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ScienceDirect
Tetrahedron
Tetrahedron 63 (2007) 1353–1359

Photocleavage studies of fluorescent amino acid conjugates bearing different types of linkages

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Received 31 October 2006; revised 24 November 2006; accepted 30 November 2006
Available online 20 December 2006

Abstract—The synthesis and photochemistry of 3-oxo-3*H*-benzopyran derivatives linked through ester, anhydride, urethane and carbonate bonds to representative L-amino acids, at the amino and carboxylic acid groups at the main chain or the hydroxyl group at the side chain, were carried out. The stability to photolysis of the resulting conjugates was studied at different wavelengths of irradiation (254, 300 and 350 nm), the anhydride and ester linkages being the most sensitive in the studied conditions.

1. Introduction

The fluorescent derivatisation of biomolecules with organic fluorophores for analytical applications represents a steadily growing field of research, especially through the development of new fluorescent ligands. Fluorescence provides higher sensitivity than the corresponding UV techniques, thus overcoming problems of low detection limits and allowing the easy monitoring of the course of organic reactions. Examples are found in many diverse areas such as cell biology and biochemistry, as fluorescent probes to investigate the functionality and action of biomolecules inside living cells,[1,2] in combinatorial chemistry, to allow the rapid screening of large libraries of compounds,[3,4] and in analytical chemistry.[5,6] In amino acid and peptide chemistry, appropriate fluorophoric moieties are also used, with 3-oxo-3*H*-benzopyrans (trivially named as coumarins) representing one of the most popular fluorogenic reagents, due to their extended spectral range, high fluorescence quantum yields, good photostability and solubility in common solvents.[7]

Photolabile protecting groups are advantageous over conventional blocking groups, because the deprotection strategy only involves radiation and can be orthogonal to chemical conditions. In addition to this quality, the use of fluorescent moieties results in an increase in their importance specially when used for the protection of colourless or nonfluorescent systems. In recent reports, 3-oxo-3*H*-benzopyran derivatives have been proposed as photolabile protecting groups for a variety of functional groups such as alcohols, aldehydes, ketones, acids and amines,[8–11] and in biological applications as caging groups for nucleotides and amino acid neurotransmitters.[12–14]

Bearing in mind our recent research in the synthesis and application of fluorescent heterocycles, namely their use in amino acid chemistry as markers and as protecting groups,[15–17] in this work we investigated the possibility of using 6-hydroxy and 6-methoxy-1-methylene-3-oxo-3*H*-benzopyrans as versatile fluorescent photolabile protecting groups for the amino, carboxyl and hydroxyl functions of organic molecules through linkages of different nature. Using amino acids as models, the synthesis and characterisation of new fluorescent amino acid conjugates were carried out. Absorption and emission properties of all compounds were measured and the results showed that these conjugates exhibited moderate to high fluorescence quantum yields and Stokes' shifts.

Photocleavage of these fully protected amino acids was achieved by using radiation of 254, 300 and 350 nm. The course of the photolysis was followed by RP-HPLC and kinetic data were also obtained.

2. Results and discussion

6-Hydroxy and 6-methoxy-1-chloromethyl-3-oxo-3*H*-benzopyrans **1a**,**b** were synthesised by a Pechmann reaction, starting from 1,3-dihydroxybenzene and 3-methoxyphenol, respectively, and ethyl chloroacetoacetate, catalysed by sulfuric acid at room temperature in good yields, by a known procedure.[18] 6-Methoxy-1-hydroxymethyl-3-oxo-3*H*-benzopyran **1c** was obtained by a similar procedure by the reaction of 3-methoxyphenol with ethyl acetoacetate. The methyl group

Keywords: 3-Oxo-3*H*-benzopyrans; Photocleavable protecting groups; Photocleavage; Amino acids; Fluorophores.
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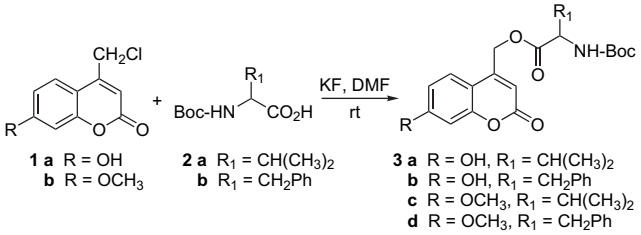
0040–4020/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2006.11.082

was oxidised to aldehyde, by reaction with selenium dioxide, which was then reacted with sodium borohydride, affording the hydroxymethyl group.[19]

Our purpose being to study the linkage of chloromethyl-3-oxo-3*H*-benzopyrans **1a**,**b** to the carboxylic function of α-amino acids by an ester bond and to compare the influence of the substituent at the oxobenzopyran, *N*-*tert*-butyloxycarbonyl-L-valine, Boc–Val–OH (**2a**) and *N*-*tert*-butyloxycarbonyl-L-phenylalanine, Boc–Phe–OH (**2b**) were chosen as models. Derivatisation at the C-terminus of **2a**,**b** with heterocycles **1a**,**b** was carried out with potassium fluoride,[20] in DMF, at room temperature, yielding fluorescent conjugates **3a–d** (Scheme 1, Table 1).



