8.33 (m, 1 H, H-7), 8.49–8.55 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 100.6 MHz): $\delta_C = 41.92$ (CH_2Cl), 55.85 (OCH_3), 96.02 (C-5), 112.58 (C-4a), 115.27 (C-3), 122.27 (C-7), 122.45 (C-10), 124.03 (C-10a), 127.50 (C-6a), 127.94 (C-9), 128.55 (C-8), 146.11 (C-10b), 150.10 (C-4), 152.23 (C-6), 160.56 (C-2). IR (KBr 1%, cm^{-1}): ν = 3,427, 2,934, 2,850, 1,709, 1,601, 1,565, 1,508, 1,476, 1,466, 1,422, 1,388, 1,274, 1,251, 1,175, 1,163, 1,143, 1,110, 1,077, 1,028, 986, 949, 922, 884, 845 and 730. UV/

Vis (ethanol, nm): λ_{max} (log ε) = 379 (3.68). HRMS (EI): calculated for $C_{15}H_{11}O_3^{35}Cl$ [M^+]: 274.0397; found: 274.0395; calculated for $C_{15}H_{11}O_3^{37}Cl$ [M^+]: 276.0367; found: 276.0370.

Synthesis of 6-methoxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran (**2**)

To a solution of 4-methoxy-1-naphthol (0.612 g, 3.51×10^{-3} mol) in 70% aqueous sulphuric acid (6 mL), ethyl acetoacetate (1.4 mL, 1.1×10^{-2} mol) was added and stirred at room temperature for 3 h. The reaction mixture was poured into ice water and stirred for 2 h to give a fine pinkish precipitate. The solid was collected by filtration, washed with cold water and dried in a vacuum oven. After purification by dry flash chromatography, using ethyl acetate/*n*-hexane, mixtures of increasing polarity as eluent, compound **2** was obtained as a beige solid (0.130 g, 15%). mp = 131.1–133.4°C. TLC (ethyl acetate/*n*-hexane, 1:1): $R_f = 0.65$. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta_H = 2.49$ (s, 3 H, CH_3), 4.04 (s, 3 H, OCH_3), 6.35 (d, J = 1.2 Hz, 1 H, H-3), 6.73 (s, 1 H, H-5), 7.70–7.58 (m, 2 H, H-8 and H-9), 8.20–8.28 (m, 1 H, H-7), 8.46–8.53 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 75.4 MHz): $\delta_C = 19.34$ (CH_3), 55.70 (OCH_3), 96.55 (C-5), 114.46 (C-3), 114.94 (C-4a), 122.12 (C-7), 122.37 (C-10), 123.90 (C-10a), 127.22 (C-6a), 127.64 (C-9 or C-8), 128.04 (C-8 or C-9), 145.23 (C-10b), 151.93 (C-6), 153.17 (C-4), 161.13 (C-2). IR (KBr 1%, cm^{-1}): ν = 3,417, 2,926, 1,725, 1,610, 1,596, 1,564, 1,506, 1,469, 1,453, 1,429, 1,386, 1,384, 1,272, 1,248, 1,234, 1,208, 1,176, 1,151, 1,110, 1,083, 1,032, 990, 944, 933, 873, 851 and 815. UV/Vis (ethanol, nm): λ_{max} (log ε) = 371 (3.57). HRMS (EI): calculated for $C_{15}H_{12}O_3$ [M^+]: 240.0786; found: 240.0791.

6-Hydroxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran (**3**)

In the same preparation, compound **3** was also obtained as a greenish solid (0.095 g; 12%). mp = 289.0–291.5°C. TLC (ethyl acetate/*n*-hexane, 1:1): $R_f = 0.35$. ^{1}H NMR (DMSO-d_6, 400 MHz): $\delta_H = 2.49$ (s, 3 H, CH_3), 6.45 (d, J = 1.6 Hz, 1 H, H-3), 7.00 (s, 1 H, H-5), 7.63–7.73 (m, 2 H, H-8 and H-9), 8.19 (dt, J = 8.4 and 1.6 Hz, 1 H, H-7), 8.30 (dt, J = 8.0 and 1.6 Hz, 1 H, H-10), 10.42 (s, 1 H, OH). ^{13}C NMR (DMSO-d_6, 100.6 MHz): $\delta_C = 12.63$ (CH_3), 100.90 (C-5), 114.07 (C-3), 115.40 (C-4a), 121.49 (C-10), 122.45 (C-7), 123.19 (C-10a), 126.32 (C-6a), 127.59 (C-9 or C-8), 127.61 (C-8 or C-9), 143.10 (C-10b), 149.51 (C-6), 153.67 (C-4) and 159.88 (C-2). IR (KBr 1%, cm^{-1}): ν = 3,650-3,186, 2,956, 2,925, 2,854, 1,680, 1,598, 1,563, 1,482, 1,422, 1,403, 1,384, 1,372, 1,348, 1,296, 1,274, 1,259, 1,236, 1,206, 1,176, 1,148, 1,094, 1,078, 1,020, 950, 881, 853, 781, 734 and 665. UV/Vis (ethanol, nm): λ_{max} (log ε) = 380 (3.71). HRMS (EI): calculated for $C_{14}H_{10}O_3$ [M^+]: 226.0630; found: 226.0633.

Compound **3** (0.098 g, 4.35×10^{-4} mol) was reacted with potassium carbonate (0.064 g, 4.65×10^{-4} mol) and methyl iodide (0.029 mL, 4.59×10^{-4} mol) in dry DMF (10 mL), at reflux for 4 h under nitrogen atmosphere. Distilled water (5 mL) was added to the reaction mixture and the aqueous phase extracted with ethyl acetate (4 × 10 mL). The organic phases were combined and washed with NaCl saturated solution, dried with magnesium sulphate, filtered and evaporated under reduced pressure. The methoxy derivative **2** was obtained as a dark brown solid (0.072 g; 70%); the experimental data confirmed its structure and were in accordance with that described above.

Synthesis of 6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-carbaldehyde (**4**)

Compound **2** (0.108 g, 4.50×10^{-4} mol) was reacted with selenium dioxide (0.299 g, 2.70×10^{-3} mol) in chlorobenzene (5 mL), at reflux for 36 h. The mixture was filtered hot, and the solvent was removed by rotary evaporation. After purification by dry flash chromatography, using ethyl acetate/*n*-hexane, mixtures of increasing polarity as eluent, compound **4** was obtained as an orange solid (0.090 g, 79%). mp = 222.5–224.8°C. TLC (chloroform/methanol, 9.5:0.5): $R_f = 0.69$. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta_H = 4.08$ (s, 3 H, OCH_3), 6.94 (s, 1 H, H-5), 7.65–7.72 (m, 2 H, H-8 and H-9), 7.89 (s, 1 H, H-3), 8.25–8.32 (m, 1 H, H-7), 8.48–8.54 (m, 1 H, H-10) and 10.16 (s, 1 H, CHO). ^{13}C NMR ($CDCl_3$, 100.6 MHz): $\delta_C = 55.86$ (OCH_3), 97.28 (C-3), 110.48 (C-4a), 122.27 (C-10), 122.40 (C-7), 123.51 (C-10a), 125.19 (C-5), 127.62 (C-6a), 127.87 (C-9), 128.92 (C-8), 144.19 (C-10b), 147.09 (C-6), 152.66 (C-4), 160.51 (C-2) and 192.13 (CHO). IR (KBr 1%, cm^{-1}): ν = 3,430, 3,066, 2,962, 2,926, 2,850, 1,731, 1,712, 1,630, 1,594, 1,561, 1,504, 1,472, 1,450, 1,418, 1,381, 1,272, 1,247, 1,183, 1,138, 1,110, 1,083, 1,028, 985, 928, 893, 867, 855 and 775. UV/Vis (ethanol, nm): λ_{max} (log ε) = 377 (3.73). HRMS (EI): calculated for $C_{15}H_{10}O_6$ [M^+]: 254.0579; found: 254.0589.

In the same preparation, 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**5**) was also obtained in 10% yield. The experimental data confirmed its structure and were in accordance with that described below.

