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ORIGINAL ARTICLE

A photoactivable amino acid based on a novel functional coumarin-6-yl-alanine

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Abstract A novel fluorescent amino acid, L-4-chloromethylcoumarin-6-yl-alanine, was obtained from tyrosine by a Pechmann reaction. The assembly of the heterocyclic ring at the tyrosine side chain could be achieved before or after incorporation of tyrosine into a dipeptide, and amino acid and dipeptide ester conjugates were obtained by coupling to a model *N*-protected alanine. The behaviour of one of the fluorescent conjugates towards irradiation was studied in a photochemical reactor at different wavelengths (254, 300, 350 and 419 nm). The photoreaction course in methanol/HEPES buffer solution (80:20) was followed by HPLC/UV monitoring. It was found that the novel unnatural amino acid could act as a fluorescent label, due to its fluorescence properties, and, more importantly, as a photoactivable unit, due to the short irradiation times necessary to cleave the ester bond between the model amino acid and the coumarin-6-yl-alanine.

Keywords Coumarin-6-yl-alanine · Coumarin · Fluorescence · Amino acid conjugates · Photolysis

Introduction

The development of functional amino acid analogues is an area of expanding interest due to their potential use as intrinsic and extrinsic probes in peptide and protein conformational studies and in bioactivity and pharmacological research. The design of fluorescent amino acids allows the construction of chromophore-labelled peptides and proteins, which can be easily studied by fluorescence spectroscopy-based techniques (Katritzky and Narindoshvili 2009). Moreover, if an extra functional group is introduced into the amino acid residue, it can also be used for coupling purposes. This feature is quite appealing when a strategy for peptide conformation restriction (for example, in the synthesis of cyclic peptides or peptidomimetics) and cross-linking is to be considered. Using certain photoactive groups that are cleaved by the action of light (Guillier et al. 2000; Corrie et al. 2005; Mayer and Heckel 2006), the ability to remove the constriction by photolysis may be an interesting feature. Photochemical cleavage is widely used to obtain control over the availability of specific molecules in areas of research such as solution and solid-phase organic synthesis, biochemical and biophysical research (for e.g. in caging strategies). Such a methodology has obvious advantages for not requiring additional chemical reagents, thus being compatible with acid or base sensitive groups, and for providing spatial and temporal resolution of the cleavage process. Reports have been published with examples such as the photoregulation of thrombin aptamer activity (Li et al. 2009), the photocontrol of helix content in short peptides (Kumita et al. 2000), and in the light-triggered assembly of protein complexes (Grunwald et al. 2010).

Coumarin derivatives have been reported as photocleavable protecting groups for molecules bearing different functional groups (Hagen et al. 2005; Geissler et al. 2005; Lin and Lawrence 2002; Shembekar et al. 2007; Gilbert et al. 2007) and as coumarins exhibit fluorescence, they may also act as fluorescent labels, enabling the direct monitoring of processes during synthesis, or the visualisation of molecules inside living cells as new extrinsic and intrinsic fluorescent probes for biologically active molecules.

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The synthesis of coumarinyl-alanines has been reported earlier by diastereoselective alkylation of chiral glycine equivalents, using different chiral auxiliaries and in multiple step synthesis with low global yields (Bennett et al. 1997; Kele et al. 2000) or using protected aspartic and glutamic acids as chiral starting materials, through a Pechmann condensation reaction between a β-ketoester intermediate with phenol derivatives, in fair yields (Brun et al. 2004).

Taking these facts into consideration, in connection with our current research interests in the development of new oxygen and nitrogen heterocycles and their applications as fluorescent labels and photoreleasable protecting groups (Piloto et al. 2006; Fonseca et al. 2007, 2010; Fernandes et al. 2008; Soares et al. 2010a, b), we now report the synthesis of a functional alanine derivative bearing a fluorescent coumarin moiety at its side chain, namely *N*-acetyl-4-chloromethylcoumarin-6-yl-alanine methyl ester. The incorporation of a fluorescent heterocycle such as coumarin in the amino acid core results in an advantageous feature for fluorescence-based biological studies and the molecule shows high chemical stability due to the fact that the fluorophore is linked by a side-chain carbon–carbon bond. In addition, given a proper choice of protecting groups that allow the sequence of its assembly and after deprotection, such an amino acid has the potential to be incorporated into peptidic structures by standard coupling procedures through its N- and C-termini, yielding a functional fluorescent peptide and the presence of a reactive chloromethyl group at the coumarin moiety provides an extra coupling site, useful for constriction strategies.