Scheme 1. Synthesis of fluorescent conjugates **3a–d**.

In order to explore the versatility of benzopyran in establishing different types of covalent bonds to diverse functional groups of organic molecules and also their stability to photolysis conditions, amino acid conjugates bearing the fluorophore connected through linkages other than ester bonds were prepared.

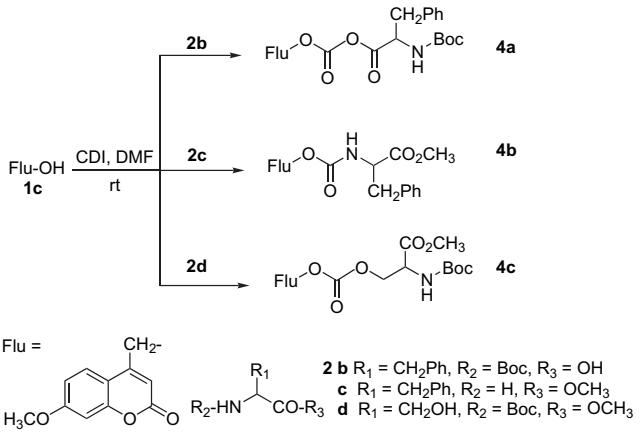
6-Methoxy-1-hydroxymethyl-3-oxo-3*H*-benzopyran **1c** was linked to Boc–Phe–OH (**2b**) and L-phenylalanine methyl ester, H–Phe–OMe (**2c**), at the main chain carboxyl and amino functions, and to *N*-*tert*-butyloxycarbonyl-L-serine methyl ester, Boc–Ser–OMe (**2d**), at the side chain hydroxyl group. The corresponding fluorescent conjugates **4a–c** were obtained by a 1,1′-carbonyldiimidazole (CDI) carbonyl transfer reaction[21] with fluorophore **1c** linked to the amino acid through a mixed carbonic anhydride (**4a**), urethane (**4b**) and carbonate (**4c**) bond (Scheme 2, Table 1).

After column chromatography on silica gel, the corresponding fluorescent derivatives were obtained in good to excellent yields (62–97%), in the case of compounds **3a–d**, and in lower yields (24–36%) for compounds **4a–c** (Table 1) and were characterised by high-resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy.

The IR spectra of labelled amino acids **3a–d** and **4a–c** showed bands due to stretching vibrations of the various

Table 1. Synthesis of fluorescent amino acid conjugates **3a–d** and **4a–c**

Entry	Label	Amino acid	Product	Yield (%)
1	**1a**	**2a**	**3a**	86
2	**1a**	**2b**	**3b**	97
3	**1b**	**2a**	**3c**	78
4	**1b**	**2b**	**3d**	62
5	**1c**	**2b**	**4a**	24
6	**1c**	**2c**	**4b**	36
7	**1c**	**2d**	**4c**	25



Scheme 2. Synthesis of fluorescent conjugates **4a–c**.

carbonyl groups of the linkage fluorophore–amino acid from 1739 to 1756 cm^{-1} (esters **3a–d**), at 1815 and 1750 cm^{-1} (anhydride **4a**), at 1720–1742 cm^{-1} (urethane **4b**) and 1682 cm^{-1} (carbonate **4c**). The spectra also showed the carbonyl bands of *N*-*tert*-butyloxycarbonyl (1673–1721 cm^{-1}) and methyl ester (1720 cm^{-1}) protecting groups, as well as the oxo group at the pyran ring (1644–1690 cm^{-1}).

^{1}H NMR spectra showed signals of the amino acid residues, such as the multiplets for the α-CH (δ 4.24–4.75 ppm), as well as the characteristic protons of the heterocyclic moiety at δ 6.12–6.37 ppm for the H-2 and δ 5.20–5.39 ppm for the methylene group. The confirmation of the presence of the newly formed linkages was also supported by ^{13}C NMR spectral signals of the carbonyl group, which were found at about δ 171 ppm for the ester (**3a–d**), at δ 171.45 and 175.33 ppm for the anhydride (**4a**), at δ 155.21 ppm for the urethane (**4b**) and δ 173.43 ppm for the carbonate (**4c**), as well as the signals of the oxo group of the heterocycle (δ 160.54 to 161.16 ppm) and the carbonyl of the Boc (at about δ 155 ppm) and methyl ester (δ 169.75 ppm) protecting groups.

