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Neurotransmitter amino acid—oxobenzo[*f*]benzopyran conjugates: synthesis and photorelease studies

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ABSTRACT

A series of fluorescent conjugates of neurotransmitter amino acids, such as β-alanine, tyrosine, 3,4-dihydroxyphenylalanine (DOPA) and glutamic acid, were prepared by reaction with a suitable fluorophore, namely 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran. The photophysical properties of the resulting ester bioconjugates were evaluated as well as the stability to photolysis at different wavelengths of irradiation (250, 300, 350 and 419 nm).

1. Introduction

Photolabile protecting groups—light-sensitive molecules that covalently bond to functional groups of molecules, which can then be released photochemically, without the need for any chemical reagent, have received remarkable interest in recent years, in organic synthesis as well as in life sciences.[1]

The use of photochemical methods as a deprotection procedure, alternatively to conventional approaches, which employ acids, bases, reducing or oxidising reagents is an interesting tool in organic synthesis.[2] The protection of signalling biomolecules with a light-sensitive group is also valuable in biological and medical research fields, as photoactivable or caged biomolecules allow temporal and spatially controlled delivery of bioactive molecules.[3] Several photoremovable protecting groups are now known for different families of compounds including alcohols,[4] amines,[5] phosphates,[6] aldehydes and ketones[7] as well as carboxylic acids.[8]

Recently, a number of moieties have been reported as new photolabile protecting groups for carboxylic acids, including 2-(dimethylamino)-5-nitrophenyl,[9] α-carboxy nitrobenzyl,[10] 3-nitro-2-naphthalenemethanol,[11] *p*-hydroxyphenacyl,[12] α-keto amides,[13] 2-hydroxy-1,2,2-triphenylethanone,[14] 1-acyl-7-nitroindolines,[15] and coumarin derivatives.[16] Some of these groups are fluorescent, which present advantages over those that are non-fluorescent, because they can also act as temporary fluorescent labels. Fluorescent labelling is suitable for analytical purposes, for being far more sensitive than common UV techniques, overcoming low detection limits. In recent years, the direction of improvement on photoreleasable groups has been towards the application of polycyclic aromatic structures, which are fluorophores in most cases, nonetheless the inconvenience of fluorescence deactivation in some photochemical processes.

Compounds possessing the carboxylic acid group are of great interest in organic chemistry and commonly need to be protected in sequential synthesis against various reagents including reactive nucleophiles, reducing agents and oxidants. Numerous carboxylic acids are also bioactive molecules and so the photochemical release of caged carboxylic compounds is of interest to basic biological research and biomedical applications.

Among the most appealing carboxylic compounds are amino acids, which play central roles both as building blocks of proteins and as intermediates in metabolism. These molecules are of the most abundant neurotransmitters in the brain. β-Alanine 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.[17]

The amino acid L-tyrosine is a precursor of dopamine—a neurotransmitter involved in controlling movement, regulating memory, sexual and reward-seeking behaviours and the regulation of pituitary hormones. 3,4-Dihydroxyphenylalanine (DOPA) has been historically considered an inert amino acid that alleviates the

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Table 1
Yields, UV–vis and fluorescence data for fluorescent neurotransmitter conjugates **3a–e** in absolute ethanol

Compound		Yield (%)	UV–vis		Fluorescence		
			λ_{max} (nm)	log ε	λ_{max} (nm)	Φ_F	Stokes' shift (nm)
3a	*Z*-β-Ala–OBba	80	347	3.88	466	0.75	119
3b	*Z*-Tyr(OtBu)–OBba	76	348	3.95	465	0.69	117
3c	*Z*-DOPA–OBba	36	349	3.83	463	0.14	114
3d	*Z*-Glu(OMe)–OBba	76	349	3.88	469	0.74	120
3e	*Z*-Glu(OBba)–OMe	29	346	4.00	464	0.75	118

symptoms of Parkinson's disease by its conversion to dopamine via the enzyme aromatic L-amino acid decarboxylase. Misu et al. have proposed that DOPA itself is a neurotransmitter and/or neuromodulator in addition to being a precursor of dopamine. It fulfils the several criteria that must be satisfied before a compound is accepted as a neurotransmitter in the homeostatic mechanism for maintaining blood pressure.[18] Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, largely distributed in all regions of the brain and being involved in cognitive functions like learning and memory. Furthermore, it is also a precursor for the synthesis of the major inhibitory neurotransmitter, the γ-aminobutyric acid (GABA).[19]