Moreover, in order to evaluate the potential application of this new amino acid in photoactivable processes, we decided to synthesize model ester conjugates by reaction with the carboxylic terminal of *N*-protected alanine, with the aim of undertaking a study of the photostability of the newly formed ester linkage to irradiation at different wavelengths.

Experimental section

General

All melting points were measured on a Stuart SMP3 melting point apparatus. 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 and 75.4 MHz for ^{13}C or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C 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 correlation techniques. Low and high resolution 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 Shimadzu SPD-GAV UV/Vis detector and a Shimadzu C-RGA Chromatopac register. All commercial reagents were used as received. Protected tyrosine **1a,b** and alanine **4a–c** derivatives were obtained by standard amino acid protecting group protocols. The L-(4-methylene)coumarin-6-yl-alaninyl group will be designated by the three letter code Mca in the NMR spectra description.

Synthesis of compounds **3** and **5–11**

N-acetyl-L-(4-chloromethyl)coumarin-6-yl-alanine methyl ester 3

N-acetyl-L-tyrosine methyl ester **1a** was dissolved (1 equiv, 0.450 g, 1.90×10^{-3} mol) in aqueous 70 % sulphuric acid (5 mL), and ethyl 4-chloro-3-oxobutanoate **2** (1.5 equiv, 0.39 mL, 2.85×10^{-3} mol) was added to the mixture. The reaction mixture was stirred at room temperature for 7 days, poured into ice, and the precipitate was filtered and dried in a vacuum oven. The resulting residue was purified by silica gel column chromatography using chloroform/methanol (100:1) as eluent. Fractions containing the product were combined and evaporated, yielding compound **3** as a beige solid (0.350 g, 55 %); mp = 172.3–173.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 1.78$ (3H, s, CH_3 Ac), 2.95 (1H, dd, *J* 9.6 and 4.4 Hz, β-CH_2), 3.11 (1H, dd, *J* 13.6 and 5.2 Hz, β-CH_2), 3.61 (3H, s, OCH_3), 4.44–4.52 (1H, m, α-H), 4.99 (2H, s, CH_2), 6.67 (1H, s, H-3), 7.37 (1H, d, *J* 8.4 Hz, H-8), 7.51 (1H, dd, *J* 8.4 and 2.0 Hz, H-7), 7.72 (1H, d, *J* 2.0 Hz, H-5), 8.35 (1H, d, *J* 7.6 Hz, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6): $\delta = 22.24$ (CH_3 Ac), 35.95 (β-CH_2), 41.13 (CH_2), 51.92 (OCH_3), 53.47 (α-C), 115.56 (C-3), 116.62 (C-8), 116.74 (C-4a), 125.58 (C-5), 133.33

(C-7), 133.67 (C-6), 150.49 (C-4), 152.15 (C-8a), 159.62 (C-2), 169.40 (C=O amide), 171.95 (C=O ester); FT-IR (KBr 1 %, cm^{-1}): ν = 3,289, 3,080, 2,961, 2,927, 2,855, 1,732, 1,646, 1,572, 1,541, 1,378, 1,357, 1,210, 1,173, 1,135, 1,057, 984, 961, 894, 736; UV/Vis (ethanol, nm): λ_{max} (log ε) = 320 (3.67); MS: *m/z* (ESI) 340 (M^++1 ^{37}Cl, 29), 338 (M^++1 ^{35}Cl, 100), HRMS: *m/z* (ESI) calc. for $C_{16}H_{17}NO_5^{37}Cl$ 340.07603, found 340.07593; calc. for $C_{16}H_{17}NO_5^{35}Cl$ 338.07898, found 338.07882.