Electronic absorption and emission spectra of 10^{-5}–10^{-6} m solutions of compounds **3a–d** and **4a–c** in degassed absolute ethanol were measured; absorption and emission maxima, molar absorptivities and fluorescence quantum yields (Φ_F) are also reported (Table 2). The Φ_F was calculated using 9,10-diphenylanthracene as standard (Φ_F=0.95 in ethanol).[22] For Φ_F determination, 9,10-diphenylanthracene was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested. The longest wavelength

Table 2. UV–vis and fluorescence data for compounds **3a–d** and **4a–c**

Compound	UV–vis		Fluorescence		Stokes' shift (nm)
	log ε	λ_{max} (nm)	λ_{em} (nm)	Φ_F[a]	
3a	4.12	325	398	0.61	73
3b	4.15	324	397	0.62	73
3c	4.14	323	392	0.28	69
3d	4.11	323	388	0.27	65
4a	3.99	323	390	0.20	67
4b	3.67	322	386	0.26	64
4c	3.70	323	389	0.22	66

[a] Mean deviation from 0.02 to 0.04.

absorption maxima of all compounds were at about 325 nm, with log ε ranging from 3.67 to 4.15. The wavelengths of maximum emission were found between 386 and 398 nm. The nature of the amino acid and of the substituent present at the fluorophore did not appear to influence the position of the wavelengths of maximum absorption and emission. All labelled amino acids **3a–d** and **4a–c** exhibited good to excellent quantum yields ($0.20<\Phi_F<0.62$) and Stokes' shifts ranging from 64 to 73 nm. It was possible to see that derivatives **3a**,**b** exhibited the highest value of fluorescence quantum yield (~0.62) and also the larger Stokes' shift (73 nm), which may be related to the electron-donating character of the hydroxy substituent at the oxobenzopyran moiety.

Furthermore, the absorption and emission spectra of compound **3b**, which had the highest value of fluorescence quantum yield in ethanol, were measured in 10 more solvents of different polarities and proton donor abilities, such as *n*-hexane, diethyl ether, 1,4-dioxane, ethyl acetate, tetrahydrofuran (THF), acetonitrile (ACN), chloroform, dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO). The wavelengths of maximum absorption and emission and Φ_F for this compound are listed in Table 3.

From the results, it can be seen that the polarity and the proton donor ability of the solvent did not influence significantly the position of the band of absorption, with the wavelength of maximum absorption ranging from 319 to 323 nm. In the case of the wavelength of maximum emission, the same behaviour was observed (from 383 to 395 nm), except for *n*-hexane (entry 1) in which the lowest value of λ_{em} was obtained (350 nm). However, a slight influence of the character of the solvent on Φ_F was observed, as lower values of Φ_F seemed to be associated with low polarity solvents, such as *n*-hexane and diethyl ether (entries 1 and 2).

Table 3. UV–vis and fluorescence data for compound **3b** in different solvents

Entry	Solvent	UV–vis	Fluorescence		Stokes' shift (nm)
		λ_{max} (nm)	λ_{em} (nm)	Φ_F[a]	
1	*n*-Hexane	320	350	0.003	30
2	Diethyl ether	319	383	0.054	64
3	1,4-Dioxane	321	392	0.15	71
4	Ethyl acetate	320	386	0.14	66
5	THF	320	388	0.10	68
6	Acetonitrile	320	390	0.19	70
7	Chloroform	323	394	0.51	71
8	DCM	322	395	0.23	73
9	DMF	322	393	0.47	71
10	DMSO	323	392	0.36	69

[a] Mean deviation from 0.01 to 0.04, except for entries 1 (0.001) and 2 (0.003).

Our main purpose being the investigation of the potential application of fluorophore **1c** as a versatile photocleavable protecting group in organic synthesis, and in order to assess the sensitivity of the various types of linkages between the fluorescent oxobenzopyrans and the amino acids, photocleavage studies of phenylalanine conjugates **3b**,**d** and **4a**,**b** and serine conjugate **4c** were carried out. Solutions of the mentioned compounds in acetonitrile (1×10^{-5} m) were irradiated in a Rayonet RPR-100 reactor, at 254, 300 and 350 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 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, at 254 and 300 nm. When the photolysis was carried out at 350 nm, the reactions were followed until less than 10% of the initial area could be detected.

Based on HPLC data, the kinetic study of the photocleavage reactions was also carried out. For each compound, 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. The rate constant (k) values were obtained by the linear least squares methodology for a straight line (Table 4). Figure 1 summarises the behaviour of phenylalanine conjugates **3b**,**d** and **4a**,**b** at 300 nm.

Concerning the influence of the wavelength of irradiation on the rate of the photocleavage reactions of compounds **3b**,**d** and **4a–c** in acetonitrile it can be seen that at 350 nm the cleavage occurs very slowly (1090–5310 min for 90% of starting material consumption) when compared to 254 and 300 nm that require similar irradiation times (30–175 and 40–130 min, respectively) to achieve the cleavage of at least 95% of the starting material.

The presence of a hydroxyl substituent at the oxobenzopyran moiety appeared to have a positive effect on the cleavage rate, as compound **3b** cleaved twice as fast than compound **3d** at 300 nm and 2.5 times faster at 350 nm. At 254 nm both compounds showed similar cleavage rates.

Table 4. Irradiation times and kinetic data for the photolysis of compounds **3b**,**d** and **4a–c** at the considered wavelengths, in acetonitrile solution (1×10^{-5} m)

Compound	254 nm		300 nm		350 nm	
	Irradiation time (min)	Rate constant, k ($\times10^{-2}$ min^{-1})[a]	Irradiation time (min)	Rate constant, k ($\times10^{-2}$ min^{-1})[a]	Irradiation time (min)	Rate constant, k ($\times10^{-2}$ min^{-1})[a]
3b	35	9.03	40	7.34	1090	0.21
3d	35	8.92	80	3.99	2730	0.10
4a	30	10.41	45	7.21	1740	0.14
4b	55	4.97	65	4.59	3190	0.07
4c	175	1.94	130	2.81	5310	0.05

[a] Correlation coefficient varied from 0.9786 to 0.9984.

