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Phototriggering of neuroactive amino acids from 5,6-benzocoumarinyl conjugates

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ABSTRACT

Uncaging of several neuroactive amino acids, namely β-alanine, tyrosine, 3,4-dihydroxyphenylalanine (DOPA) and glutamic acid from the corresponding 5,6-benzocoumarinyl conjugates was carried out by irradiation at different wavelengths and in different solvent systems. The release of the various amino acids was faster in ACN/HEPES buffer mixtures and for the tyrosine conjugate, an increase in the photolysis reaction rates and the quantitative uncaging of the amino acid was associated with increasing water content in the solvent mixture.

1. Introduction

Coumarins are structural units present in many natural compounds and a large number of their natural and synthetic derivatives exhibit diverse biological and pharmacological activities, but more recently they have gained recognition for their application as photocleavable protecting groups for the caging of biomolecules. They have been used in cell biology and biophysical studies with a range of chemical functionalities, such as alcohols,[1] amines,[2] phosphates,[3] carboxylic acids,[4] aldehydes and ketones.[5] Fused coumarins, 5,6- and 7,8-benzocoumarins and their derivatives have also been reported in photocleavage studies,[6–8] and their electronic properties along with their photophysical characteristics make them promising for biological applications.[9,10]

Photo-uncaging produces rapid, sequential release of biomolecules for finely tuned stimulation of biological events, so light-activable groups can be used as a powerful tool in the spatio-temporal controlled release of selected bioactive molecules from inactive (caged) forms by phototrigerring.[11] Important caged biomolecules include neurotransmitters, nucleotides, peptides, proteins, DNA and RNA.[12]

Having in mind these facts and as a continuation of our work related with the investigation of alternative heterocyclic photolabile protecting groups,[6–8,13–16] we now report the evaluation of the release under irradiation of neuroactive amino acids β-alanine, tyrosine, 3,4-dihydroxyphenylalanine and glutamic acid from the corresponding 5,6-benzocoumarinyl ester conjugates. Photo-uncaging studies were carried out at 254, 300, 350 and 419 nm in mixtures of aqueous HEPES buffer and organic solvents in different ratios and cleavage kinetic data obtained by HPLC/UV monitoring.

2. Results and discussion

5,6-Benzocoumarin– neuroactive amino acid conjugates **2a**–**e** were obtained by cleavage of *N*-benzyloxycarbonyl (*Z*) or *N*-*tert*-butyloxycarbonyl (Boc) groups under usual chemical deprotection conditions from the corresponding precursors **1a**–**e** with β-alanine, tyrosine, 3,4-dihydroxyphenylalanine and glutamic acid (Scheme 1). The 5,6-benzocoumarinyl moiety will be designated in this report by a three-letter code (Bba) for simplicity of naming the various fluorescent conjugates, as indicated in 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 **2a**–**e** showed bands due to stretching vibrations of the different carbonyl groups present at the benzocoumarin–amino acid conjugates from 1625 to 1752 cm^{-1}. ^{1}H NMR spectra showed signals of the amino acid residues, such as the α-CH (δ 3.80–4.35 ppm) and the α-, β- and γ-CH$_2$ (δ 1.99–3.01 ppm), in addition to the benzocoumarin methylene group (δ 5.09–6.04 ppm). Also the characteristic H-2 of the 5,6-benzocoumarin ring was present (δ 6.60–6.74 ppm). As it was expected, the deprotection procedures did not affect the integrity of the ester linkage between the amino acid and the benzocoumarin, as confirmed by ^{13}C NMR spectra signals of its carbonyl group, at δ 168.69–177.67 ppm.

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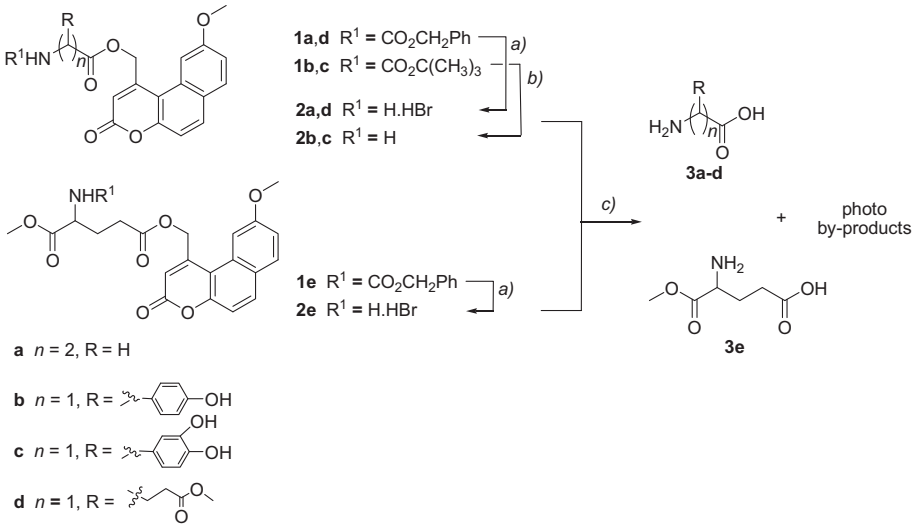