Coumarin-6-yl-alanine ester conjugate ***5***

Compound **3** (1 equiv, 0.050 g, 1.48 × 10^{-4} mol) was dissolved in dry *N*,*N*-dimethylformamide (3 mL). *N*-*t*-Butyloxycarbonyl)-L-alanine **4a** (1 equiv, 0.025 g, 1.48 × 10^{-4} mol) and potassium fluoride (3 equiv, 0.028 g, 4.45 × 10^{-4} mol) were added to the mixture. The reaction mixture was stirred at room temperature for 3 days. The mixture was filtered and the solvent was removed by rotary evaporation. The crude solid was recrystallized from methanol and diethyl ether and conjugate **5** was obtained as a light yellow solid (0.067 g, 95 %); mp = 174.0–175.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.31 (3H, d, *J* 7.5 Hz, β-CH_3 Ala), 1.38 (9H, s, (C(CH_3)$_3$), 1.77 (3H, s, CH_3 Ac), 2.92 (1H, dd, *J* 9.6 and 3.9 Hz, β-CH_2 Mca), 3.12 (1H, dd, *J* 14.1 and 5.4 Hz, β-CH_2 Mca), 3.61 (3H, s, OCH_3), 4.17–4.02 (1H, m, α-H Ala), 4.48–4.53 (1H, m, α-H Mca), 5.42 (2H, s, CH_2), 6.48 (1H, s, H-3), 7.36 (1H, d, *J* 8.7 Hz, H-8), 7.48–7.50 (2H, m, H-7 and NH Ala), 7.62 (1H, d, *J* 1.6 Hz, H-5), 8.35 (1H, d, *J* 7.8 Hz, NH Mca); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 16.69 (β-CH_3 Ala), 22.19 (CH_3 Ac), 28.11 (C(CH_3)$_3$) 35.84 (β-CH_2 Mca), 49.15 (α-C Ala), 51.87 (OCH_3), 53.39 (α-C Mca), 61.39 (CH_2), 78.41 (C(CH_3)$_3$), 111.75 (C-3), 116.31 (C-4a), 116.43 (C-8), 125.08 (C-5), 133.16 (C-7), 133.69 (C-6), 149.98 (C-4), 151.73 (C-8a), 155.44 (C=O urethane), 159.50 (C-2), 169.36 (C=O amide), 172.00 (C=O methyl ester), 172.64 (C=O ester); FT-IR (KBr 1 %, cm^{-1}): ν = 3,315, 3,295, 3,084, 2,996, 2,985, 2,938, 1,755, 1,731, 1,688, 1,666, 1,575, 1,551, 1,534, 1,436, 1,372, 1,350, 1,316, 1,287, 1,235, 1,174, 1,160, 1,133, 1,070, 1,013, 965, 834; UV/Vis (ethanol, nm): λ_{max} (log ε) = 318 (3.58); MS: *m/z* (ESI) 491 (M^++1, 27); HRMS: *m/z* (ESI) calc. for $C_{24}H_{31}N_2O_9$ 491.20241, found 491.20289.