4-(Hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**5**)

Compound **4** (0.098 g, 3.85×10^{-4} mol) was reacted with sodium borohydride (0.016 g, 4.28×10^{-4} mol) in

chloroform (7 mL) and ethanol (17 mL) for 3 days at room temperature. After purification by dry flash chromatography, using ethyl acetate/*n*-hexane, mixtures of increasing polarity as eluent, compound **5** was obtained as a light yellow solid (0.046 g, 47%). mp = 219.1–220.7°C. TLC (ethyl acetate/*n*-hexane, 7:3): $R_f = 0.60$. ^{1}H NMR (DMSO-d_6, 400 MHz): $\delta_H = 4.02$ (s, 3 H, OCH_3), 4.86 (dd, J = 4.2 and 1.2 Hz, 2 H, CH_2OH), 5.73 (t, J = 4.2 Hz, 1 H, OH), 6.55 (t, J = 1.2 Hz, 1 H, H-3), 6.93 (s, 1 H, H-5), 7.67–7.75 (m, 2 H, H-8 and H-9), 8.19 (dd, J = 8.0 and 1.5 Hz, 1 H, H-7), 8.32 (dd, J = 7.6 and 1.6 Hz, 1 H, H-10). ^{13}C NMR (DMSO-d_6, 100.6 MHz): $\delta_C = 56.01$ (OCH_3), 59.58 (CH_2OH), 97.12 (C-5), 110.22 (C-3), 112.78 (C-4a), 121.60 (C-10), 121.90 (C-7), 123.03 (C-10a), 126.28 (C-6a), 127.92 (C-9), 128.23 (C-8), 144.17 (C-10b), 151.08 (C-6), 157.37 (C-4) and 160.12 (C-2). IR (KBr 1%, cm^{-1}): ν = 3,410, 2,924, 2,853, 1,707, 1,602, 1,567, 1,506, 1,474, 1,451, 1,422, 1,384, 1,355, 1,315, 1,275, 1,250, 1,143, 1,117, 1,097, 1,061, 987, 950, 856, 762, 736 and 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 365 (3.69). HRMS (EI): calculated for $C_{15}H_{12}O_4$ [M^+]: 256.0736; found: 256.0745.

General procedure for the synthesis of conjugates **7a–d**

4-(Chloromethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **1** (1 equiv) was dissolved in dry DMF (2 or 3 mL), potassium fluoride (~3 equiv) and the corresponding amino acid neurotransmitter **6a–d** (1 equiv) was added. The reaction mixture was stirred at room temperature for 1 or 3 days. The solvent was removed by rotary evaporation under reduced pressure, and the crude residue was purified by column chromatography using mixtures of ethyl acetate and light petroleum or *n*-hexane as eluent.

N-(Benzyloxycarbonyl)-L-glycine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester (**7a**)

Compound **1** (0.063 g, 2.30×10^{-4} mol), DMF (2 mL), KF (0.040 g, 6.85×10^{-3} mol) and Z-Gly-OH (0.054 g, 1.21×10^{-4} mol) were used, and the reaction time was 1 day. Mixtures of increasing polarity of ethyl acetate/light petroleum were used as the dry flash chromatography eluent to give compound **7a** as a yellow solid (0.054 g, 53%). mp = 173.5–175.1°C. TLC (ethyl acetate/light petroleum, 4:6): $R_f = 0.48$. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta_H = 4.04$ (s, 3 H, OCH_3), 4.16 (d, J = 6.0 Hz, 2 H, CH_2 Gly), 5.16 (s, 2 H, CH_2 Z), 5.34 (br s, 1 H, α-NH Gly), 5.43 (s, 2 H, CH_2), 6.56 (s, 1 H, H-3), 6.61 (s, 1 H, H-5), 7.28–7.40 (m, 5 H, 5 × Ar–H Z), 7.64–7.72 (m, 2 H, H-8 and H-9), 8.25–8.30 (m, 1 H, H-7), 8.48–8.54 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 100.6 MHz): $\delta_C = 42.80$ (CH_2 Gly), 55.90 (OCH_3), 62.37 (CH_2), 67.35 (CH_2 Z), 95.17 (C-5), 112.05 (C-4a), 112.95 (C-3), 122.27 (C-7), 122.41 (C-10), 123.97 (C-10a), 127.41 (C-6a), 127.99 (C-9 and Ar–C), 128.15 (Ar–C), 128.28 (Ar–C), 128.52 (Ar–C), 128.55 (C-8 and Ar–C), 135.99 (Ar–C), 145.79 (C-10b), 148.86 (C-4), 152.38 (C-6), 156.32 (C=O urethane), 160.55 (C-2) and 169.47 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,315, 2,925, 1,735, 1,687, 1,600, 1,538, 1,475, 1,455, 1,422, 1,386, 1,291, 1,197, 1,110, 1,085, 1,060, 989 and 750. UV/Vis (ethanol, nm): λ_{max} (log ε) = 376 (3.72). HRMS (EI): calculated for $C_{25}H_{22}NO_7$ [M^+]: 448.14095; found: 448.13908.

N-(Benzyloxycarbonyl)-L-alanine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester (**7b**)

Compound **1** (0.070 g, 2.55×10^{-4} mol), DMF (3 mL), KF (0.045 g, 7.75×10^{-4} mol) and Z-Ala-OH (0.058 g, 2.59×10^{-4} mol) were used and the reaction time was 1 day. Mixtures of increasing polarity of ethyl acetate/*n*-hexane were used as the dry flash chromatography eluent to give compound **7b** as a yellow solid (0.101 g, 86%). mp = 153.3–154.7°C. TLC (ethyl acetate/*n*-hexane, 1:1): $R_f = 0.87$. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta_H = 1.53$ (d, J = 7.2 Hz, 3 H, β-CH_3 Ala), 4.00 (s, 3 H, OCH_3), 4.49–4.59 (m, 1 H, α-CH Ala), 5.07–5.17 (m, 2 H, CH_2 Z), 5.28–5.47 (m, 3 H, CH_2 and α-NH Ala), 6.52 (s, 1 H, H-3), 6.53 (s, 1 H, H-5), 7.25–7.40 (m, 5 H, 5 × Ar–H Z), 7.60–7.70 (m, 2 H, H-8 and H-9), 8.23 (d, J = 9.2 Hz, 1 H, H-7) and 8.45 (d, J = 9.2 Hz, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 100.6 MHz): $\delta_C = 18.23$ (β-CH_3 Ala), 49.77 (α-CH Ala), 55.79 (OCH_3), 62.36 (CH_2), 67.09 (CH_2 Z), 95.11 (C-5), 111.98 (C-4a), 112.78 (C-3), 122.21 (C-10), 122.27 (C-7), 123.83 (C-10a), 127.27 (C-6a), 127.88 (C-9 and Ar–C), 128.09 (Ar–C), 128.18 (C-8 and Ar–C), 128.40 (Ar–C), 128.48 (Ar–C), 136.02 (Ar–C), 145.59 (C-10b), 148.94 (C-4), 152.20 (C-6), 155.67 (C=O urethane), 160.47 (C-2) and 172.37 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,312, 2,928, 1,758, 1,714, 1,598, 1,566, 1,551, 1,506, 1,473, 1,455, 1,422, 1,387, 1,345, 1,311, 1,276, 1,252, 1,211, 1,178, 1,161, 1,116, 1,085, 1,073, 1,030, 986, 959, 856 and 815. UV/Vis (ethanol, nm): λ_{max} (log ε) = 375 (3.72). HRMS (EI): calculated for $C_{26}H_{24}NO_7$ [M^+]: 462.15467; found: 462.15473.