Scheme 1. Synthesis of conjugates **2a**–**e** and photo-release of neuroactive amino acids **3a**–**e**. Reagents and conditions: (a) 45% hydrobromic acid/acetic acid, rt; (b) trifluoroacetic acid/DCM (1:2), rt; (c) hν, MeOH/HEPES buffer or ACN/HEPES buffer in different proportions.

Table 1
Yields, UV/vis absorption and fluorescence data for neuroactive amino acid conjugates **2a**–**e** in absolute ethanol, MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions

Compound		Yield (%)	EtOH					MeOH/HEPES (80:20)					ACN/HEPES (80:20)				
			λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]
2a	H-β-Ala-OBba	98	344	4.02	465	0.86	121	344	3.94	473	0.83	129	342	3.94	470	0.50	128
2b	H-Tyr-OBba	76	344	3.98	460	0.81	116	344	3.97	474	0.70	130	341	3.94	464	0.26	123
2c	H-DOPA-OBba	31	345	3.92	459	0.29	114	343	3.76	473	0.48	130	342	4.03	467	0.09	125
2d	H-Glu(OMe)-OBba	95	346	3.93	460	0.75	114	344	4.27	473	0.75	129	342	4.07	469	0.47	127
2e	H-Glu(OBba)-OMe	51	345	3.92	458	0.84	113	344	3.76	474	0.79	130	342	4.06	468	0.47	126

[a] In nm.

In order to obtain the parameters necessary for the monitoring of the photolysis reaction, as well as the fluorescence properties, the UV/vis characterisation was carried out in absolute ethanol and in different solvent mixtures. The absorption and emission spectra of conjugates **2a**–**e** in degassed solutions in absolute ethanol, MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) were measured (Table 1, Fig. 1). By comparison of the absorption maxima, no significant differences were observed in these solvents (between 341 and 346 nm), although for the water containing solvent systems, there was a slight bathochromic shift (4–16 nm) in the wavelength of maximum emission, relative to the data collected in absolute ethanol. The Stokes' shift was between 113 and 130 nm, which is an advantageous property in fluorescence techniques as it will minimise self-quenching phenomena.

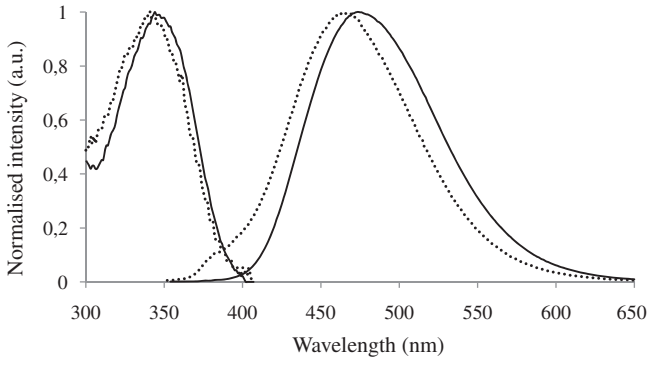

Fig. 1. Normalised UV/vis absorption and fluorescence spectra of conjugate **2b** in MeOH/HEPES buffer (80:20) (full line, λ_{exc}=344 nm), and ACN/HEPES buffer (80:20) (spaced line, λ_{exc}=341 nm) solutions.

Relative fluorescence quantum yields were determined using 9,10-diphenylanthracene in ethanol as standard,[17] and it was found that all conjugates were highly emissive in ethanol (Φ_F from 0.75 to 0.86), with the exception of the lower value obtained for the DOPA conjugate **2c** (Φ_F=0.29). The same trend was previously reported by us for the precursor conjugates **1a**–**e** bearing a *N*-benzyloxycarbonyl protecting group. High Φ_F values were also obtained for **2a**–**e** in MeOH/HEPES buffer (80:20), with that of **2c** increasing to 0.48, whereas in ACN/HEPES buffer (80:20) the quantum yield was lower for all conjugates.