N-acetyl-L-(3-carboxymethyl)benzofuran-5-yl-alanine ***6***

Compound **3** (0.100 g, 2.96 × 10^{-4} mol) was dissolved in 1 M NaOH (5 mL) and the mixture was stirred by heating at reflux for 17 h. The reaction mixture was acidified with a few drops of 6 M HCl, and the aqueous phase was extracted with ethyl acetate (3 × 10 mL). The organic layers were combined and the solvent was removed by rotary evaporation. Compound **6** was obtained as beige oil (0.075 g, 83 %); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.76 (3H, s, CH_3 Ac), 2.88–3.13 (2H, m, β-CH_2), 3.64 (2H, s, CH_2), 4.35–4.41 (1H, m, α-H), 7.17 (1H, dd, *J* 8.4 and 1.6 Hz, H-6), 7.42 (1H, d, *J* 0.8 Hz, H-4), 7.44 (1H, d, *J* 8.4 Hz, H-7), 7.83 (1H, s, H-2), 8.17 (1H, d, *J* 8.4 Hz, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 22.37 (CH_3 Ac), 28.99 (CH_2), 36.75 (β-CH_2), 54.13 (α-C), 110.88 (C-7), 113.87 (C-3), 120.26 (C-4), 125.66 (C-6), 127.80 (C-3a), 132.08 (C-5), 143.71 (C-2), 153.52 (C-7a), 169.43 (C=O amide), 171.92 (C=O acid), 173.23 (C=O acid); FT-IR (KBr 1 %, cm^{-1}): ν = 3,301, 2,928, 1,723, 1,651, 1,615, 1,557, 1,539, 1,475, 1,446, 1,379, 1,338, 1,279, 1,256, 1,230, 1,217, 1,187, 1,161, 1,124, 1,091, 1,023, 971, 954, 943, 884, 871, 752, 508; UV/Vis (ethanol, nm): λ_{max} (log ε) = 314 (3.72); MS: *m/z* (ESI) 306 (M^++1, 35); HRMS: *m/z* (ESI) calc. for $C_{15}H_{16}NO_6$ 306.09721, found 306.09697.

N-acetyl-L-alaninyl-L-tyrosine methyl ester ***7***

N-acetyl-L-alanine **4b** (1 equiv, 0.200 g, 1.53 × 10^{-3} mol) was dissolved in dry *N*,*N*-dimethylformamide (3 mL). Anhydrous 1-hydroxybenzotriazole (1.1 equiv, 0.206 g, 1.68 × 10^{-3} mol), *N*,*N*′-dicyclohexylcarbodiimide (1 equiv, 0.315 g, 1.53 × 10^{-3} mol) and L-tyrosine methyl ester **1b** (1 equiv, 0.354 g, 1.53 × 10^{-3} mol), were added to the mixture with intervals of 10 min. The reaction mixture was stirred at room temperature for 1 day. The mixture was filtered and the solvent was removed by rotary evaporation. The crude residue was dissolved in acetone and left in the freezer for some days. The mixture was filtered and the solvent was removed by rotary evaporation. The solid mixture was used in the next reaction without further purification. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.11–1.14 (3H, m, β-CH_3 Ala), 1.79 (3H, s, CH_3 Ac), 3.80–3.20 (2H, m, β-CH_2 Tyr), 3.56 (3H, s, OCH_3), 4.20–4.40 (2H, m, α-H Ala and Tyr), 6.64 (2H, d, *J* 8.4 Hz, H-3 and H-5), 6.97 (2H, d, *J* 8.4 Hz, H-2 and H-6), 7.97 (1H, d, *J* 8.1 Hz, NH Ala), 8.17 (1H, d, *J* 7.2 Hz, NH Tyr), 9.20 (1H, broad s, OH).

N-acetyl-L-alaninyl-L-(4-chloromethyl)-coumarin-6-yl-alanine methyl ester ***8***

Compound **7** (1 equiv, 0.470 g, 1.53 × 10^{-3} mol) was dissolved in aqueous 70 % sulphuric acid (5 mL) and ethyl 4-chloro-3-oxobutanoate **2** (2 equiv, 0.45 mL, 3.05 × 10^{-3} mol) was added to the mixture. The reaction mixture was stirred at room temperature for 3 weeks, poured into ice, and the precipitate formed was filtered and dried in a vacuum oven. Recrystallization from methanol and diethyl ether afforded dipeptide **8** as a brown solid (0.100 g, 16 %); mp = 182.0–183.4 °C; ^{1}H NMR (400 MHz, MeOH-d_4):