Our recently reported results involve the design and application of fluorescent oxygen heterocycles, as novel labels and photoreleasable protecting groups for the carboxylic acid function of amino acids.[16c–d,20] In previous work, we prepared conjugates of GABA-neurotransmitter amino acid, with a set of polyaromatic and polyheteroaromatic fluorescent reagents, based on naphthalene, pyrene and oxobenzopyran derivatives (trivially known as coumarins).[20c] The evaluation of their photophysical properties and their behaviour towards photocleavage revealed that an angular fused oxobenzopyran derivative proved to be the most fluorogenic derivatisation reagent and the most effective as photocleavable protecting group under irradiation at 350 nm. Photolysis at around 350 nm is preferable in studies involving biomolecules as it avoids cell damage due to short-wavelength light, and so we decided to study the caging and uncaging of several other relevant neurotransmitter amino acids, using the 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran fluorophore, in a MeOH/HEPES solution (80:20).

2. Results and discussion

2.1. Synthesis

1-Chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran **1** was synthesised through a Pechmann reaction, between 7-methoxy-2-naphthol and ethyl 4-chloroacetoacetate, catalysed by sulfuric acid at room temperature, as reported earlier.[20b] This fluorophore 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.

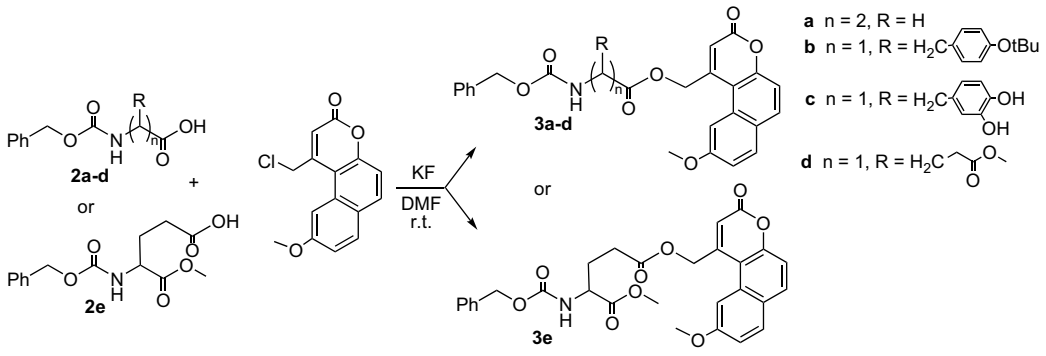
Our purpose being the investigation of the behaviour to photolysis conditions of the ester linkage between fluorophore **1** and the different neurotransmitters, we prepared the corresponding conjugates for their evaluation under irradiation at 254, 300, 350 and 419 nm. Derivatisation at the carboxylic acid group of *N*-benzyloxycarbonyl-protected amino acid neurotransmitters, such as β-alanine **2a**, tyrosine **2b**, 3,4-dihydroxyphenylalanine (DOPA) **2c** and glutamic acid, at the main and side chain, **2d** and **2e**, respectively, with fluorophore **1** was carried out in DMF, at room temperature, by using potassium fluoride.[21]

The expected bioconjugates **3a–e** (Scheme 1, Table 1) were obtained in low to good yields (29–80%) and characterised by the usual spectroscopic techniques.

The IR spectra of conjugates **3a–e** showed bands due to stretching vibrations of the ester carbonyl group of the fluorophore-amino acid linkage from 1712 to 1729 cm^{-1}. ^1H NMR spectra showed signals of the amino acid residues, such as the α-CH (δ 4.42–4.55 ppm, for **3b–e**) or α-CH_2 (δ 2.76 ppm, for **3a**), as well as the characteristic protons of the fluorophore methylene group (δ 5.58–5.67 ppm). The confirmation of the presence of the newly formed ester linkage was also supported by ^{13}C NMR spectra signals of the carbonyl group, which were found between δ 171.18 and 171.80 ppm.