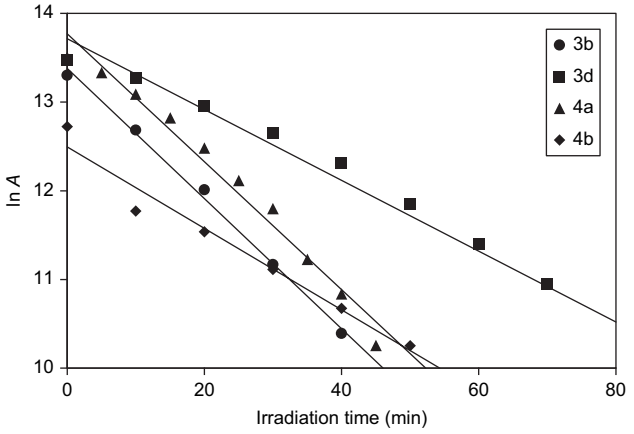


Figure 1. Plot of ln *A* versus irradiation time for the photolysis of phenylalanine conjugates **3b**,**d** and **4a**,**b** at 300 nm.

Regarding the nature of the linkage of fluorophore to the amino acid, we found out that when the linkage is done through a carbonate bond (compound **4c**), the cleavage is slower at all considered wavelengths of irradiation, when compared to the other types of linkages.

Considering compounds **3d** and **4a**, in which the same oxobenzopyran was linked to the C-terminal of phenylalanine through different linkages (ester and anhydride, respectively), our results indicated that the anhydride bond cleaved faster at the considered wavelengths. For compounds **4a** and **4b**, in which the same fluorophore was linked to the C- and N-terminal, through an anhydride and a urethane bond, respectively, it could be seen that the urethane bond was more stable to radiation, cleaving in a slower rate.

Considering the three types of possible linkages (ester, anhydride and urethane) between the same fluorophore (6-methoxy-1-methylene-3-oxo-3*H*-benzopyran) and the main chain amino or carboxyl terminal of the amino acid (compounds **3d**, **4a** and **4b**), the relative sensitivity of bond cleavage follows the order anhydride>ester>urethane, at 254 and 350 nm, whereas at 300 nm the order becomes anhydride>urethane>ester.

We also studied the influence of the solvent on the course of the photocleavage reaction for compound **3b** at 300 nm, by using other solvents like methanol and a THF/H_2O mixture, 4:1. The data collected revealed that in both cases the cleavage occurred faster than that in acetonitrile, revealing that for this compound protic solvents improved the photocleavage rate, a dramatic increase being obtained in the case of THF/H_2O mixture (Table 5).

From the obtained results, 6-hydroxy and 6-methoxy-1-methylene-3-oxo-3*H*-benzopyrans can be considered as suitable and versatile photolabile protecting groups for the amino, carboxyl and hydroxyl functions of amino acids, on account of the short irradiation times, which are desirable in the process of photodeprotection, to prevent side reactions in other functionalities present in the molecule that is being cleaved. Although irradiation times at 254 nm are slightly shorter for all compounds except **4c**, the wavelength of 300 nm seems more suitable to perform such deprotection reactions because the radiation will have a less detrimental effect on the rest of the molecule. Irradiation at 350 nm would be less appropriate because of the required long irradiation periods.

Table 5. Photolysis and kinetic data for compound **3b** in different solvents

Solvent	Irradiation time (min)	Rate constant, k ($\times 10^{-2}$ min^{-1})[a]
ACN	40	3.19
MeOH	25	4.68
THF/H_2O, 4:1	10	12.31

[a] Correlation coefficient varied from 0.9855 to 0.9934.

3. Conclusions

A series of fluorescent amino acid conjugates were synthesised in good to excellent yields by a straightforward procedure, between 6-hydroxy and 6-methoxy-1-chloromethyl-3-oxo-3*H*-benzopyrans and the C-terminus of *N*-Boc-protected L-valine and L-phenylalanine, through an ester bond. Another series of fluorescent amino acid conjugates, bearing the same fluorophore (6-methoxy-1-methylene-3-oxo-3*H*-benzopyran) connected by different linkages to the N- and C-terminus of L-phenylalanine (carbonic anhydride and urethane) and to the hydroxyl group at the side chain of L-serine (carbonate), were also obtained in moderate yields.

The photocleavage studies of the fluorescent amino acid conjugates with 1-methylene-3-oxo-3*H*-benzopyrans at 254, 300 and 350 nm showed that these heterocycles could be used as photolabile protecting groups through different linkages, the most suitable wavelength of irradiation being 300 nm as a result of short irradiation times. The type of linkage between the heterocycle and the amino acid was found to be determinant to the time of irradiation required to cleave the link: the anhydride and ester bonds were more sensitive to radiation whereas the carbonate bond was the most stable, at all considered wavelengths of irradiation.