$\delta = 1.31$ (3H, d, J 7.2 Hz, β-CH_3 Ala), 1.94 (3H, s, CH_3 Ac), 3.10–3.32 (2H, m, β-CH_2 Mca), 4.30–4.35 (1H, m, α-H Ala), 4.77–4.81 (1H, m, α-H Mca), 4.96 (2H, s, CH_2), 6.64 (1H, s, H-3), 7.34 (1H, d, J 8.8 Hz, H-8), 7.52 (1H, dd, J 8.8 and 2.0 Hz, H-7), 7.70 (1H, d, J 1.6 Hz, H-5); ^{13}C NMR (100.6 MHz, MeOH-d_4): $\delta = 17.84$ (β-CH_3 Ala), 22.21 (CH_3 Ac), 37.65 (β-CH_2 Mca), 42.15 (CH_2), 50.36 (α-C Ala), 52.90 (OCH_3), 54.71 (α-H Mca), 116.11 (C-3), 118.07 (C-8), 118.59 (C-4a), 126.51 (C-5), 134.74 (C-7), 134.91 (C-6), 152.68 (C-4), 154.04 (C-8a), 162.39 (C-2), 172.87 (C=O ester), 173.07 (C=O amide Ac), 175.05 (C=O amide); FT-IR (KBr 1 %, cm^{-1}): $\nu = 3{,}288$, 3,084, 2,937, 1,737, 1,648, 1,572, 1,550, 1,493, 1,439, 1,384, 1,352, 1,285, 1,251, 1,176, 1,058, 960, 907, 830, 735, 709, 661, 581, 514, 523; UV/vis (ethanol, nm): λ_{max} (log ε): 320 (3.62) MS: m/z (ESI): 411 (M^++1 ^{37}Cl, 23), 409 (M^++1 ^{35}Cl, 70); HRMS: m/z (ESI) calc. for $C_{19}H_{22}N_2O_6$ ^{37}Cl 411.11341, found 411.11377; calc. for $C_{19}H_{22}N_2O_6$ ^{35}Cl 409.11609, found 409.11589.

Alaninyl-coumarin-6-yl-alanine ester conjugate ***9***

Dipeptide **8** (1 equiv, 0.032 g, 7.83×10^{-4} mol) was dissolved in dry *N*,*N*-dimethylformamide (3 mL). *N*-acetyl-L-alanine **4b** (1 equiv, 0.011 g, 7.83×10^{-4} mol) and potassium fluoride (3 equiv, 0.014 g, 2.35×10^{-3} mol) were added to the mixture. The reaction mixture was stirred at room temperature for 2 days. The mixture was filtered and the solvent was removed by rotary evaporation. Recrystallization from methanol and diethyl ether resulted in dipeptide conjugate **9** as a beige solid (0.025 g, 63 %); ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 1.10$–1.13 (3H, m, β-CH_3 Ala), 1.33–1.35 (3H, m, β-CH_3 Ala), 1.74 (3H, s, CH_3 Ac), 1.85 (3H, s, CH_3 Ac), 2.96–3.16 (2H, m, β-CH_2 Mca), 3.61 (3H, s, OCH_3), 4.22–4.28 (1H, m, α-H Ala), 3.34–4.41 (1H, m, α-H Ala), 4.48–4.57 (1H, m, α-H Mca), 5.37–5.47 (2H, m, CH_2), 6.44 (1H, s, H-3), 7.33 (1H, d, J 8.4 Hz, H-8), 7.48–7.51 (1H, m, H-7), 7.60 (1H, d, J 1.6 Hz, H-5), 7.92 (1H, d, J 7.6 Hz, NH Ala), 8.29 (1H, d, J 8.0 Hz, NH Mca), 8.43 (1H, d, J 6.8 Hz, NH Ala); ^{13}C NMR (100.6 MHz, DMSO-d_6): $\delta = 16.71$ (β-CH_3 Ala), 17.96 (β-CH_3 Ala), 22.11 (CH_3 Ac), 22.30 (CH_3 Ac), 35.76 (β-CH_2 Mca), 47.71 (α-C Ala), 47.85 (α-C Ala), 51.93 (OCH_3), 53.15 (α-C Mca), 61.49 (CH_2), 111.90 (C-3), 116.39 (C-8), 125.12 (C-5), 133.27 (C-7), 133.53 (C-6), 150.14 (C-4), 151.75 (C-4a), 159.29 (C-2), 162.29 (C-8a), 168.84 (C=O ester), 169.47 (C=O amide Ac), 172.27 (C=O amide Ac), 172.60 (C=O ester), 174.26 (C=O amide); FT-IR (KBr 1 %, cm^{-1}): $\nu = 3{,}290$, 3,068, 2,984, 2,934, 1,726, 1,654, 1,634, 1,574, 1,536, 1,445, 1,375, 1,305, 1,264, 1,208, 1,159, 1,042, 1,014, 972, 940, 876, 835, 754, 732, 665, 514; UV/Vis (ethanol, nm): λ_{max} (log ε) = 319 (3.51); MS: m/z (ESI) 504 (M^++1, 100); HRMS: m/z (ESI) calc. for $C_{24}H_{30}N_3O_9$ 504.19766 found 504.19663.