2.2. Evaluation of the photophysical properties of conjugates 3a–e

The UV–vis absorption and emission spectra of degassed 10^{-5} to 10^{-6} M solutions in absolute ethanol of conjugates **3a–e** were measured, absorption and emission maxima, molar absorptivities and fluorescence quantum yields are also reported (Table 1). Fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard (Φ_F=0.95 in ethanol).[22] 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. Conjugates **3a–e** exhibited moderate to excellent fluorescence quantum yields ($0.14<\Phi_F<0.75$), and large Stokes' shifts between 114 and 120 nm. The DOPA conjugate **3c** showed the lower fluorescence quantum yield, through fluorescence deactivation probably due to the possibility of establishing extra H-bonds through the free hydroxyl groups to the solvent. All the studied neurotransmitters are not easily detectable by UV–vis absorption and have no or low intrinsic fluorescence and thus, the introduction of a fluorophore like the considered oxobenzobenzopyran **1** proved to be a very appropriate strategy to enhance the photophysical properties of the



Scheme 1. Synthesis of fluorescent neurotransmitter ester conjugates **3a–e**.

resulting conjugates. Moreover, the fluorescence quantum yield of the fluorophore in its free form was low (Φ_F=0.03)[16c] and had a dramatic increase upon connection via a ester linkage to the amino acid.

2.3. Photolysis studies of neurotransmitter conjugates 3a–e

Considering that the main goal of this work was to study the possibility of using fluorophore **1** as a fluorescent photocleavable protecting group in the caging of relevant biomolecules like neurotransmitters, photolysis studies of conjugates **3a–e** were carried out. Solutions of the mentioned compounds in methanol/HEPES buffer 80:20 were irradiated in a Rayonet RPR-100 reactor, at 254, 300, 350 and 419 nm, in order to determine the best cleavage conditions. 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 three 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 2).

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 (Fig. 1). The corresponding rate constants were calculated and are presented in Table 2.

Concerning the influence of the wavelength of irradiation on the rate of photocleavage reactions of conjugates **3a–e** in methanol/HEPES buffer 80:20 solution, it was found that the irradiation times were quite comparable at 254 and 350 nm, irradiation at 350 nm being preferable for reasons explained earlier. For all the wavelengths of irradiation considered, the irradiation times for conjugate **3b** were always shorter and so the photocleavage at 419 nm was also tested. Although a large increase (ca. 7 h) in the irradiation time was observed (473 min, k=6.3×10^{-3} min^{-1}), the wavelength of 419 nm can still be considered useful for certain practical applications.

Taking into consideration the influence of the structure of the neurotransmitter on the photocleavage rates, we could see that the β-alanine conjugate **3a** had the largest irradiation times for all wavelengths of irradiation. The presence of polar side chains in the other studied neurotransmitters appeared to have an influence on the decrease of the irradiation times, as shorter irradiation times were necessary for all the other conjugates **3b–e** compared to that of conjugate **3a**. The *O*-blocked tyrosine conjugate **3b** cleaved faster in all wavelengths of irradiation, while that the closely related DOPA conjugate **3c** cleaved slower (ca. 3–6 times), suggesting a detrimental effect of the free hydroxyl groups (**3b**) on the irradiation time, when compared to the hydroxyl-protected compound (**3c**). Comparison of the data obtained for the glutamic acid conjugates **3d** and **3e**, bearing the fluorophore at the main chain and at the side chain, respectively, revealed that cleavage of the ester linkage at the side chain was always faster, in the conditions tested.

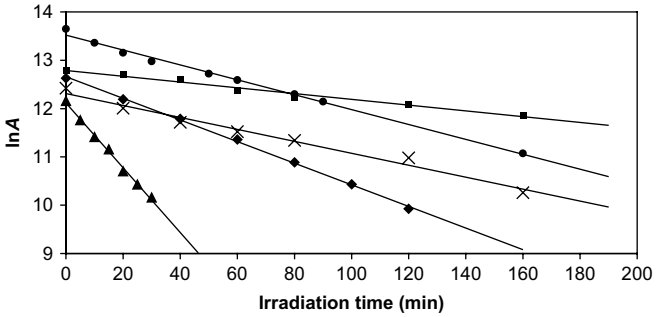


Figure 1. Plot of ln A versus irradiation time for the photolysis of conjugates **3a** (■), **3b** (▲), **3c** (●), **3d** (×) and **3e** (◆) at 350 nm in MeOH/HEPES (80:20) solution.

As reported before,[16c] the *N*-benzyloxycarbonyl urethane-type blocking group was stable in the tested conditions, no cleavage being detected. The photochemical quantum yields were calculated as previously described[23] and were lower than 0.01. The efficiency of the photocleavage process was not high, due to fluorescence deactivation and other photophysical processes, which limit the overall quantum yield of deprotection, as well as the low power of the lamps used and the fact that using an open chamber reactor can result in a larger dissipation of radiation. Nevertheless, considering the short irradiation times, 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran **1** can be considered an additional alternative to other established photocleavable protecting groups with application in neurotransmitter chemistry.