Bearing in mind the photophysical properties displayed by these compounds, they can be considered as versatile fluorogenic reagents as well as photolabile protecting groups for organic molecules, including amino acids and other biomolecules.

4. Experimental

4.1. General

All melting points were measured on a Gallenkamp 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 Perkin Elmer FTIR-1600 using KBr discs. UV–vis spectra were run on a Hitachi U-2000 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian 300 spectrometer in $CDCl_3$ at 300 MHz 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. ^{13}C NMR spectra were run in the same instrument at 75.4 MHz using the solvent peak as internal reference. 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 HMBC and HMQC 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 Spex Fluorolog 1680 spectrometer. Photolyses were carried out using a Rayonet RPR-100 chamber reactor equipped with eight lamps of different wavelengths (254, 300 or 350 nm, 14 W each). HPLC analysis was performed using a Lichrospher 100 RP18 (5 μm) column in an HPLC system composed by a Jasco PU-980 pump, a UV–vis Shimadzu SPD-GAV detector and a Shimadzu C-RGA Chromatopac register.

Compounds **1a–c** were synthesised according to published procedures[18,19] and their spectral data compared well with that of the literature.

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

1-Chloromethyl-3-oxo-3*H*-benzopyrans **1a**,**b** (1 equiv) were dissolved in dry DMF (2 mL), potassium fluoride (3 equiv) and Boc–Val–OH, **2a**, or Boc–Phe–OH, **2b** (1.1 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.

4.2.1. *N*-(*tert*-Butyloxycarbonyl)-L-valine (6-hydroxy-3-oxo-3*H*-benzopyran-1-yl) methyl ester (3a). Starting from **1a** (0.210 g, 1 mmol) and **2a** (0.239 g, 1.1 mmol), the eluent used in the chromatography was $CHCl_3$/*n*-hexane, 80:20, and $CHCl_3$. Compound **3a** was obtained as a colourless oil (0.336 g, 86%). ^1H NMR ($CDCl_3$): δ=0.92 (d, *J*=6.9 Hz, 3H, γ-CH_3), 1.01 (d, *J*=6.9 Hz, 3H, γ-CH_3), 1.41 (s, 9H, C(CH_3)$_3$), 2.18–2.24 (m, 1H, β-H), 4.24–4.29 (m, 1H, α-H), 5.11 (d, *J*=8.7 Hz, 1H, NH), 5.25 (d, *J*=6.3 Hz, 2H, CH_2), 6.25 (s, 1H, H-2), 6.80 (dd, *J*=2.4 and 9.0 Hz, 1H, H-7), 6.83 (d, *J*=2.4 Hz, 1H, H-5), 7.30 (d, *J*=9.0 Hz, 1H, H-8), 10.01 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$): δ=17.55 (γ-*C*H_3), 19.16 (γ-*C*H_3), 28.27 (C(*C*H_3)$_3$), 30.94 (β-C), 58.78 (α-C), 61.89 (CH_2), 80.31 (*C*(CH$_3$)$_3$), 103.62 (C-5), 109.47 (C-2), 109.70 (C-8a), 113.51 (C-7), 124.53 (C-8), 148.77 (C-1), 155.45 (C-4a), 156.00 (C=O urethane), 161.16 (C-3), 161.21 (C-6), 171.83 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3380–3300 (br, OH and NH), 2966, 2931, 2876, 1739 (C=O), 1710 (C=O), 1664 (C=O), 1632, 1573, 1519, 1505, 1455. UV–vis (ethanol, nm): λ_{max} (log ε)=325 (4.12). MS (EI, %): *m*/*z*=335 ([M]$^{+\bullet}$−*t*Bu, 37), 176 (41), 147 (20), 116 (18), 72 (100). HRMS (EI) calcd for $C_{16}H_{17}NO_7$ ([M]$^{+\bullet}$−*t*Bu): 335.1005; found: 335.0993.

4.2.2. *N*-(*tert*-Butyloxycarbonyl)-L-phenylalanine (6-hydroxy-3-oxo-3*H*-benzopyran-1-yl) methyl ester (3b). Starting from **1a** (0.420 g, 2 mmol) and **2b** (0.583 g, 2.2 mmol), the eluent used in the chromatography was DCM/MeOH, 100:1, and DCM/MeOH, 95:5. Compound **3b** was obtained as a yellowish solid (0.850 g, 97%). Mp=125.0–129.3 °C. ^1H NMR ($CDCl_3$): δ=1.43 (s, 9H, C(CH_3)$_3$), 3.11 (d, *J*=6.6 Hz, 2H, β-CH_2), 4.64–4.68 (m, 1H, α-H), 5.02 (d, *J*=7.5 Hz, 1H, NH), 5.20 (s, 2H, CH_2), 6.12 (s, 1H, H-2), 6.81 (dd, *J*=2.4 and 8.7 Hz, 1H, H-7), 6.86 (d, *J*=2.4 Hz, 1H, H-5), 7.11–7.15 (m, 2H, 2×Ph–*H*), 7.24–7.29 (m, 4H, H-8 and 3×Ph–*H*), 9.28 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$): δ=28.19 (C(*C*H_3)$_3$), 38.08 (β-CH_2), 54.72 (α-C), 61.92 (CH_2), 80.63 (*C*(CH$_3$)$_3$), 103.46 (C-5), 109.22 (C-2), 109.62 (C-8a), 113.56 (C-7), 124.52 (C-8), 127.29 (C-4 Ph), 128.69 (C-3 and C-5 Ph), 129.02 (C-2 and C-6 Ph), 135.35 (C-1 Ph), 148.60 (C-1), 155.25 (C-4a), 156.00 (C=O urethane), 161.16 (C-3), 161.29 (C-6), 171.38 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3500–3200 (br, OH), 3347 (NH), 2979, 2931, 1740 (C=O), 1713 (C=O), 1682 (C=O), 1615, 1571, 1525, 1454. UV–vis (ethanol, nm): λ_{max} (log ε)=324 (4.15). MS (EI, %): *m*/*z*=383 ([M]$^{+\bullet}$−*t*Bu, 35), 366 (15), 322 (20), 248 (37), 192 (24), 176 (38), 175 (18), 147 (43), 131 (74), 120 (65), 91 (100). HRMS (EI) calcd for $C_{20}H_{17}NO_7$ ([M]$^{+\bullet}$−*t*Bu): 383.1005; found: 383.1003.