N-(Benzyloxycarbonyl)-L-β-alanine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester (**7c**)

Compound **1** (0.087 g, 3.16×10^{-4} mol), DMF (2 mL), KF (0.055 g, 9.49×10^{-4} mol) and Z-β-Ala-OH (0.074 g, 3.29×10^{-4} mol) were used and the reaction time was 3 days. Mixtures of increasing polarity of ethyl acetate/*n*-hexane were used as the dry flash chromatography eluent to give compound **7c** as a yellow solid (0.099 g, 70%).

mp = 119.9–122.6°C. TLC (ethyl acetate/*n*-hexane, 1:1): $R_f = 0.64$. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta_H = 2.74$ (t, J = 6.0 Hz, 2 H, α-CH_2 β-Ala), 3.56 (q, J = 6.0 Hz, 2 H, β-CH_2 β-Ala), 3.94 (s, 3 H, OCH_3), 5.10 (s, 2 H, CH_2 Z), 5.26 (s, 2 H, CH_2), 5.41 (br s, 1 H, NH), 6.44 (s, 1 H, H-3), 6.46 (s, 1 H, H-5), 7.24–7.38 (m, 5 H, 5 × Ar–H Z), 7.56–7.66 (m, 2 H, H-8 and H-9), 8.12–8.20, (m, 1 H, H-7) and 8.34–8.42 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 75.4 MHz): $\delta_C = 34.34$ (α-CH_2 β-Ala), 36.46 (β-CH_2 β-Ala), 55.68 (OCH_3), 61.55 (CH_2), 66.71 (CH_2 Z), 94.90 (C-5), 111.85 (C-4a), 112.25 (C-3), 122.11 (C-7 and C-10), 123.66 (C-10a), 127.09 (C-6a), 127.77 (Ar–C), 128.00 (C-9), 128.05 (C-8), 128.27 (2 × Ar–C), 128.41 (2 × Ar–C), 136.26 (Ar–C), 145.36 (C-10b), 149.20 (C-4), 152.02 (C-6), 156.23 (C=O urethane), 160.45 (C-2), 171.42 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,346, 2,936, 1,721, 1,643, 1,614, 1,599, 1,565, 1,529, 1,507, 1,474, 1,455, 1,422, 1,385, 1,312, 1,274, 1,249, 1,170, 1,143, 1,112, 1,087, 1,029, 989, 951, 860 and 770. UV/Vis (ethanol, nm): λ_{max} (log ε) = 375 (3.75). HRMS (EI): calculated for $C_{26}H_{24}NO_7$ [M^+]: 462.15600; found: 462.15473.

N-(Benzyloxycarbonyl)-L-γ-aminobutyric acid (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester (**7d**)

Compound **1** (0.097 g, 3.55×10^{-4} mol), DMF (3 mL), KF (0.065 g, 1.12×10^{-3} mol) and Z-GABA-OH (0.087 g, 3.67×10^{-4} mol) were used, and the reaction time was 3 days. Mixtures of increasing polarity of ethyl acetate/light petroleum were used as the dry flash chromatography eluent to give compound **7d** as a yellow solid (0.095 g, 56%). mp = 144.5–146.4°C. TLC (ethyl acetate/light petroleum, 1:1): $R_f = 0.74$. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta_H = 1.84$–2.0 (m, 2 H, β-CH_2 GABA), 2.56 (t, J = 7.2 Hz, 2 H, α-CH_2 GABA), 3.30 and 3.33 (2 × d, J = 6.4 and 6.8 Hz, 2 H, γ-CH_2 GABA), 4.01 (s, 3 H, OCH_3), 4.96 (br s, 1 H, α-NH GABA), 5.10 (s, 2 H, CH_2 Z), 5.34 (s, 2 H, CH_2), 6.55 (s, 1 H, H-3), 6.58 (s, 1 H, H-5), 7.28–7.38 (m, 5 H, 5 × Ar–H Z), 7.62–7.70 (m, 2 H, H-8 and H-9), 8.26 (d, J = 9.2 Hz, 1 H, H-7) and 8.49 (d, J = 9.6 Hz, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 100.6 MHz): $\delta_C = 25.17$ (β-CH_2 GABA), 31.16 (α-CH_2 GABA), 40.22 (γ-CH_2 GABA), 55.85 (OCH_3), 61.51 (CH_2), 66.72 (CH_2 Z), 95.15 (C-5), 112.08 (C-4a), 112.36 (C-3), 122.22 (C-7), 122.34 (C-10), 123.92 (C-10a), 127.31 (C-6a), 127.91 (Ar–C), 128.05 (Ar–C), 128.09 (C-9 and Ar–C), 128.40 (Ar–C), 128.47 (C-8 and Ar–C), 136.41 (Ar–C), 145.63 (C-10b), 149.58 (C-4), 152.24 (C-6), 156.45 (C=O urethane), 160.68 (C-2) and 172.36 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,310, 2,927, 1,737, 1,715, 1,692, 1,652, 1,565, 1,556, 1,505, 1,455, 1,385, 1,321, 1,266, 1,249, 1,174, 1,137, 1,110, 1,083, 1,030, 945, 828, 772, 737 and 665. UV/Vis (ethanol, nm): λ_{max} (log ε) = 373 (3.66). HRMS (EI): calculated for $C_{27}H_{26}NO_7$ [M^+]: 476.1717; found: 476.1704.

General procedure for the removal of *N*-benzyloxycarbonyl group in conjugates **7a–c**

A 45% solution of hydrobromic acid in acetic acid (20 μL) and acetic acid (1 mL) were added to the amino acid conjugates **7a–c**, with stirring, at room temperature. The reaction mixture was maintained in these conditions for 3 days, and the process was followed by TLC (ethyl acetate/*n*-hexane; 6:4). During this time, additional amounts of 45% solution of hydrobromic acid in acetic acid were added, and the total volume for each compound was mentioned below. When the reaction was completed, cold diethyl ether was added (0.5 mL), and the precipitate filtered off and washed with the same solvent to give a light yellow solid.

L-Glycine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester hydrobromide (**8a**)

Starting from conjugate **7a** (0.025 g, 5.59×10^{-5} mol) and using 45% solution of hydrobromic acid in acetic acid (110 μL), compound **8a** was obtained (0.004 g, 18%). mp = 194.8–195.9°C. TLC (ethyl acetate/methanol, 4:3): $R_f = 0.54$. ^{1}H NMR (CD_3OD, 400 MHz): $\delta_H = 4.13$ (s, 5 H, α-CH_2 Gly and OCH_3), 5.74 (s, 2 H, CH_2), 6.66 (s, 1H, H-3), 6.95 (s, 1 H, H-5), 7.70–7.80 (m, 2 H, H-8 and H-9), 8.31–8.37 (m, 1 H, H-7) and 8.46–8.51 (m, 1 H, H-10). ^{13}C NMR (CD_3OD, 100.6 MHz): $\delta_C = 41.07$ (α-CH_2 Gly), 56.60 (OCH_3), 64.28 (CH_2), 97.09 (C-5), 113.03 (C-3), 113.72 (C-4a), 122.94 (C-10), 123.45 (C-7), 125.08 (C-10a), 128.82 (C-6a), 129.23 (C-9), 129.73 (C-8), 146.65 (C-10b), 151.92 (C-4), 153.84 (C-6), 162.66 (C-2) and 168.32 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,515, 3,414, 2,992, 2,926, 2,719, 2,651, 1,729, 1,596, 1,567, 1,505, 1,475, 1,453, 1,423, 1,387, 1,317, 1,276, 1,253, 1,172, 1,143, 1,111, 1,086, 1,061, 985, 963, 923, 871, 840, 733 and 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 374 (3.49). HRMS (EI): calculated for $C_{17}H_{15}NO_5$ [M^+]: 313.09505; found: 313.09504.