Regarding our synthesised conjugates, which are benzyl-type carboxylic acid esters and if we consider the conventional deprotection methods usually employed in the deprotection of carboxylic benzyl esters, such as catalytic hydrogenolysis and acid or base hydrolysis, we can suggest that photocleavage is an attractive method to achieve deprotection of this type of compounds. In terms of reaction time, chemical methods can take longer than the irradiation times presented in this report, with additional reagents being necessary.

3. Conclusions

Fluorescent neurotransmitter amino acid ester conjugates **3a–e** were prepared in moderate to good yields by using a simple synthetic method involving a chloromethylated oxobenzobenzopyran precursor and the carboxylic acid group of a series of *N*-benzyloxycarbonyl-protected neurotransmitter amino acids. The photophysical studies confirmed that the 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran **1** is a suitable fluorogenic reagent for the derivatisation of non-fluorescent molecules: the relative fluorescence quantum yields of the bioconjugates were determined and found to be moderate to excellent.

Regarding the photocleavage studies of the fluorescent conjugates, in methanol/HEPES buffer solution (80:20), at 254, 300 and

Table 2
Irradiation times (in min) and rate constants (×10^{-2} min^{-1}) for the photolysis of compounds **3a–e** at different wavelengths in MeOH/HEPES (80:20) solution

Compound		254 nm		300 nm		350 nm	
		Irr time	k	Irr time	k	Irr time	k
3a	*Z*-β-Ala–OBba	418	0.72	957	0.31	513	0.59
3b	*Z*-Tyr(OtBu)–OBba	53	5.69	64	4.80	44	6.70
3c	*Z*-DOPA–OBba	302	0.94	250	1.15	183	1.57
3d	*Z*-Glu(OMe)–OBba	217	1.26	547	0.53	233	1.24
3e	*Z*-Glu(OBba)–OMe	178	1.72	271	1.13	135	2.24

350 nm, it was possible to conclude that the structure of the neurotransmitter had an influence on the irradiation times and, especially in the case of the tyrosine conjugate **3b**, can be considered useful for practical applications. Owing to the irradiation times, photolysis at 350 nm would be the most convenient for the uncaging of the compounds studied.

In summary, the synthesised conjugates required short irradiation times for photocleavage to occur, making them appropriate for using as photolabile protecting groups for organic molecules, including amino acids and other relevant biomolecules, in addition to their usefulness in fluorescent labelling due to the high Stokes' shifts and good fluorescent quantum yields.

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 using KBr discs. UV–vis 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 parts per million using δ_H 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 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 10 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.

4.2. General procedure for the synthesis of compounds 3a–e

1-Choromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran, Bba-Cl (1 equiv) was dissolved in dry DMF (2 mL), potassium fluoride (3 equiv) and the corresponding amino acid neurotransmitters **1a–e** (1 equiv) were added. The reaction mixture was stirred at room temperature for 3–4 days. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography in silca gel using dichloromethane as eluent.

*4.2.1. N-(Benzyloxycarbonyl)-L-β-alanine (9-methoxy-3-oxo-3H-benzo[f]benzopyran-1-yl) methyl ester, Z-β-Ala–OBba (**3a**)*