4.2.3. *N*-(*tert*-Butyloxycarbonyl)-L-valine (6-methoxy-3-oxo-3*H*-benzopyran-1-yl) methyl ester (3c). Starting from **1b** (0.100 g, 0.45 mmol) and **2a** (0.106 g, 0.50 mmol), the eluent used in the chromatography was $CHCl_3$. Compound **3c** was obtained as a yellow solid (0.140 g, 78%). Mp=77.8–86.3 °C. ^1H NMR ($CDCl_3$): δ=0.92 (d, *J*=6.9 Hz, 3H, γ-CH_3), 1.01 (d, *J*=6.9 Hz, 3H, γ-CH_3), 1.46 (s, 9H, C(CH_3)$_3$), 2.16–2.24 (m, 1H, β-H), 3.89 (s, 3H, OCH_3), 4.30–4.36 (m, 1H, α-H), 5.00 (d, *J*=9.0 Hz, 1H, NH), 5.33 (s, 2H, CH_2), 6.37 (s, 1H, H-2), 6.87 (d, *J*=2.7 Hz, 1H, H-5), 6.88 (dd, *J*=2.7 and 8.4 Hz, 1H, H-7), 7.42 (d, *J*=8.4 Hz, 1H, H-8). ^{13}C NMR ($CDCl_3$): δ=17.53 (γ-CH_3), 19.14 (γ-CH_3), 28.24 (C(*C*H_3)$_3$), 30.97 (β-C), 55.76 (OCH_3), 58.74 (α-C), 61.82 (CH_2), 80.14 (*C*(CH$_3$)$_3$), 101.21 (C-5), 110.43 (C-2), 110.48 (C-8a), 112.64 (C-7), 124.46 (C-8), 148.51 (C-1), 155.53 (C-4a), 155.60 (C=O urethane), 160.67 (C-3), 162.90 (C-6), 171.85 (C=O ester). IR (KBr 1%, cm^{-1}): ν=3346 (NH), 2963, 2876, 1750 (C=O), 1721 (C=O), 1684 (C=O), 1619, 1559, 1529, 1463, 1450. UV–vis (ethanol, nm): λ_{max} (log ε)=323 (4.11). MS (FAB, %): *m*/*z*=406 ([M+H]$^{+\bullet}$, 15), 405 (M$^{+\bullet}$, 65), 332 (39), 262 (19), 190 (100), 189 (16), 161 (29), 116 (63), 72 (79). HRMS (EI) calcd for $C_{21}H_{27}NO_7$ [M]$^{+\bullet}$: 405.1788; found: 405.1751.

4.2.4. *N*-(*tert*-Butyloxycarbonyl)-L-phenylalanine (6-methoxy-3-oxo-3*H*-benzopyran-1-yl) methyl ester (3d). Starting from **1b** (0.100 g, 0.45 mmol) and **2b** (0.130 g, 0.50 mmol), the eluent used in the chromatography was $CHCl_3$. Compound **3d** was obtained as a yellow solid (0.125 g, 62%). Mp=118.9–121.2 °C. ^1H NMR ($CDCl_3$): δ=1.43 (s, 9H, C(CH_3)$_3$), 3.11 (d, *J*=6.3 Hz, 2H, β-CH_2), 3.88 (s, 3H, OCH_3), 4.63–4.70 (m, 1H, α-H), 5.02 (d, *J*=7.2 Hz, 1H, NH), 5.23 (s, 2H, CH_2), 6.18 (s, 1H, H-2), 6.83–6.85 (m, 2H, H-5 and H-7), 7.12–7.14 (m, 2H, 2×Ph–*H*), 7.25–7.28 (m, 3H, 3×Ph–*H*), 7.32 (d, *J*=9.6 Hz, 1H, H-8). ^{13}C NMR ($CDCl_3$): δ=28.20 (C(*C*H_3)$_3$), 38.26 (β-CH_2), 54.69 (α-C), 55.75 (OCH_3), 61.92 (CH_2), 80.29 (*C*(CH$_3$)$_3$), 101.13 (C-5), 110.47 (C-8a), 110.52 (C-2), 112.59 (C-7), 124.47 (C-8), 127.28 (C-4 Ph),