L-Alanine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl)methyl ester hydrobromide (**8b**)

Starting from conjugate **7b** (0.035 g, 7.59×10^{-5} mol) and using 45% solution of hydrobromic acid in acetic acid (130 μL), compound **8b** was obtained (0.022 g, 71%). mp = 198.2–198.8°C. TLC (ethyl acetate/methanol, 4:3): $R_f = 0.74$. ^{1}H NMR (CD_3OD, 400 MHz): $\delta_H = 1.70$ (d, J = 7.2 Hz, 3 H, β-CH_3 Ala), 4.11 (s, 3 H, OCH_3),

4.40–4.50 (m, 1 H, α-CH Ala), 5.70 (s, 2 H, CH_2), 6.61 (s, 1 H, H-3), 6.90 (s, 1 H, H-5), 7.70–7.78 (m, 2 H, H-8 and H-9), 8.28–8.34 (m, 1 H, H-7) and 8.42–8.47 (m, 1 H, H-10). ^{13}C NMR (CD_3OD, 100.6 MHz): $\delta_C = 16.29$ (β-CH_3 Ala), 49.90 (α-CH Ala), 56.90 (OCH_3), 64.53 (CH_2), 97.06 (C-5), 113.14 (C-3), 113.68 (C-4a), 122.91 (C-10), 123.44 (C-7), 125.01 (C-10a), 128.77 (C-6a), 129.22 (C-9), 129.72 (C-8), 146.62 (C-10b), 151.80 (C-4), 153.78 (C-6), 162.59 (C-2) and 170.67 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,426, 2,924, 1,727, 1,698, 1,638, 1,613, 1,597, 1,566, 1,505, 1,474, 1,453, 1,423, 1,386, 1,315, 1,274, 1,249, 1,204, 1,176, 1,145, 1,111, 1,085, 1,031, 987, 960, 857, 815, 787, 733 and 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 373 (3.49). HRMS (EI): calculated for $C_{18}H_{17}NO_7$ [M^+]: 327.11071; found: 327.11072.

L-β-Alanine (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl) methyl ester hydrobromide (**8c**)

Starting from conjugate **7c** (0.040 g, 8.67 × 10^{-5} mol) and using 45% solution of hydrobromic acid in acetic acid (60 μL), compound **8c** was obtained (0.0158 g, 45%). mp = 204.3–205.4°C. TLC (ethyl acetate/methanol, 4:3): $R_f = 0.26$. ^{1}H NMR (CD_3OD, 400 MHz): $\delta_H = 3.02$ (t, J = 6.4 Hz, 2 H, α-CH_2 β-Ala), 3.30–3.40 (m, 2 H, β-CH_2 β-Ala), 4.08 (s, 3 H, OCH_3), 5.58 (s, 2 H, CH_2), 6.57 (s, 1 H, H-3), 6.85 (s, 1 H, H-5), 7.66–7.77 (m, 2 H, H-8 and H-9), 8.23–8.30 (m, 1 H, H-7) and 8.37–8.45 (m, 1 H, H-10). ^{13}C NMR (CD_3OD, 100.6 MHz): $\delta_C = 32.20$ (α-CH_2 β-Ala), 36.33 (β-CH_2 β-Ala), 56.55 (OCH_3), 63.42 (CH_2), 97.02 (C-5), 112.81 (C-3), 113.77 (C-4a), 122.88 (C-10), 123.41 (C-7), 124.97 (C-10a), 128.69 (C-6a), 129.15 (C-9), 129.05 (C-8), 146.49 (C-10b), 152.49 (C-4), 153.69 (C-6), 162.71 (C-2) and 171.59 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,437, 3,010, 2,923, 1,741, 1,699, 1,631, 1,607, 1,592, 1,565, 1,504, 1,473, 1,444, 1,422,1,383, 1,336, 1,312, 1,275, 1,251, 1,197, 1,172, 1,150, 1,114, 1,088, 1,032, 1,017, 992, 959, 927, 877, 729 and 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 371 (3.33). HRMS (EI): calculated for $C_{18}H_{17}NO_5$ [M^+]: 327.11071; found: 327.11074.

N-[(6-Methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl)methyloxycarbonyl]-L-β-alanine methyl ester (**10a**)

To a solution of CDI (0.059 g, 3.64 × 10^{-4} mol) in dry DMF (1 mL), 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**5**) (0.060 g, 2.43 × 10^{-4} mol) dissolved in dry DMF (3 mL) was added, and the reaction mixture was stirred at room temperature for 3 h. After the addition of β-alanine methyl ester (**9a**) (0.025 g, 2.44 × 10^{-4} mol) the mixture was kept reacting in the same conditions for 12 h. The solvent was evaporated and purification by dry flash chromatography, using chloroform/methanol, mixtures of increasing polarity as eluent, gave compound **10a** as a light yellow solid (0.038 g, 42%). mp = 175.7–177.9°C. TLC (chloroform/methanol, 9.5:0.5): $R_f = 0.75$. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta_H = 2.62$ (t, J = 5.7 Hz, 2 H, α-CH_2 β-Ala), 3.50–3.62 (m, 2 H, β-CH_2 β-Ala), 3.74 (s, 3 H, OCH_3 β-Ala), 4.04 (s, 3 H, OCH_3), 5.38 (s, 2 H, CH_2), 5.59 (t, J = 5.7 Hz, 1 H, NH β-Ala), 6.56 (s, 1 H, H-3), 6.65 (s, 1 H, H-5), 7.64–7.72 (m, 2 H, H-8 and H-9), 8.24–8.32 (m, 1 H, H-7) and 8.48–8.56 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 75.4 MHz): $\delta_C = 34.02$ (α-CH_2 β-Ala), 36.74 (β-CH_2 β-Ala), 51.9 (OCH_3 β-Ala), 55.87 (OCH_3), 62.01 (CH_2), 95.29 (C-5), 112.10 (C-4a), 112.21 (C-3), 122.22 (C-10), 122.40 (C-7), 123.97 (C-10a), 127.31 (C-6a), 127.88 (C-9), 128.37 (C-8), 145.63 (C-10b), 150.52 (C-4), 152.24 (C-6), 155.27 (C=O urethane), 160.90 (C-2) and 172.68 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3,324, 3,081, 2,925, 2,854, 1,739, 1,711, 1,612, 1,598, 1,562, 1,554, 1,505, 1,474, 1,451, 1,420, 1,382, 1,324, 1,312, 1,252, 1,197, 1,174, 1,145, 1,110, 1,083, 1,052, 1,016, 986, 952, 890, 876, 811 and 734. UV/Vis (ethanol, nm): λ_{max} (log ε) = 374 (3.70). HRMS (EI): calculated for $C_{20}H_{20}NO_7$ [M^+]: 386.12455; found: 386.12343.

N-[(6-Methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl)methyloxycarbonyl]-L-β-alanine (**10b**)

The same procedure described before for the synthesis of compound **10a** was followed using CDI (0.046 g, 2.84 × 10^{-4} mol) in DMF (1 mL), 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**5**) (0.020 g, 7.81 × 10^{-5} mol) in DMF (1 mL) (stirring time 24 h), and β-alanine (**9b**) (0.014 g, 1.56 × 10^{-4} mol) (stirring time 24 h). After evaporation of the solvent, the crude mixture was dissolved in methanol and the white precipitated filtered off. The methanol solution evaporated and purified by preparative chromatography (ethyl acetate/methanol, 9:1) to give compound **10b** as a light yellow solid (0.0242 g, 82%). mp = 224.9–225.9°C. TLC (ethyl acetate/methanol, 8:2): $R_f = 0.61$. ^{1}H NMR (DMSO-d6, 400 MHz): $\delta_H = 2.43$ (t, J = 6.8 Hz, 2 H, α-CH_2 β-Ala), 3.20-3.30 (m, 2 H, β-CH_2 β-Ala), 4.05 (s, 3 H, OCH_3), 5.44 (s, 2 H, CH_2), 6.50 (s, 1 H, H-3), 7.00 (s, 1 H, H-5), 7.66 (t, J = 5.2 Hz, 1 H, α-NH), 7.71–7.79 (m, 2 H, H-8 and H-9), 8.20–8.26 (m, 1 H, H-7) and 8.33–8.38 (m, 1 H, H-10). ^{13}C NMR (DMSO-d6, 100.6 MHz): $\delta_C = 34.07$ (α-CH_2 β-Ala), 36.66 (β-CH_2 β-Ala), 56.11 (OCH_3), 61.44 (CH_2), 97.36 (C-5), 111.25 (C-3), 112.50 (C-4a), 121.70 (C-10), 122.00 (C-7), 123.04 (C-10a), 126.46 (C-6a), 128.15 (C-9), 128.57 (C-8), 144.51 (C-10b), 151.27 (C-6), 152.44 (C-4), 155.31 (C=O urethane), 159.76 (C-2) and 172.72 (CO_2H). IR (KBr 1%, cm^{-1}): ν = 3,269, 3,081, 2,992, 2,972, 2,933, 1,741, 1,714, 1,690, 1,613, 1,572, 1,505, 1,476, 1,461,

1,426, 1,386, 1,305, 1,274, 1,254, 1,197, 1,180, 1,147, 1,114, 1,085, 1,046, 992, 956, 888, 844, 771, 738 and 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 373 (3.52). HRMS (EI): calculated for $C_{19}H_{17}NO_7$ [M^+]: 339.11071; found: 339.11072.