The sensitivity of the 5,6-benzocoumarin–neuroactive amino acid conjugates towards UV/vis irradiation was evaluated by exposing solutions of the mentioned compounds to radiation at 254, 300, 350 and 419 nm, and monitorised by HPLC/UV. Given our previous results with other heterocyclic conjugates for photolysis in different solvent systems with varying aqueous content,[18] solutions of **2a**–**e** were prepared in MeOH/HEPES buffer (80:20). Peak areas (*A*) of conjugates **2a**–**e** revealed a gradual decrease with the irradiation time until less than 5% of the initial area was detected (Table 2). The plots of ln *A* versus irradiation time showed a linear correlation suggesting a first order reaction, obtained by the linear least squares methodology for a straight line. Rate constants were calculated and are presented in Table 2.

In MeOH/HEPES buffer (80:20), it was found that the irradiation times were long at the tested wavelengths. Nonetheless, for all conjugates the best results were obtained at 254 nm with the cleavage being more than two times faster than at 300 or 350 nm. For each set of results at a particular wavelength, the structure of the neuroactive amino acid was not significantly influential on the reaction rates, although some tendencies were visible: photolysis of the glutamic acid conjugates bearing the 5,6-benzocoumarinyl

Table 2
Irradiation times (t_{irr}, in hours), rate constants (k, in 10^{-2} h^{-1}) and photochemical quantum yields (Φ_{phot}, $\times 10^{-3}$) for the photolysis of conjugates **2a**–**e** at different wavelengths in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions

Compound		MeOH/HEPES (80:20)									ACN/HEPES (80:20)								
		254 nm			300 nm			350 nm			254 nm			300 nm			350 nm		
		t_{irr}	k	Φ_{phot}	t_{irr}	k	Φ_{phot}	t_{irr}	k	Φ_{phot}	t_{irr}	k	Φ_{phot}	t_{irr}	k	Φ_{phot}	t_{irr}	k	Φ_{phot}
2a	H-β-Ala-OBba	8.9	0.57	0.019	21.0	0.24	0.015	29.5	0.17	0.003	5.1	0.98	0.023	4.1	1.25	0.015	5.0	1.05	0.019
2b	H-Tyr-OBba	13.6	0.35	0.011	29.5	0.16	0.007	27.8	0.13	0.003	2.6	1.89	0.039	1.3	3.76	0.034	3.4	1.55	0.032
2c	H-DOPA-OBba	9.2	0.37	0.020	27.9	0.17	0.008	19.3	0.18	0.008	5.2	0.98	0.014	14.8	0.34	0.002	10.3	0.49	0.008
2d	H-Glu(OMe)-OBba	14.3	0.55	0.005	31.5	0.18	0.004	38.3	0.26	0.001	4.6	1.10	0.021	6.2	0.80	0.012	6.6	0.78	0.011
2e	H-Glu(OBba)-OMe	7.5	0.65	0.026	20.1	0.25	0.013	33.1	0.15	0.005	3.8	1.39	0.019	6.5	0.76	0.006	4.6	1.14	0.015

photolabile group at the main chain, **2d**, or at the side chain, **2e**, revealed that cleavage of the ester bond at the side chain was faster for all wavelengths of irradiation, and the DOPA conjugate **2c** photolysis was faster than that of tyrosine conjugate **2b**.

Photolysis was also carried out with mixtures of methanol with water content higher than 20%, in an attempt to improve the photoreaction rates. For tyrosine conjugate **2b** in a 50:50 mixture at 300 nm, it was possible to achieve the quantitative release of tyrosine within 3.2 h, which represents a significant decrease in the irradiation time (ca. nine times). For the other conjugates, the reaction rates were not improved with the increase in the water content, when compared to the results obtained in a 80:20 mixture.

Taking into consideration the above mentioned findings, it was decided to test an aprotic organic solvent, namely acetonitrile, which is also suitable for photocleavage studies.[19] Therefore, solutions of conjugates **2a**–**e** in ACN/HEPES buffer (80:20) were irradiated in the same conditions. As can be seen from the data collected in Table 2, the resulting irradiation times were significantly decreased, especially at 300 and 350 nm, when compared to those obtained in MeOH/HEPES buffer (80:20).

Concerning the influence of the wavelength of irradiation on the rate of the photocleavage reactions of conjugates **2a**–**e** in ACN/HEPES buffer (80:20) solution, it was found that the shorter irradiation times were observed at 300 nm for conjugates **2a** and **2b** and at 254 nm for conjugates **2c**–**e**. By comparison of the results at 300 and 350 nm, it was noticeable that the release of β-alanine **3a**, tyrosine **3b** and glutamic acid **3d** was faster at 300 nm, contrary to the release of DOPA **3c** and glutamic acid **3e**, with shorter irradiation times at 350 nm. No significant difference in the release of glutamic acid methyl ester (**3d** and **3e**) was seen in the cleavage of the conjugate's ester bond at its main (**2d**) or side chain (**2e**) at 300 nm (Fig. 2).