Starting from compound **1** (0.100 g, 0.364 mmol) and *Z*-β-Ala–OH (**2a**) (0.081 g, 0.364 mmol), compound **3a** was obtained as a yellow solid (0.135 g, 80%). Mp=121.4–123.0 °C. ^{1}H NMR ($CDCl_3$): δ=2.76 (t, *J* 6.0 Hz, 2H, CH_2), 3.53 (q, *J* 6.0 Hz, 2H, CH_2), 3.95 (s, 3H, OCH_3), 5.10 (s, 2H, CH_2 Z), 5.35 (br s, 1H, NH), 5.67 (s, 2H, CH_2 Bba), 6.63 (s, 1H, H-2), 7.21 (dd, *J* 8.8 and 2 Hz, 1H, H-8), 7.27–7.35 (m, 6H, 5×Ph-*H* and H-5), 7.42 (br s, 1H, H-10), 7.82 (d, *J* 8.8 Hz, 1H, H-7), 7.90 (d, *J* 8.8 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$): δ=34.45 (CH_2), 36.46 (CH_2), 55.43 (OCH_3), 64.44 (CH_2 Bba), 66.82 (CH_2 Z), 105.79 (C-10), 111.81 (C-4b), 113.11 (C-2), 115.30 (C-5), 116.45 (C-8), 126.35 (C-6a), 128.49 (2×Ph-*C*), 130.17 (3×Ph-*C*), 130.53 (C-6b), 131.34 (C-7), 133.80 (C-6), 136.29 (C1 Ph), 150.50 (C-1), 155.56 (C-4a), 156.25 (C=O urethane), 159.63 (C-9), 160.13 (C-3), 171.53 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3378, 3064, 3033, 2926, 2854, 1722, 1625, 1552, 1519, 1455, 1445, 1364, 1337, 1231, 1168, 1139, 1075, 1059, 1020, 935, 838, 737, 697. UV–vis (MeOH/HEPES 80:20, nm): λ_{max} (log ε)=349 (4.08), 277 (3.94), 235 (4.54). HRMS (EI): calcd for $C_{26}H_{23}NO_7$ [M^+] 461.1475; found 461.1476.

*4.2.2. N-(Benzyloxycarbonyl)-O-(tert-butyloxycarbonyl)-L-tyrosine (9-methoxy-3-oxo-3H-benzo[f]benzopyran-1-yl) methyl ester, Z-Tyr(OtBu)–OBba (**3b**)*

Starting from compound **1** (0.100 g, 0.364 mmol) and *Z*-Tyr(OtBu)–OH·DCHA (**2b**) (0.201 g, 0.364 mmol), compound **3b** was obtained as a yellow solid (0.169 g, 76%). Mp=73.0–74.1 °C. ^{1}H NMR ($CDCl_3$): δ=1.30 (s, 9H, $C(CH_3)_3$), 3.10–3.19 (m, 2H, β-CH_2), 3.95 (s, 3H, OCH_3), 4.74–4.79 (m, 1H, α-H), 5.09 (m, 2H, CH_2 Z), 5.13 (d, *J* 7.6 Hz, 1H, NH), 5.66 (m, 2H, CH_2 Bba), 6.63 (s, 1H, H-2), 6.88 (d, *J* 8.4 Hz, 2H, H-3′and H-5′), 7.02 (d, *J* 8.4 Hz, 2H, H-2′and H-6′), 7.23 (dd, *J* 8.8 and 2.4 Hz, 1H, H-8), 7.32–7.34 (m, 6H, 5×Ph-*H* and H-5), 7.41 (br s, 1H, H-10), 7.83 (d, *J* 9.2 Hz, 1H, H-7), 7.91 (d, *J* 8.8 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$): δ=28.76 ($C(CH_3)_3$), 37.35 (β-CH_2), 55.13 (α-C), 55.44 (OCH_3), 64.96 (CH_2 Bba), 67.21 (CH_2 Z), 78.47 ($C(CH_3)_3$), 105.50 (C-10), 111.76 (C-4b), 113.32 (C-2), 115.29 (C-5), 116.65 (C-8), 124.26 (C-3′ and C-5′), 126.33 (C-6a), 128.17 (2×Ph-*C*), 128.23 (Ph-*C*), 128.49 (2×Ph-*C*), 129.53 (C-2′and C-6′), 129.75 (C1′), 130.50 (C-6b), 131.35 (C-7), 133.81 (C-6), 135.96 (C-1 Ph), 149.86 (C-1), 154.74 (C-4′), 155.58 (C-4a), 155.69 (C=O urethane), 159.71 (C-9), 159.97 (C-3), 171.18 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3336, 3065, 3032, 2975, 2929, 2853, 1725, 1625, 1553, 1519, 1507, 1455, 1445, 1427, 1365, 1339, 1232, 1162, 1105, 1061, 1025, 898, 838, 696. UV–vis (MeOH/HEPES 80:20, nm): λ_{max} (log ε)=351 (4.03), 273 (3.82), 235 (4.55). HRMS (EI): calcd for $C_{36}H_{35}NO_8$ [M^+] 609.2364; found 609.2363.