General photolysis procedure

A 1×10^{-4} M methanol/HEPES (80:20) solution of compounds **7a–d**, **8a–c** and **10a**, **b** (5 mL) were placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 254, 300, 350 and 419 ± 10 nm.

Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 3:1, at a flow rate of 0.8 mL/min for all compounds, previously filtered through a Millipore, type HN 0.45-µm filter and degassed by ultrasound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: **7a**, 6.6; **7b**, 6.5; **7c**, 6.7; **7d**, 6.0; **8a**, 4.2; **8b**, 4.2; **8c**, 4.2; **10a**, 4.7; **10b**, 2.3 min).

Results and discussion

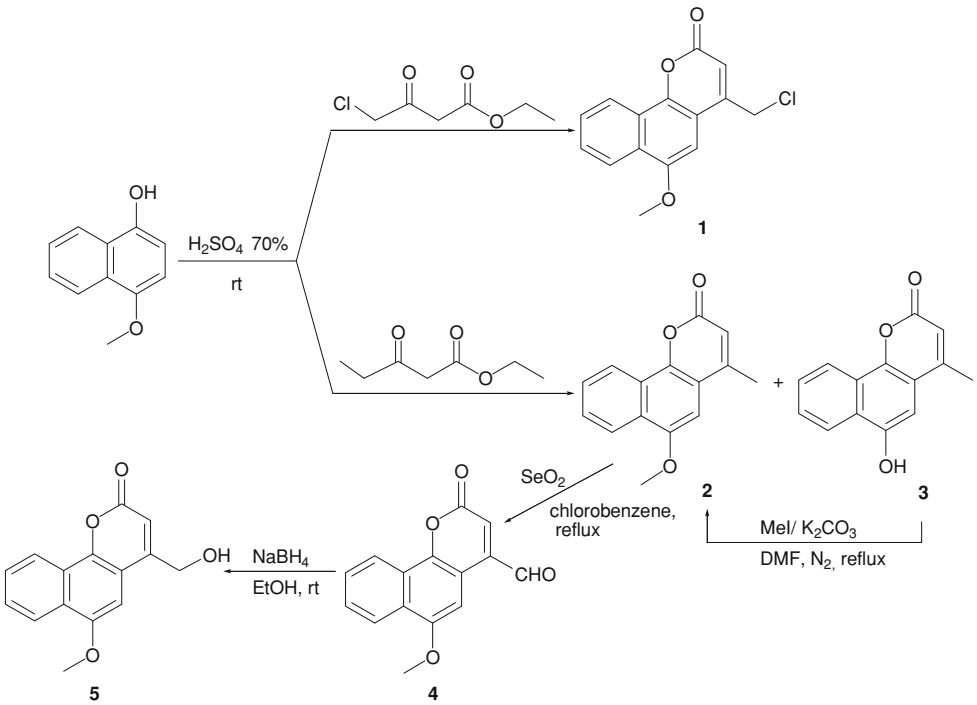
Synthesis

4-Methoxy-1-naphthol was reacted with ethyl 4-chloroacetoacetate or ethyl acetoacetate through a Pechmann reaction, catalysed by sulphuric acid at room temperature, yielding 4-(chloromethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **1** or 6-methoxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran **2** (Gangadasu et al. 2004, synthesised by a different method) together with the unexpected demethylated derivative, 6-hydroxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran **3**, the latter being methylated with methyl iodide to obtain the desired methoxy derivative **2**.

By reaction of compound **2** with selenium dioxide, the methyl group was oxidised to the aldehyde **4**, which was then reacted with sodium borohydride, affording the 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **5** (Scheme 1; Table 1). It should be noted that compound **5** was also obtained as a by-product (10%) in the reaction with selenium dioxide by an allylic hydroxylation (Paquete 1995). Fluorophores **1** and **5** will be designated in this report by a three letter code (Flu) for simplicity of naming the various fluorescent conjugates, as indicated in Table 3.

The functionalised fluorophores **1** and **5** were used in the preparation of several neurotransmitter amino acids conjugates through different linkages, namely ester and urethane, by reaction at their carboxylic acid or amino functions, respectively.

N-Benzyloxycarbonyl-protected amino acids, such as glycine (**6a**), alanine (**6b**), *β*-alanine (**6c**) and *γ*-aminobutyric acid (**6d**), were derivatised at the C-terminus with chloromethylated benzo[*h*]benzopyran **1** in the presence of potassium fluoride (Tjoeng and Heavner 1981) in DMF, at room temperature resulting in the expected bioconjugates



Scheme 1 Synthesis of functionalised 6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyrans **1–5**

Table 1 Yields and UV/visible data for precursors **1–5** and conjugates **7a–d**, **8a–c** and **10a**, **b** in absolute ethanol and methanol/HEPES buffer (80:20) solution

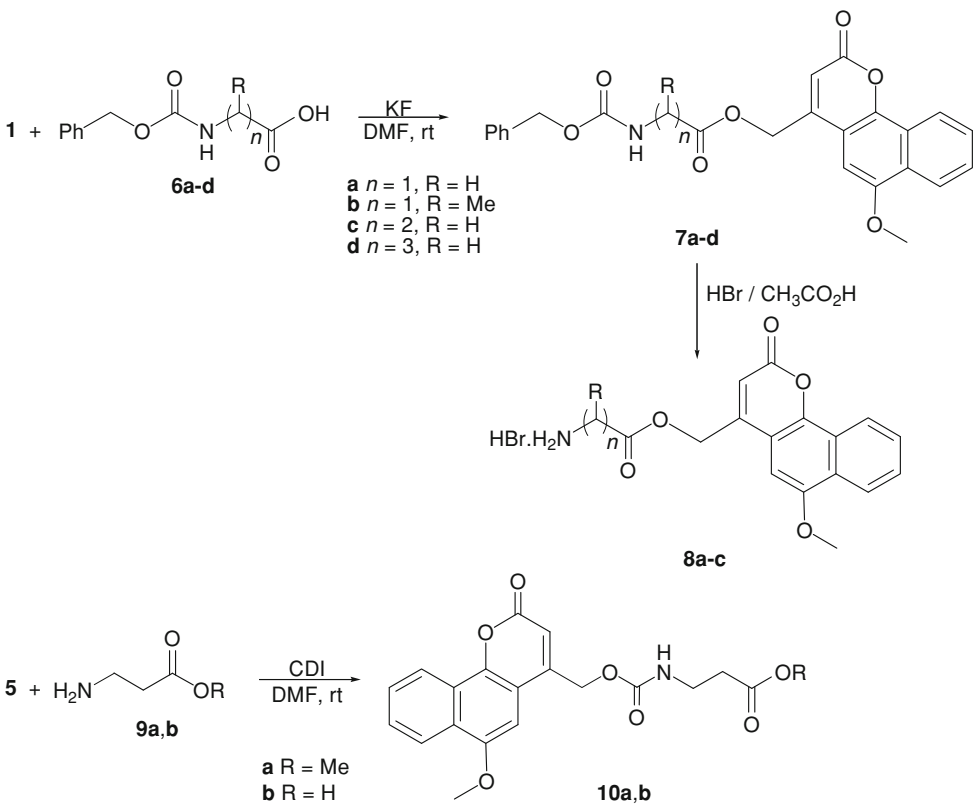
Compound	Yield (%)	EtOH		MeOH/HEPES buffer (80:20)	
		λ_{max} (nm)	log ε	λ_{max} (nm)	log ε
1	45	379	3.68	383	3.70
2	15	371	3.57	371	3.68
3	12	380	3.71	377	3.73
4	79	377	3.73	380	3.61
5	47	365	3.69	371	3.73
7a	53	376	3.72	373	3.73
7b	86	375	3.72	374	3.72
7c	70	375	3.75	376	3.78
7d	56	373	3.66	370	3.60
8a	18	374	3.39	372	3.62
8b	71	373	3.49	373	3.64
8c	45	371	3.33	370	3.63
10a	42	374	3.70	374	3.71
10b	82	373	3.52	371	3.62

7a–d (Scheme 2; Table 1) in good yields (53–86%). Furthermore, the *N*-benzyloxycarbonyl-protecting group was removed by acidolysis with hydrobromic acid in acetic acid yielding the corresponding conjugates **8a–c** bearing the neurotransmitter and the photosensitive fluorophore. This approach constitutes the most suitable model to evaluate the usefulness of the new fluorescent heterocycle as photoreleasable protecting group for neurotransmitter amino acids.