Furthermore, considering the influence of the structure of the

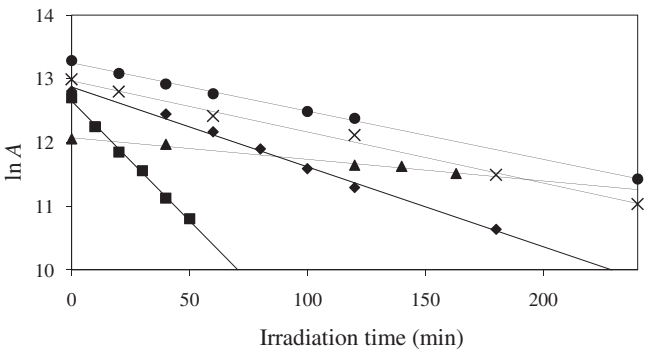

Fig. 2. Plot of ln A versus irradiation time for the photolysis of compounds **2a** (◆), **2b** (■), **2c** (▲), **2d** (×) and **2e** (●) at 300 nm in ACN/HEPES buffer (80:20) solution.

neurotransmitter on the photocleavage rates, it was possible to see that the DOPA conjugate **2c** had the largest irradiation times for all wavelengths. In contrast, the irradiation times for the cleavage of tyrosine conjugate **2b** were always shorter, being the best result at 300 nm (1.3 h). In this solvent system, cleavage at 419 nm was also attempted resulting in a long, unpractical irradiation time. Also, photolysis at 300 nm in mixtures of acetonitrile with higher water content were tested, the best results being obtained for the tyrosine conjugate **2b** in 50:50 (1.0 h), 20:80 (0.7 h) and 10:90 (0.6 h) ACN/HEPES buffer mixtures. For this conjugate, there was a visible increase in the reaction rate with the increasing water content.

The photochemical quantum yields of the photocleavage process in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions of compounds **2a**–**e** were also calculated based on half-lives ($t_{1/2}$), molar absorptivities (ε) and the incident photon flux (I_0), determined by potassium ferrioxalate actinometry[20,21] (at 254, 300, 350 and 419 nm, 1.22×10^{17}, 2.54×10^{17}, 2.63×10^{17} and 2.61×10^{17} photons s^{-1} cm^{-2}, respectively) (Table 2).

Taking into account the determined photochemical quantum yields, it was found that the photocleavage process was not as efficient as desirable, probably due to the dissipation of part of the absorbed energy via fluorescence pathways that compete with the photocleavage reaction, as well as the type of reactor used (open chamber reactor) and the low power of the lamps (14–35 W).

3. Conclusions

The release of the various neuroactive amino acids was achieved by irradiation of the corresponding 5,6-benzocoumarinyl ester conjugates at different wavelengths and in diverse solvent systems. Photolysis was faster in ACN/HEPES buffer mixtures and for the tyrosine conjugate, an increase in the photolysis reaction rates and the quantitative uncaging of the amino acid was associated with increasing water content in the solvent mixture.

4. Experimental section

4.1. 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. UV/vis absorption spectra (200–700 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 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 Me_4Si=0 ppm as reference and J values are given in hertz. Assignments were made by comparison of chemical shifts, peak multiplicities and J values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. High resolution mass spectrometry analyses were

performed at the 'C.A.C.T.I.—Unidad de Espectrometria de Masas', at University of Vigo, Spain.