*4.2.3. N-(Benzyloxycarbonyl)-3,4-dihydroxy-L-phenylalanine (9-methoxy-3-oxo-3H-benzo[f]benzopyran-1-yl) methyl ester, Z-DOPA–OBba (**3c**)*

Starting from compound **1** (0.100 g, 0.364 mmol) and *Z*-DOPA–OH (**2c**) (0.121 g, 0.364 mmol), compound **3c** was obtained as a yellow oily solid (0.087 g, 36%). ^{1}H NMR ($CDCl_3$): δ=2.90–2.97 (m, 1H, β-CH_2), 3.08–3.15 (m, 1H, β-CH_2), 3.92 (s, 3H, OCH_3), 4.70–4.77 (m, 1H, α-H), 5.10 (s, 2H, CH_2 Z), 5.49 (d, *J* 8.7 Hz, 1H, NH), 5.58 (d, *J* 5.1 Hz, 2H, CH_2 Bba), 6.29 (s, 1H, H-2), 6.55 (dd, *J* 8.1 and 1.5 Hz, 1H, H-6′), 6.70 (d, *J* 1.8 Hz, 1H, H-2′), 6.76 (d, *J* 7.8 Hz, 1H, H-5′), 7.19 (d, *J* 7.2 and 2.1 Hz, 1H, H-8), 7.27–7.40 (m, 6H, 5×Ph-*H* and H-5), 7.37 (d, *J* 2.1 Hz, H-10), 7.78 (d, *J* 9.0 Hz, H-7), 7.85 (d, *J* 8.7 Hz, H-6). ^{13}C NMR ($CDCl_3$): δ=37.76 (β-CH_2), 55.42 (α-C), 55.49 (OCH_3), 64.78 (CH_2 Bba), 67.29 (CH_2 Z), 105.84 (C-10), 111.83 (C-4b), 112.16 (C-2), 115.05 (C-5), 116.10 (C-5′), 116.26 (C-2′), 116.66 (C-8), 121.57 (C-6′), 127.47 (C-1′), 128.16 (2×Ph-*C*), 128.26 (Ph-*C*), 128.50 (2×Ph-*C*), 130.31 (C-6b), 131.33 (C-7), 134.00 (C-6), 135.89 (C1 Ph), 143.79 (C-4′), 144.45 (C-3′), 151.04 (C-1), 155.22 (C-4a), 155.89 (C=O urethane), 159.70 (C-9), 161.27 (C-3), 171.55 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3415 (br), 2927, 1712, 1702, 1625, 1552, 1519, 1445, 1363, 1340, 1284, 1233, 1216, 1177, 1114, 1062, 1025, 839, 751, 697, 666. UV–vis (MeOH/HEPES, 80:20, nm): λ_{max} (log ε)=350 (3.91), 280 (3.84), 236 (4.55). HRMS (EI): calcd for $C_{32}H_{27}NO_9$ [M^+] 569.1686; found 569.1690.

*4.2.4. 2-(N-Benzyloxycarbonyl)amino-5-methyl-1-(9-methoxy-3-oxo-3H-benzo[f]benzo pyran-1-yl) methyl pentanedioate, Z-Glu(OMe)–OBba (**3d**)*

Starting from compound **1** (0.100 g, 0.364 mmol) and *Z*-Glu(OMe)–OH (**2d**) (0.107 g, 0.364 mmol), compound **3d** was

obtained as a light brown solid (0.147 g, 76%). Mp=83.2–84.5 °C. ^{1}H NMR ($CDCl_3$): δ=2.02–2.15 (m, 1H, γ-CH_2), 2.24–2.33 (m, 1H, γ-CH_2), 2.45–2.50 (m, 2H, β-CH_2), 3.64 (s, 3H, OCH_3 Glu), 3.92 (s, 3H, OCH_3 Bba), 4.52–4.59 (m, 1H, α-H), 5.10 (s, 2H, CH_2 Z), 5.63 (s, 2H, CH_2 Bba), 5.75 (d, *J* 8.1 Hz, 1H, NH), 6.62 (s, 1H, H-2), 7.18 (dd, *J* 9.0 and 2.1 Hz, 1H, H-8), 7.22–7.31 (m, 7H, 5×Ph-*H*, H-5 and H-10), 7.77 (d, *J* 9.3 Hz, 1H, H-7), 7.84 (d, *J* 8.7 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$): δ=26.88 (γ-CH_2), 29.84 (β-CH_2), 51.83 (OCH_3 Glu), 53.54 (α-C), 55.36 (OCH_3 Bba), 64.94 (CH_2 Bba), 67.14 (CH_2 Z), 105.53 (C-10), 111.57 (C-4b), 112.83 (C-2), 115.12 (C-5), 116.49 (C-8), 126.19 (C-6a), 128.12 (3×Ph-*C*), 128.40 (2×Ph-*C*), 130.35 (C-6b), 131.26 (C-7), 133.71 (C-6), 135.91 (C1 Ph), 150.02 (C-1), 155.39 (C-4a), 156.00 (C=O urethane), 159.59 (C-9), 159.97 (C-3), 171.25 (C=O ester), 172.96 (C=O Glu). IR (KBr 1%, cm^{-1}): ν=3329, 3067, 3033, 2952, 1729, 1692, 1625, 1595, 1553, 1519, 1445, 1381, 1365, 1267, 1231, 1124, 1055, 1026, 985, 941, 840, 821, 728, 697. UV–vis (MeOH/HEPES, 80:20, nm): λ_{max} (log ε)=351 (4.00), 274 (3.79), 239.0 (4.54). HRMS (EI): calcd for $C_{29}H_{27}NO_9$ [M^+] 533.1686; found 533.1683.