128.68 (C-3 and C-5 Ph), 129.07 (C-2 and C-6 Ph), 135.45 (C-1 Ph), 148.08 (C-1), 155.06 (C═O urethane), 155.50 (C-4a), 160.54 (C-3), 162.83 (C-6), 171.44 (C═O ester). IR (KBr 1%, cm^{-1}): ν=3349 (NH), 3087, 3063, 3025, 2978, 2934, 2872, 2840, 2631, 2419, 2048, 1942, 1756 (C═O), 1717 (C═O), 1690 (C═O), 1614, 1560, 1532, 1497, 1455. UV–vis (ethanol, nm): λ_{max} (log ε)=323 (4.14). MS (EI, %): *m*/*z*=453 ($[M]^{+\bullet}$, 3), 398 (25), 397 (100), 380 (34), 379 (15), 337 (20), 336 (46), 262 (59), 206 (56), 190 (69), 189 (20), 161 (27), 131 (68), 120 (59), 91 (68). HRMS (EI) calcd for $C_{25}H_{27}NO_7$ $[M]^{+\bullet}$: 453.1788; found: 453.1798.

4.2.5. 2-(*tert*-Butyloxycarbonylamino)-3-phenylpropanoic [(6-methoxy-3-oxo-3*H*-benzopyran-1-yl) methyl carbonic] anhydride (4a). 1-Hydroxymethyl-6-methoxy-3-oxo-3*H*-benzopyran **1c** (0.150 g, 0.728 mmol), Boc–Phe–OH, **2b** (0.386 g, 1.46 mmol) and CDI (0.236 g, 1.46 mmol) were dissolved in dry DMF (4 mL). The reaction mixture was stirred for 10 min and then triethylamine (0.5 mL) was added. The reaction mixture was stirred at room temperature for 24 h and the solvent was removed by rotary evaporation under reduced pressure. The crude residue was purified by column chromatography, using $CHCl_3$ and $CHCl_3$/MeOH, 100:1, as eluent. Compound **4a** was obtained as a light yellow solid (0.086 g, 24%). Mp=71.0–73.5 °C (dec). ^1H NMR ($CDCl_3$): δ=1.43 (s, 9H, C(CH_3)$_3$), 3.10–3.12 (m, 2H, β-CH_2), 3.88 (s, 3H, OCH_3), 4.63–4.70 (m, 1H, α-H), 5.00 (d, *J*=7.0 Hz, 1H, NH), 5.24 (s, 2H, CH_2), 6.18 (s, 1H, H-2), 6.80–6.87 (m, 2H, H-5 and H-7), 7.14–7.18 (m, 2H, 2×Ph–*H*), 7.25–7.40 (m, 4H, H-8 and 3×Ph–*H*). ^{13}C NMR ($CDCl_3$): δ=28.21 (C(CH_3)$_3$), 38.27 (β-CH_2), 54.70 (α-C), 55.76 (OCH_3), 61.93 (CH_2), 80.30 ($C(CH_3)_3$), 101.15 (C-5), 110.49 (C-8a), 110.54 (C-2), 112.61 (C-7), 124.48 (C-8), 127.29 (C-4 Ph), 128.69 (C-3 and C-5 Ph), 129.08 (C-2 and C-6 Ph), 135.46 (C-1 Ph), 148.09 (C-1), 155.10 (C═O urethane), 155.52 (C-4a), 160.56 (C-3), 162.85 (C-6), 171.45 (C═O), 175.33 (C═O). IR (KBr 1%, cm^{-1}): ν=3302 (NH), 1815 (C═O), 1750 (C═O), 1683 (C═O), 1670 (C═O). UV–vis (ethanol, nm): λ_{max} (log ε)=323 (3.99). HRMS (EI) calcd for $C_{26}H_{27}NO_9$ $[M]^{+\bullet}$: 497.1686; found: 497.1693.