4.2. Synthesis procedure for conjugates 1b and 1c

4.2.1. Synthesis of N-(tert-butyloxycarbonyl)-L-tyrosine (9-methoxy-(5,6)-benzocoumarin-1-yl) methyl ester, Boc-Tyr-OBba ***1b***. 1-Choromethyl-9-methoxy-(5,6)-benzocoumarin, Bba-Cl (0.083 g, 0.302 mmol) was dissolved in dry DMF (3 mL), potassium fluoride (0.053 g, 0.907 mmol) and Boc-Tyr-OH (0.085 g, 0.302 mmol) were added. The reaction mixture was stirred at room temperature for 3 days. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography in silica gel using dichloromethane/methanol 100:1 as eluent, to give compound **1b** as a yellow solid (0.118 g, 96%). Mp=96.6–98.2 °C. ^{1}H NMR (DMSO-d_6): δ=1.32 (s, 9H, C(CH_3)$_3$), 2.81–2.89 (m, 2H, β-CH_2), 3.94 (s, 3H, OCH_3), 4.22–4.25 (m, 1H, α-CH), 5.79 (d, *J* 6.0 Hz, 2H, CH_2 Bba), 6.62 (d, *J* 8.4 Hz, 2H, H-3 and H-5 Tyr), 6.63 (s, 1H, H-2), 7.01 (d, *J* 8.4 Hz, 2H, H-2 and H-6 Tyr), 7.29 (dd, *J* 9.2 and 2.4 Hz, 1H, H-8),7.40 (d, *J* 8.8 Hz, 1H, H-5), 7.46 (d, *J* 7.6 Hz, 1H, α-NH), 7.50 (d, *J* 1.6 Hz, 1H, H-10), 8.00 (d, *J* 8.8 Hz, 1H, H-7), 8.14 (d, *J* 8.8 Hz, 1H, H-6). ^{13}C NMR (DMSO-d_6): δ=28.05 (C(CH_3)$_3$), 35.45 (β-CH_2), 55.30 (OCH_3), 55.82 (α-C), 64.41 (CH_2 Bba), 78.45 (C(CH_3)$_3$), 105.96 (C-10), 111.53 (C-4b), 112.24 (C-2), 114.79 (C-5), 114.95 (C-3 and C-5 Tyr), 116.83 (C-8), 125.97 (C-6a), 127.30 (C-1 Tyr), 129.98 (C-2 and C-6 Tyr), 130.16 (C-6b), 131.21 (C-7), 133.94 (C-6), 151.51 (C-1), 154.89 (C-4a), 155.49 (CONH), 155.96 (C-4 Tyr), 159.15 (C-9 and C-3), 171.73 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3383, 2977, 2934, 1714, 1625, 1517, 1446, 1366, 1272, 1233, 1186, 1105, 1062, 1023, 989, 944, 838, 729. HRMS (ESI): calcd for $C_{29}H_{30}NO_8$ [M^++H]: 520.19720; found: 520.19745.

4.2.2. Synthesis of N-(tert-butyloxycarbonyl)-3,4-dihydroxy-L-phenylalanine (9-methoxy-(5,6)-benzocoumarin-1-yl) methyl ester, Boc-DOPA-OBba ***1c***. Starting from Bba-Cl (0.112 g, 0.41 mmol), DMF (3 mL), potassium fluoride (0.071 g, 1.22 mmol) and Boc-BOPA-OH (0.121 g, 0.41 mmol), following the same procedure as described for **1b**, compound **1c** was obtained as a yellow solid (0.166 g, 76%). Mp=113.1–114.7 °C. ^{1}H NMR (DMSO-d_6): δ=1.32 (s, 9H, C(CH_3)$_3$), 2.48–2.50 (m, 2H, β-CH_2), 3.95 (s, 3H, OCH_3), 4.18–4.23 (m, 1H, α-CH), 5.80 (d, *J* 3.6 Hz, 2H, CH_2 Bba), 6.46 (dd, *J* 8.0 and 2.0 Hz, 1H, H-6 DOPA), 6.60 (d, *J* 8.8 Hz, 1H, H-5 DOPA), 6.61 (d, *J* 1.6 Hz, 1H, H-2 DOPA), 6.65 (s, 1H, H-2), 7.30 (dd, *J* 9.2 and 2.4 Hz, 1H, H-8), 7.41 (d, *J* 8.8 Hz, 1H, H-5), 7.42 (d, *J* 8.0 Hz, 1H, α-NH), 7.50 (d, *J* 1.2 Hz, 1H, H-10), 8.01 (d, *J* 8.8 Hz, 1H, H-7), 8.15 (d, *J* 8.8 Hz, 1H, H-6), 8.68 (s, 1H, OH), 8.71 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ=28.06 (C(CH_3)$_3$), 35.66 (β-CH_2), 55.31 (OCH_3), 55.88 (α-C), 64.41 (CH_2 Bba), 78.47 (C(CH_3)$_3$), 105.95 (C-10), 111.54 (C-4b), 112.16 (C-2), 114.80 (C-5), 115.29 (C-5 DOPA), 116.38 (C-2 DOPA), 116.85 (C-8), 119.74 (C-6 DOPA), 125.97 (C-6a), 128.02 (C-1 DOPA), 130.17 (C-6b), 131.22 (C-7), 133.95 (C-6), 143.89 (C-4 DOPA), 144.91 (C-3 DOPA), 151.58 (C-1), 154.90 (C-4a), 155.50 (CONH), 159.16 (C-3 and C-9), 171.76 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3378, 2977, 2935, 1712, 1625, 1552, 1519, 1445, 1367, 1282, 1233, 1162, 1115, 1062, 1022, 950, 840, 821, 804, 730. HRMS (ESI): calcd for $C_{29}H_{30}NO_9$ [M^++H]: 536.19210; found: 536.19221.