4-(Hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **5** was reacted with the N-terminus of β-alanine methyl ester (**9a**) and free β-alanine (**9b**), by a 1,1′-carbonyldiimidazole (CDI) carbonyl transfer reaction (D'Addona and Bochet 2001), in DMF, at room temperature, yielding the corresponding urethane-linked conjugates **10a** (42%) and **10b** (82%) (Scheme 2; Table 1). All compounds synthesised were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy.

The IR spectra of conjugates **7a–d**, **8a–c**, **10a**, **b** showed bands due to stretching vibrations of the different carbonyl groups present at the fluorophore-amino acid conjugates from 1,643 to 1,758 cm^{-1}. ^{1}H NMR spectra showed signals of the amino acid residues, such as the α-CH_2 (δ 4.16 ppm, **7a** or 4.13 ppm, **8a**), α-CH (δ 4.49–4.59 ppm, **7b** or δ 4.40–4.50 ppm, **8b**); α-, β-CH_2 (δ 2.72, 3.57 ppm, **7c**; δ 3.02, 3.30–3.40 ppm, **8c**), as well as α-, β-, γ-CH_2 (δ 2.56, 1.84–2.0, 3.30–3.33 ppm, **7d**). The fluorophore methylene group was also visible for all conjugates (δ 5.26–5.74 ppm). Also the characteristic aromatic protons, H-3 and H-5, of the oxobenzopyran ring were present at 6.44–6.66 and 6.46–6.95 ppm, respectively.

Scheme 2 Synthesis of fluorescent neurotransmitter conjugates **7a–d**, **8a–c**, **10a**, **b**



The confirmation of the presence of the newly formed ester (**7a–d**) and urethane (**10a**, **b**) linkages were also supported by ^{13}C NMR spectra signals of the carbonyl group, which were found at δ 169.47–172.37 ppm and at about 155.30 ppm, respectively.

Evaluation of the photophysical properties of conjugates **7a–d**, **8a–c** and **10a**, **b**

The UV/visible absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol and in a methanol/HEPES buffer (80:20) solution of conjugates **7a–d**, **8a–c** and **10a**, **b**, in comparison with precursors **1** and **5** were measured, absorption and emission maxima, molar absorptivities and relative fluorescence quantum yields are also reported (Tables 1, 2). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard ($\Phi_F = 0.95$ in ethanol) (Morris et al. 1976). For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

By comparison of absorption maxima for all compounds in both solvents, no significant changes were observed (λ_{max} in the range 365–380 nm, in ethanol, and 370–383 nm, in methanol/HEPES buffer). Furthermore, upon linkage of benzobenzopyran **1** to amino acids **6a–d**, a slight hypsochromic shift (up to 8 nm in ethanol and up to 13 nm in methanol/HEPES buffer) was observed for conjugates **7a–d** and their corresponding Z-deprotected form **8a–c**.

For benzobenzopyran **5** and conjugates **10a**, **b**, no shift (for **10b** in methanol/HEPES) or a bathochromic shift was observed both in ethanol and in methanol/HEPES buffer solution.

Bioconjugates **7a–d** and **8a–c** exhibited good fluorescence quantum yields in ethanol ($0.43 < \Phi_F < 0.63$) and in methanol/HEPES buffer ($0.28 < \Phi_F < 0.53$), which are higher than the value obtained for the corresponding chloromethylated precursor **1**, being at least ten times (9–13) in ethanol and up to fifty times (28–53) in methanol/HEPES buffer. In the case of conjugates **10a**, **b**, when compared to the fluorophore **5**, it was observed that the higher value was displayed by compound **10b** ($\Phi_F = 0.71$) in ethanol.

Compounds **7a–d**, **8a–c** and **10a**, **b** displayed emission maxima at about 472 nm (in ethanol) and in the range 469–484 nm (in methanol/HEPES buffer), with large Stokes' shifts (ca. 100 nm), which is an important feature in fluorescent labelling for bioapplications. Moreover, comparison between precursors **1**, **5** and the corresponding conjugates **7c**, **10a** and **10b**, in methanol/HEPES buffer solution revealed longer wavelengths of maximum emission than in ethanol.

By comparison of fluorescence maxima for benzobenzopyrans **1**, **5** and the corresponding conjugates **7a–d**, **8a–c** and **10a**, **b** in ethanol, a shift to longer wavelengths (9–12 nm) occurred. In the case of methanol/HEPES buffer there was no considerable variation except for a bathochromic shift for conjugates **7c** (13 nm), **10a** and **10b** (8 nm). Figure 1 illustrates the absorption and fluorescence normalised spectra of tags **1**, **5** and the β-alanine

Table 2 Fluorescence data for precursors **1–5** and conjugates **7a–d**, **8a–c** and **10a**, **b** in absolute ethanol and methanol/HEPES buffer (80:20) solution

Compound	EtOH			MeOH/HEPES buffer (80:20)		
	λ_{em} (nm)	Φ_F	Stokes' shift (nm)	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
1	461	0.05	82	471	0.01	88
2	463	0.33	72	447	0.32	76
3	459	0.17	79	466	0.02	89
4	461	0.49	84	472	0.30	92
5	460	0.45	95	469	0.45	98
7a	473	0.48	97	474	0.34	101
7b	472	0.56	98	472	0.31	98
7c	471	0.63	96	484	0.28	108
7d	473	0.60	100	470	0.38	100
8a	471	0.43	97	469	0.51	97
8b	471	0.50	98	471	0.53	98
8c	470	0.63	99	469	0.43	99
10a	472	0.31	98	477	0.44	103
10b	471	0.71	98	477	0.47	106

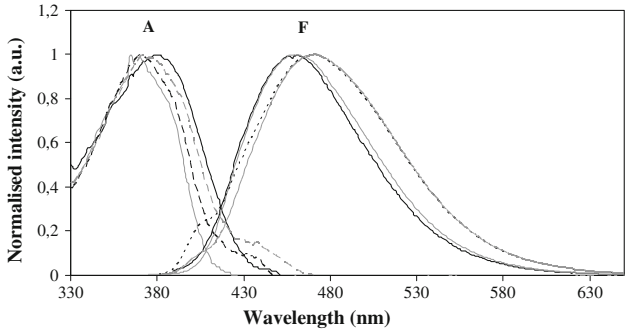


Fig. 1 Normalised UV/visible absorption (*A*) and fluorescence (*F*) spectra of precursors **1**, **5** and conjugates **8c**, **10b** in ethanol (**1**, $\lambda_{exc} = 379$ nm; **5**, $\lambda_{exc} = 365$ nm; **8c**, $\lambda_{exc} = 375$ nm; **10b**, $\lambda_{exc} = 374$ nm) (**1**, *black full line*; **5**, *grey full line*; **8c**, *black spaced line*; **10b**, *grey spaced line*)

bioconjugates **8c** and **10b** in ethanol. The shape of the spectra in methanol/HEPES buffer solution was similar.

The neurotransmitters used as models in this report are representative of aliphatic amino acids, which do not absorb or emit in the visible region, not being easily detectable by absorption and fluorescence techniques. As such, the linkage of a fluorophore like the considered oxobenzo[*h*]benzopyran proved to be a very suitable approach to enhance the photophysical properties of biomolecules of this type.