*4.2.5. 4-(N-Benzyloxycarbonyl)amino-5-methyl-1-(9-methoxy-3-oxo-3H-benzo[f]benzo pyran-1-yl) methyl pentanedioate, Z-Glu(OBba)–OMe (**3e**)*

Starting from compound **1** (0.070 g, 0.255 mmol) and *Z*-Glu(OMe)–OH (**2e**) (0.076 g, 0.255 mmol), compound **3e** was obtained as a light brown solid (0.040 g, 29%). Mp=101.1–103.6 °C. ^{1}H NMR ($CDCl_3$): δ=1.98–2.10 (m, 1H, γ-CH_2), 2.28–2.39 (m, 1H, γ-CH_2), 2.53–2.71 (m, 2H, β-CH_2), 3.78 (s, 3H, OCH_3 Glu), 3.95 (s, 3H, OCH_3 Bba), 4.50 (q, *J* 5.1 Hz 1H, α-H), 5.11 (s, 2H, CH_2 Z), 5.50 (d, *J* 8.1 Hz, 1H, NH), 5.66 (s, 2H, CH_2 Bba), 6.65 (s, 1H, H-2), 7.22 (dd, *J* 8.7 and 2.4 Hz, 1H, H-8), 7.27–7.35 (m, 6H, 5×Ph-*H* and H-5), 7.40 (s, 1H, H-10), 7.82 (d, *J* 9.0 Hz, 1H, H-7), 7.90 (d, *J* 8.7 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$): δ=27.63 (γ-CH_2), 29.90 (β-CH_2), 52.65 (OCH_3 Bba), 53.01 (α-C), 55.39 (OCH_3 Glu), 64.28 (CH_2 Bba), 67.12 (CH_2 Z), 105.71 (C-10), 111.82 (C-4b), 112.61 (C-2), 115.26 (C-5), 116.53 (C-8), 126.31 (C-6a), 128.05 (Ph-*C*), 128.17 (2×Ph-*C*), 128.46 (2×Ph-*C*), 130.54 (C-6b), 131.29 (C-6), 133.72 (C-7), 135.99 (C1 Ph), 150.75 (C-1), 155.48 (C-4a), 155.94 (C=O urethane), 159.61 (C-9), 160.18 (C-3), 171.80 (C=O ester), 172.09 (C=O Glu). IR (KBr 1%, cm^{-1}): ν=3328, 3081, 2954, 1724, 1693, 1625, 1553, 1521, 1455, 1446, 1407, 1380, 1365, 1277, 1230, 1179, 1053, 1024, 985, 958, 942, 902, 838. UV–vis (MeOH/HEPES 80:20, nm): λ_{max} (log ε)=347 (4.00), 273 (3.94), 235 (4.55). HRMS (EI): calcd for $C_{29}H_{27}NO_9$ [M^+] 533.1686; found 533.1689.

4.3. General photolysis procedure

A 1×10^{-3} M (compound **3c**) or 1×10^{-4} M MeOH/HEPES (80:20) solution of compounds **3a**–**b**,**d**–**e** (5 mL) was 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 0.6 (compound **3c**), 0.8 (compounds **3a**,**d**–**e**) or 1.2 (compound **3b**) mL/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 compound (retention time: **3a**, 5.3; **3b**, 8.1; **3c**, 5.6; **3d**, 5.3; **3e**, 5.4 min).

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