4.3. Synthesis procedures for conjugates 2a–e

4.3.1. Synthesis of β-alanine (9-methoxy-(5,6)-benzocoumarin-1-yl) methyl ester hydrobromide, HBr·H-β-Ala-OBba ***2a***. A 45% solution of hydrobromic acid in acetic acid (36 μL), and acetic acid (0.5 mL) were added to *N*-(benzyloxycarbonyl)-β-alanine-(9-methoxy-(5,6)-benzocoumarin-1-yl)methyl ester, *Z*-β-Ala-OBba **1a**[7] (0.040 g, 0.087 mmol) with stirring, at room temperature. The reaction mixture was maintained in these conditions for four days, and the process was followed by TLC (dichloromethane/methanol 50:1). When the reaction was completed, cold diethyl ether was added (1 mL), and the precipitate filtered off and washed with the same solvent to give **2a** as a yellow solid (0.035 g, 98%). Mp=189.0–190.8 °C. ^{1}H NMR (DMSO-d_6): δ=2.60–2.70 (m, 2H, CH_2), 3.01 (t, *J* 7.2 Hz, 2H, CH_2), 3.94 (s, 3H, OCH_3), 5.13 (d, *J* 0.8 Hz, 2H, CH_2 Bba), 6.72 (s, 1H, H-2), 7.27 (dd, *J* 8.8 and 2.4 Hz, 1H, H-8), 7.38 (d, *J* 8.8 Hz, 1H, H-5), 7.74 (d, *J* 2.4 Hz, 1H, H-10), 7.98 (d, *J* 9.2 Hz, 1H, H-7), 8.10 (d, *J* 8.8 Hz, 1H, H-6). ^{13}C NMR (DMSO-d_6): δ=31.70 (CH_2), 34.87 (CH_2), 51.71 (OCH_3), 62.54 (CH_2 Bba), 106.49 (C-10), 111.77 (C-2), 112.25 (C-4b), 114.84 (C-5), 116.69 (C-8), 125.95 (C-6a), 130.52 (C-6b), 130.98 (C-7), 133.51 (C-6), 154.78 (C-4a), 158.19 (C-1), 159.04 (C-9), 159.81 (C-3), 170.85 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3378, 3288, 3218, 3089, 2965, 2260, 1717, 1683, 1427, 1554, 1519, 1427, 1375, 1365, 1337, 1279, 1203, 1131, 1068, 1050, 1025, 987, 857, 722. HRMS (ESI): calcd for $C_{18}H_{18}NO_5$ [M^++H]: 328.11795; found: 328.11723.

4.3.2. Synthesis of L-tyrosine (9-methoxy-(5,6)-benzocoumarin-1-yl) methyl ester, H-Tyr-OBba ***2b***. To a solution of Boc-Tyr-OBba **1b** (0.062 g, 0.119 mmol) in dichloromethane (2 mL), trifluoroacetic acid (1 mL) was added and the reaction mixture was stirred at room temperature for 24 h. The process was followed by TLC (dichloromethane/methanol 50:1). After evaporation, aqueous buffer solution (pH 8) was added (5 mL) to the resulting residue followed by extraction with ethyl acetate. Compound **2b** was obtained as a yellow solid (0.046 g, 76%). Mp=146.8–147.9 °C. ^{1}H NMR (DMSO-d_6): δ=2.74–2.90 (m, 2H, β-CH_2), 3.80–3.90 (m, 1H, α-CH), 3.94 (s, 3H, OCH_3), 5.70–5.85 (m, 2H, CH_2 Bba), 6.53 (br s, 2H, NH_2), 6.60 (s, 1H, H-2), 6.61 (d, *J* 8.8 Hz, 2H, H-3 and H-5 Tyr), 6.96 (d, *J* 8.8 Hz, 2H, H-2 and H-6 Tyr), 7.30 (dd, *J* 9.0 and 2.4 Hz, 1H, H-8), 7.41 (d, *J* 9.2 Hz, 1H, H-5), 7.52 (d, *J* 2.0 Hz, 1H, H-10), 8.02 (d, *J* 9.2 Hz, 1H, H-7), 8.15 (d, *J* 8.8 Hz, 1H, H-6), 9.21 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ=38.62 (β-CH_2), 55.32 (OCH_3), 55.40 (α-C), 64.35 (CH_2 Bba), 106.05 (C-10), 111.80 (C-4b), 112.52 (C-2), 114.86 (C-5), 115.06 (C-3 and C-5 Tyr), 116.81 (C-8), 126.01 (C-6a), 126.63 (C-1 Tyr), 130.06 (C-6b), 130.15 (C-2 and C-6 Tyr), 131.29 (C-7), 134.02 (C-6), 151.38 (C-1), 154.94 (C-4a), 156.11 (C-4 Tyr), 159.16 (C-9), 159.22 (C-3), 172.90 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3365, 3225, 1712, 1625, 1552, 1517, 1445, 1364, 1233, 1200, 1178, 1140, 1062, 1023, 949, 839, 722. HRMS (ESI): calcd for $C_{24}H_{22}NO_6$ [M^++H]: 420.14416; found: 420.14481.