Photolysis studies of neurotransmitter conjugates **7a–d, 8a–c** and **10a**, **b**

The sensitivity of benzo[*h*]benzopyran conjugates **7a–d**, **8a–c** and **10a**, **b** towards UV–visible irradiation was evaluated by exposing solutions of the mentioned compounds in methanol/HEPES buffer (80:20) solution in a Rayonet RPR-100 reactor at 254, 300, 350 and 419 nm. The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material versus irradiation time were obtained for each compound at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time, and were the average of 3 runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 3).

For each compound and based on HPLC data, the plot of ln *A* versus irradiation time showed a linear correlation for the disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line. The corresponding rate constants were calculated and are presented in Table 3.

Concerning the influence of the wavelength of irradiation on the rate of photocleavage reactions of conjugates **7a–d** and **10a** in methanol/HEPES buffer (80:20) solution, it was found that irradiation times at 300 and 350 nm were comparable, the best results being obtained at 350 nm.

Taking into consideration the influence of the structure of the neurotransmitter on the photocleavage rates, we could see that the *β*-alanine conjugate **7c** had the largest irradiation times for 300 and 350 nm. In contrast, the irradiation times for alanine and GABA conjugates **7b** and **7d** were always shorter and very similar, and as a result, photocleavage at 419 nm was also tested for these two compounds. Although for compound **7b** the irradiation time was at about two times larger (57 min) than that obtained at 350 nm and that for **7d** was six times larger (245 min), these values indicate that for these neurotransmitter conjugates the wavelength of 419 nm still represents a practical possibility if photodeprotection should be considered.

Table 3 Irradiation times (in minutes) and rate constants ($\times 10^{-2}$ min^{-1}) for the photolysis of conjugates **7a–d**, **8a–c**, **10a**, **b** and **11a**, **b** (Fernandes et al. 2008a, b) at different wavelengths in methanol/HEPES buffer (80:20) solution

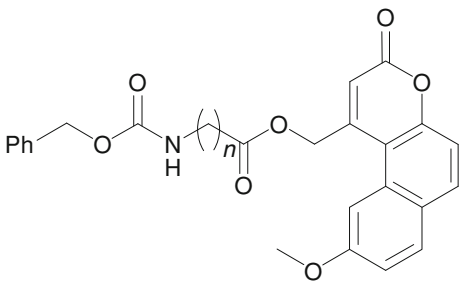
Compound		254 nm		300 nm		350 nm		419 nm	
		Irr time	*k*	Irr time	*k*	Irr time	*k*	Irr time	*k*
7a	Z-Gly-OFlu	93	3.24	144	2.09	60	5.02	–	–
7b	Z-Ala-OFlu	48	5.45	49	6.17	30	9.94	57	5.36
7c	Z-*β*-Ala-OFlu	52	6.16	592	0.48	438	0.62	–	–
7d	Z-GABA-OFlu	52	5.35	45	6.62	39	6.77	245	1.21
8a	H-Gly-OFlu	–	–	–	–	469	0.67	–	–
8b	H-Ala-OFlu	–	–	–	–	441	0.66	–	–
8c	H-*β*-Ala-OFlu	–	–	–	–	370	0.80	–	–
10a	Flu-*β*-Ala-OMe	75	4.10	140	1.50	140	1.87	–	–
10b	Flu-*β*-Ala-OH	–	–	–	–	63	3.91	–	–
11a	Z-*β*-Ala-OBba	418	0.72	957	0.31	513	0.59	–	–
11b	Z-GABA-OBba	169	1.75	124	2.51	84	3.78	–	–

At this point, a comparison could be made between the data collected for benzo[*h*]benzopyran conjugates **7c** and **7d** here reported and previously described benzo[*f*]benzopyran *β*-alanine and GABA conjugates **11a** and **11b** (Fernandes et al. 2008a, b). These compounds differ in the fused framework of the tricyclic heterocycle system (Table 3; Fig. 2). Considering the results for compounds **7c**, **d** and **11a**, **b** presented in Table 3, photorelease of conjugates **7c** and **d** occurred with shorter irradiation times at 300 and 350 nm (up to three times) as well as at 254 nm (up to eight times). In both series, *β*-alanine conjugates were associated with slower cleavages. These results confirmed that the newly synthesised 4-(chloromethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **1** has advantages as a photocleavable protecting group when compared with the previously reported one.

As a continuation of this research, photolysis of conjugates **8a–c** and **10b**, which have only the light-sensitive moiety linked to the neurotransmitter, was carried out in the same conditions at 350 nm (Fig. 3). It was found that for compounds **8a–c** irradiation times were large (370–469 min), being for *β*-alanine conjugate **8c** inferior to that of the release of Z-protected amino acid from ester conjugate **7c**. Cleavage of compound **10b** was the fastest; the free *β*-alanine was released within at about half irradiation time than the *β*-alanine methyl ester from conjugate **10a**.

By comparison of *β*-alanine conjugates **7c** and **10a**, as well as **8c** and **10b** which differ in the type of linkage between the fluorophore and the neurotransmitter, it was found that at 300 and 350 nm the urethane bond (**10a**, **b**) cleaved significantly faster than the ester bond (**7c** and **8c**).

Furthermore, the release of Z-protected amino acids (**6a–d**), *β*-alanine methyl ester (**9a**), as well as the free residues, as the expected photolysis products of conjugates **7a–d**, **10a** and **8a–c**, **10b**, respectively, was also followed by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solutions with concentrations ranging from 1.05×10^{-2} to 1.47×10^{-2} M.



11 a *n* = 2 Z-β-Ala-OBba
b *n* = 3 Z-GABA-OBba

Fig. 2 Structure of 2-oxo-2*H*-benzo[*f*]benzopyran neurotransmitter conjugates **11a**, **b** (Fernandes et al. 2008a, b)

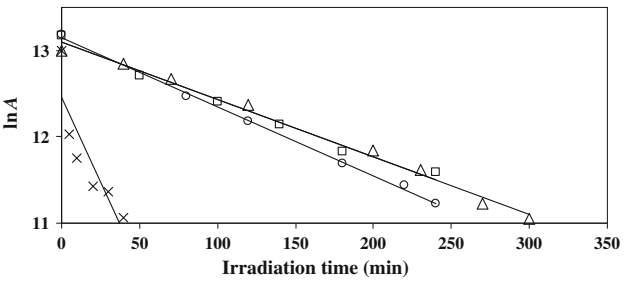


Fig. 3 Plot of ln *A* versus irradiation time for the photolysis at 350 nm of conjugates **8a** (*triangles*), **8b** (*squares*), **8c** (*circles*) and **10b** (*multiplication symbol*) in methanol/HEPES buffer (80:20) solution

Upon irradiation at the usual wavelengths, a new set of signals appeared related to aliphatic protons of the amino acid residue and the N- or C-termini protecting groups (in case of cleavage of conjugates **7a–c** and **10a**). After a variable irradiation period (depending on the wavelength of irradiation and structure of the conjugate) the signals due to the amino acid residue in the conjugate form disappeared completely and were replaced by the corresponding set of signals of Z-protected amino acids (Z-Aaa-OH, for **7a–d**), *β*-alanine methyl ester (H-*β*-Ala-OMe, for **10a**) or the free neurotransmitter amino acids (H-Aaa-OH, for **8a–c** and **10b**). These observations confirmed the quantitative release of the amino acid, without removal of the Z-protecting group, when it was present, which is an interesting result considering its linkage similarity with the new reported urethane-bonded benzopyran. Moreover, signals due to by-products related to the fluorophore were also detected in the ^{1}H NMR spectra.

As example, Figs. 4 and 5 show ^{1}H NMR spectra for photolysis at 350 nm of *β*-alanine conjugates **8c** and **10b**, in comparison with that of the expected released *β*-alanine. As mentioned before, upon irradiation it was visible a decrease in triplets related to α-CH_2 and β-CH_2 (δ 3.04 and 3.33 ppm, **8c**; 2.60 and 3.48, **10b**), as well as the singlet for the benzylic-type CH_2 of the fluorophore (δ 5.62 ppm, **8c**; 5.49 ppm, **10b**). In both cases, the released *β*-alanine gave rise to a new set of signals, namely α-CH_2 (δ at about 2.70 ppm) and β-CH_2 (δ at about 3.20 ppm).