4.2.6. *N*-[(6-Methoxy-3-oxo-3*H*-benzopyran-1-yl)methyloxycarbonyl]-L-phenylalanine methyl ester (4b). 1-Hydroxymethyl-6-methoxy-3-oxo-3*H*-benzopyran **1c** (0.150 g, 0.728 mmol) and CDI (0.130 g, 0.80 mmol) were dissolved in dry DMF (5 mL). The reaction mixture was stirred for 1 h. L-Phenylalanine methyl ester hydrochloride **2c** (0.345 g, 1.60 mmol) was neutralised with triethylamine (0.25 mL, 1.80 mmol) in DMF. The resulting solid was filtered and the filtrate was added to previous reaction mixture, which was then stirred at room temperature for 20 h. A further addition of triethylamine (1 mL, 7.17 mmol) and CDI (0.186 g, 0.863 mmol) was done and the mixture was stirred for another 68 h. The solvent was removed by rotary evaporation under reduced pressure and the crude oily residue was purified by column chromatography using $CHCl_3$ and $CHCl_3$/MeOH, 100:1, as eluent. Compound **4b** was obtained as a yellow oily solid (0.108 g, 36%). ^1H NMR ($CDCl_3$): δ=3.06–3.09 (m, 2H, β-CH_2), 3.73 (s, 3H, OCH_3 ester), 3.90 (s, 3H, OCH_3), 4.70–4.75 (m, 1H, α-H), 4.80–4.90 (m, 1H, NH), 5.39 (s, 2H, CH_2), 6.20 (s, 1H, H-2), 7.03–7.18 (m, 3H, H-5, H-7 and 1×Ph–*H*), 7.20–7.35 (m, 4H, 4×Ph–*H*), 7.43 (d, *J*=8.5 Hz, 1H, H-8). ^{13}C NMR ($CDCl_3$): δ=38.01 (β-CH_2), 52.80 (OCH_3 ester), 52.90 (α-C), 55.61 (OCH_3), 62.38 (CH_2), 102.02 (C-5), 111.10 (C-8a), 111.20 (C-2), 112.91 (C-7), 124.59 (C-8), 127.16 (C-4 Ph), 127.99 (C-5 Ph), 128.43 (C-3 Ph), 128.95 (C-2 Ph), 129.50 (C-6 Ph), 135.90 (C-1 Ph), 146.87 (C-1), 155.21 (C═O urethane), 155.50 (C-4a), 161.02 (C-3), 162.80 (C-6), 169.75 (C═O ester). IR (KBr 1%, cm^{-1}): ν=3354 (NH), 3029, 2950, 2853, 1742–1720 (C═O), 1632 (C═O), 1614, 1562, 1515, 1497, 1454, 1441. UV–vis (ethanol, nm): λ_{max} (log ε)=322 (3.67). HRMS (EI) calcd for $C_{22}H_{21}NO_7$ $[M]^{+\bullet}$: 411.1318; found: 411.1326.

4.2.7. *N*-(*tert*-Butyloxycarbonyl)-*O*-{[(6-methoxy-3-oxo-3*H*-benzopyran-1-yl) methoxy]carbonyl}-L-serine methyl ester (4c). 1-Hydroxymethyl-6-methoxy-3-oxo-3*H*-benzopyran **1c** (0.177 g, 0.859 mmol), CDI (0.154 g, 0.948 mmol) and Boc–Ser–OMe, **2d** (0.350 g, 1.724 mmol) were dissolved in dry DMF (5 mL). The reaction mixture was stirred at room temperature for 66 h, triethylamine (0.5 mL) was added and the mixture was stirred for another 6 h. The solvent was removed by rotary evaporation under reduced pressure and the crude brown oil was purified by column chromatography, using $CHCl_3$ and $CHCl_3$/MeOH, 100:1, as eluent. Compound **4c** was obtained as a light yellow oily solid (0.097 g, 25%). ^1H NMR ($CDCl_3$): δ=1.40 (s, 9H, C(CH_3)$_3$), 3.71 (s, 3H, OCH_3 ester), 3.85 (s, 3H, OCH_3), 4.01–4.04 (m, 2H, β-CH_2), 4.63–4.67 (m, 1H, α-H), 5.24 (m, 1H, NH), 5.39 (s, 2H, CH_2), 6.18 (s, 1H, H-2), 6.75–6.82 (m, 2H, H-5 and H-7), 7.43 (d, *J*=8.5 Hz, 1H, H-8). ^{13}C NMR ($CDCl_3$): δ=28.22 (C(CH_3)$_3$), 39.13 (β-CH_2), 53.90 (α-C), 52.40 (OCH_3 ester), 55.76 (OCH_3), 61.82 (CH_2), 80.15 ($C(CH_3)_3$), 101.18 (C-5), 110.47 (C-8a), 110.49 (C-2), 112.63 (C-7), 124.48 (C-8), 148.59 (C-1), 155.20 (C═O urethane), 155.52 (C-4a), 160.66 (C-3), 162.91 (C-6), 171.35 (C═O ester), 173.43 (C═O carbonate). IR (KBr 1%, cm^{-1}): ν=3295 (NH), 1682 (C═O), 1673 (C═O), 1651 (C═O), 1645 (C═O). UV–vis (ethanol, nm): λ_{max} (log ε)=323 (3.70). HRMS (EI) calcd for $C_{21}H_{25}NO_{10}$ $[M]^{+\bullet}$: 451.1478; found: 451.1490.

4.3. General photolysis procedure

A 1×10^{-5} m solution in acetonitrile of the compound to be tested (20 mL) was placed in a quartz tube and irradiated in the reactor at the desired wavelength. Aliquots of 100 μL were taken at regular intervals and analysed by HPLC. The eluent was acetonitrile/water, 3:1, at a flow rate of 0.8 mL/min, previously filtered through a Milipore, 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: **3b**, 3.9; **3d**, 5.8; **4a**, 5.7; **4b**, 4.1; **4c**, 3.4 min).

Acknowledgements

We thank the Fundação para a Ciência e Tecnologia (Portugal) for their financial support to the Centro de Química (Universidade do Minho).

References and notes

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