4.3.3. Synthesis of 3,4-dihydroxy-L-phenylalanine (9-methoxy-(5,6)-benzocoumarin-1-yl) methyl ester, H-DOPA-OBba ***2c***. Starting from Boc-DOPA-OBba **1c** (0.166 g, 0.31 mmol), dichloromethane (1 mL) and trifluoroacetic acid (0.5 mL), following the same procedure as described for compound **2b**, compound **2c** was obtained as a yellow solid (0.041 g, 31%). Mp=277.2–278.5 °C. ^{1}H NMR (DMSO-d_6): δ =2.83–2.86 (m, 2H, β-CH_2), 3.92 (s, 3H, OCH_3), 3.98–4.02 (m, 1H, α-CH), 5.11 (s, 2H, CH_2 Bba), 6.41 (dd, *J* 8.0 and 2.0 Hz, 1H, H-6 DOPA), 6.54 (d, *J* 2.0 Hz, 1H, H-2 DOPA), 6.64 (d, *J* 8.0 Hz, 1H, H-5 DOPA), 6.70 (s, 1H, H-2), 7.25 (d, *J* 8.8 and 2.4 Hz, 1H, H-8), 7.37 (d, *J* 8.8 Hz, 1H, H-5), 7.72 (d, *J* 2.4 Hz, 1H, H-10), 7.92 (d, *J* 8.8 Hz, 1H, H-7), 8.08 (d, *J* 8.8 Hz, 1H, H-6), 8.93 (br s, 2H, 2×OH). ^{13}C NMR (DMSO-d_6): δ=36.53 (β-CH_2), 54.14 (α-C), 55.51 (OCH_3), 62.79 (CH_2 Bba), 106.71 (C-10), 112.00 (C-2), 112.49 (C-4b), 115.08 (C-5), 115.89 (C-5 DOPA), 116.79 (C-2 DOPA), 116.94 (C-8), 120.40 (C-6 DOPA), 125.68 (C-1 DOPA), 126.19 (C-6a), 130.74 (C-6b), 131.28 (C-7), 133.84 (C-6), 144.71 (C-4 DOPA), 145.44 (C-3 DOPA), 155.01 (C-4a), 158.45 (C-1), 159.30 (C-9), 160.25 (C-3), 170.85 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3220, 2260, 1752, 1709, 1625, 1552, 1492, 1446, 1232, 1197, 1140, 1062, 1025, 956, 839, 801, 723. HRMS (ESI): calcd for $C_{24}H_{22}NO_7$ [M^++H]: 436.13908; found: 436.13901.

4.3.4. Synthesis of 2-amino-5-methyl-1-(9-methoxy-(5,6)-benzocoumarin-1-yl)methyl pentanedioate hydrobromide, HBr·H-Glu(OMe)-OBba ***2d***. Starting from 2-(N-benzyloxycarbonyl)amino-5-methyl-