Conclusions

The synthesis of a novel fused benzopyran, namely a 6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran, was achieved in good yield by a one-step procedure, in the case of the 4-chloromethyl derivative **1**, and through a few steps of simple synthetic modifications in the case of the 4-hydroxymethyl derivative **5**. In order to evaluate the behaviour of this polyaromatic heterocycle as new

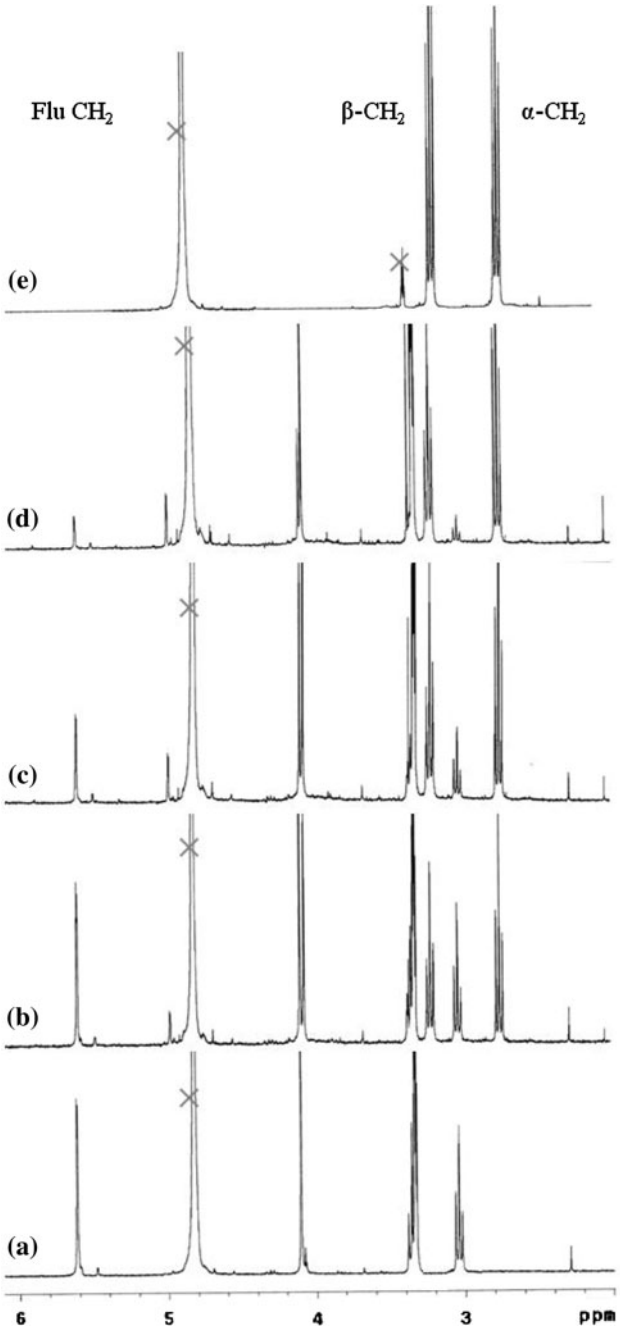


Fig. 4 ^{1}H NMR spectra in methanol-d_4/D_2O (80:20) of the photolysis of conjugate H-β-Ala-OFlu **8c** ($C = 1.05 \times 10^{-2}$ M) at 350 nm: **a** before irradiation; **b** after irradiation for 35 min; **c** after irradiation for 60 min; **d** after irradiation for 90 min; **e** H-β-Ala-OH

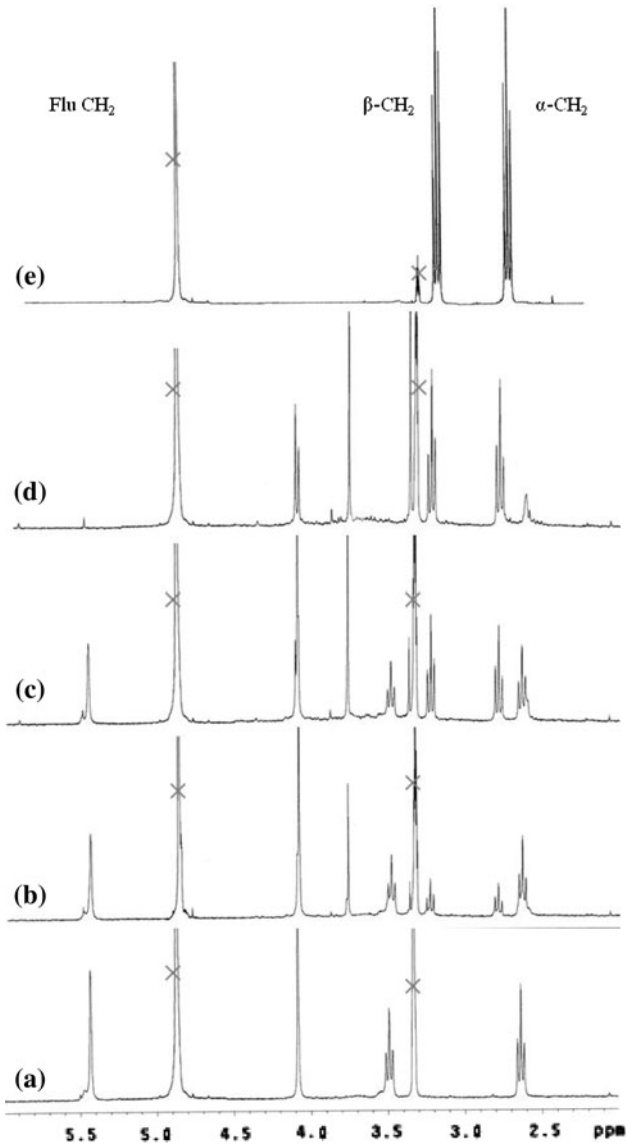


Fig. 5 ^{1}H NMR spectra in methanol-d_4/D_2O (80:20) of the photolysis of conjugate Flu-β-Ala-OH **10b** ($C = 1.47 \times 10^{-2}$ M) at 350 nm: **a** before irradiation; **b** after irradiation for 20 min; **c** after irradiation for 40 min; **d** after irradiation for 140 min; **e** H-β-Ala-OH

photoreleasable protecting group, tags **1** and **5** were efficiently used in the synthesis of fluorescent neurotransmitter amino acid ester **7a–d** and urethane **10a**, **b** conjugates, by reaction with the carboxylic acid or amino terminals of *N*-benzyloxycarbonyl-protected and the methyl ester, or the free form (in case of **10b**), of excitatory and inhibitory neurotransmitters such as glycine, alanine, β-alanine and γ-aminobutyric acid.

A study of the photophysical properties of the conjugates was carried out in ethanol and methanol/HEPES buffer (80:20) solution, and the results suggested that precursors 2-oxo-2*H*-benzo[*h*]benzopyran **1** and **5** are suitable fluorophores for the derivatisation of non-fluorescent molecules, yielding bioconjugates with high relative fluorescence quantum yields.

Regarding the photocleavage studies of the fluorescent conjugates, in methanol/HEPES buffer (80:20) solution, at 254, 300 and 350 nm, it was possible to conclude that the structure of the neurotransmitter had an influence on the irradiation times. Although a suitable wavelength of irradiation could be chosen for all neurotransmitters

conjugates, Z-protected alanine and GABA amino acids cleaved faster in all wavelengths considered. Owing to the irradiation times, photolysis at 419 nm can also be applicable for some of the compounds studied. In order to restore the biological activity as neurotransmitter, conjugates having only the amino acid linked to the light-sensitive protecting group **8a–c** (Z-protecting groups were removed before irradiation in compounds **7a–c**) and **10b** were irradiated at 350 nm, and it was found that the best results were observed for the uncaging of β-alanine from the urethane-conjugate.

Although there are differences in photolysis times, quantitative release of all the neurotransmitters studied was proved to occur in the tested conditions. Furthermore, the newly reported benzo[*h*]benzopyran can be considered an enhancement of our previously fluorescent fused heterocycles studied as photolabile protecting groups.

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