1-(9-methoxy-(5,6)-benzocoumarin-1-yl) methyl pentanedioate, Z-Glu(OMe)-OBba **1d**[7] (0.020 g, 0.038mmol), 45% solution of hydrobromic acid in acetic acid (27 μL), and acetic acid (0.4 mL), following the same procedure as described for **2a**, compound **2d** was obtained as a yellow solid (0.017 g, 95%). Mp=167.0–168.3 °C. ^{1}H NMR (DMSO-d_6): δ=1.99–2.18 (m, 2H, CH_2), 2.45–2.61 (m, 2H, CH_2), 3.56 (s, 3H, OCH_3 Glu), 3.95 (s, 3H, OCH_3), 4.32–4.35 (m, 1H, α-CH), 5.90–6.04 (m, 2H, CH_2 Bba), 6.74 (s, 1H, H-2), 7.33 (dd, *J* 8.8 and 2 Hz, 1H, H-8), 7.42 (d, *J* 8.8 Hz, 1H, H-5), 7.49 (d, *J* 2.0 Hz, 1H, H-10), 8.04 (d, *J* 9.2 Hz, 1H, H-7), 8.17 (d, *J* 8.8 Hz, 1H, H-6). ^{13}C NMR (DMSO-d_6): δ=25.25 (CH_2), 28.70 (CH_2), 51.32 (α-C), 51.56 (OCH_3 Glu), 55.37 (OCH_3), 65.47 (CH_2 Bba), 106.30 (C-10), 111.42 (C-4b), 111.89 (C-2), 114.87 (C-5), 116.67 (C-8), 126.05 (C-6a), 130.08 (C-6b), 131.41 (C-7), 134.16 (C-6), 151.11 (C-1), 154.94 (C-4a), 159.22 (C-9), 159.31 (C-3), 168.69 (CO_2CH_2), 172.17 (CO_2CH_3). IR (KBr 1%, cm^{-1}): ν=3395, 3222, 2962, 1712, 1687, 1626, 1552, 1520, 1444, 1238, 1203, 1139, 1089, 1039, 1018, 839, 801, 723. HRMS (ESI): calcd for $C_{21}H_{22}NO_7$ [M$^+$+H]: 400.13908; found: 400.13889.

*4.3.5. Synthesis of 4-amino-5-methyl-1-(9-methoxy-(5,6)-benzocoumarin-1-yl)methyl pentanedioate hydrobromide, HBr·H-Glu(OBba)-OMe **2e**.* Starting from 4-(N-benzyloxycarbonyl)amino-5-methyl-1-(9-methoxy-(5,6)-benzocoumarin-1-yl) methyl pentanedioate, Z-Glu(OBba)-OMe **1e**[7] (0.174 g, 0.362 mmol), 45% solution of hydrobromic acid in acetic acid (63 μL), and acetic acid (1 mL), following the same procedure as described for **2a** (reaction time: two days), compound **2e** was obtained as a yellow solid (0.088 g, 51%). Mp=224.0–225.2 °C. ^{1}H NMR (DMSO-d_6): δ=1.90–2.0 (m, 1H, β-CH_2 Glu), 2.05–2.15 (m, 2H, γ-CH_2 Glu), 2.25–2.35 (m, 1H, β-CH_2 Glu), 3.64 (s, 3H, OCH_3 Glu), 3.91 (s, 3H, OCH_3), 4.15–4.20 (m, 1H, α-CH), 5.09 (s, 2H, CH_2 Bba), 6.69 (s, 1H, H-2), 7.24 (dd, *J* 9.0 and 2.0 Hz, 1H, H-8), 7.35 (d, *J* 9.2 Hz, 1H, H-5), 7.69 (d, *J* 2.0 Hz, 1H, H-10), 7.95 (d, *J* 8.8 Hz, 1H, H-7), 8.08 (d, *J* 9.2 Hz, 1H, H-6). ^{13}C NMR (DMSO-d_6): δ=24.67 (β-CH_2), 29.87 (γ-CH_2), 52.34 (OCH_3 Glu), 54.96 (α-C), 55.53 (OCH_3), 62.75 (CH_2 Bba), 106.69 (C-10), 111.96 (C-2), 112.46 (C-4b), 115.06 (C-5), 116.92 (C-8), 126.18 (C-6a), 130.72 (C-6b), 131.28 (C-7), 133.83 (C-6), 154.98 (C-4a), 158.46 (C-1), 159.28 (C-9), 160.24 (C-3), 173.60 (CO_2CH_3), 177.67 (CO_2CH_2). IR (KBr 1%, cm^{-1}): ν=3354, 2982, 1693, 1627, 1590, 1548, 1524, 1440, 1422, 1384, 1351, 1308, 1240, 1216, 1191, 1177, 1094. HRMS (ESI): calcd for $C_{21}H_{22}NO_7$ [M$^+$+H]: 400.13908; found: 400.13870.

4.4. Photolysis general

Photolyses were carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 254, 300, 350 and 419±10 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 μm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software.

4.4.1. General photolysis procedure. A 1×10^{-4} M solution of conjugates **2a**–**e** (5 mL) in HEPES buffer with methanol or acetonitrile, in varying proportions, was placed in a quartz tube and irradiated in the reactor at the desired wavelength. HEPES buffer solution was prepared in distilled water with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (10 mM), NaCl (120 mM), KCl (3 mM), $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and pH adjusted to 7.2. 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 (retention time at about 3.0 min), previously filtered through a Millipore, type HN 0.45 μm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each conjugate.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2011.01.090.

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