N-acetyl-L-tyrosinyl-L-alanine methyl ester ***10***

N-acetyl-L-tyrosine **1c** (0.300 g, 1.34×10^{-3} mol) was dissolved in dry *N*,*N*-dimethylformamide (3 mL). Anhydrous 1-hydroxybenzotriazole (1.1 equiv, 0.200 g, 1.48×10^{-3} mol), *N*,*N*′-dicyclohexylcarbodiimide (1 equiv, 0.277 g, 1.34×10^{-3} mol) and L-alanine methyl ester **4c** (1 equiv, 0.188 g, 1.34×10^{-3} mol) were added to the mixture with intervals of 10 min. The reaction mixture was stirred at room temperature for 2 days. The mixture was filtered and the solvent was removed by rotary evaporation. The crude residue was dissolved in acetone and left in the freezer for some days. The first precipitate formed was filtered and the solvent volume reduced. A new precipitate was collected and dipeptide **10** was obtained as a white solid (0.233 g, 56 %); mp = 100.3–102.2 °C; ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.27$ (3H, d, J 7.2 Hz, β-CH_3 Ala), 1.73 (3H, s, CH_3 Ac), 2.53–2.89 (2H, m, β-CH_2 Tyr), 3.60 (3H, s, OCH_3), 4.21–4.30 (1H, m, α-H Ala), 4.38–4.46 (1H, m, α-H Tyr), 6.63 (2H, d, J 8.4 Hz, H-3 and H-5), 7.03 (2H, d, J 8.1 Hz, H-2 and H-6), 7.99 (1H, d, J 8.7 Hz, NH Tyr), 8.42 (1H, d, J 6.9 Hz, NH Ala), 9.18 (1H, broad s, OH); ^{13}C NMR (75.4 MHz, DMSO-d_6): 16.86 (β-CH_3 Ala), 22.46 (CH_3 Ac), 36.86 (β-CH_2 Tyr), 47.53 (α-C Ala), 51.85 (OCH_3), 53.91 (α-C Tyr), 114.80 (C-3 and C-5), 128.02 (C-4), 130.04 (C-2 and C-6), 155.72 (C-1), 169.03 (C=O amide Ac), 171.60 (C=O amide), 172.94 (C=O ester); FT-IR (KBr 1 %, cm^{-1}): $\nu = 3{,}538$, 3,342, 3,271, 3,074, 2,978, 2,928, 1,717, 1,676, 1,652, 1,600, 1,557, 1,519, 1,457, 1,435, 1,374, 1,298, 1,281, 1,245, 1,187, 1,162, 1,107, 1,057, 981, 960, 925, 872, 845, 832, 814, 756, 723, 707; UV/Vis (ethanol, nm): λ_{max} (log ε) = 303 (3.17); MS: m/z (ESI) 309 (M^++1, 100); HRMS: m/z (ESI) calc. for $C_{15}H_{21}N_2O_6$ 309.14450, found 309.14360.

N-acetyl-L-(4-chloromethyl)-coumarin-6-yl-alaninyl-L-alanine methyl ester ***11***

Dipeptide **10** (1 equiv, 0.300 g, 9.73×10^{-4} mol) was dissolved in aqueous 70 % sulphuric acid (5 mL) and ethyl 4-chloro-3-oxobutanoate **2** (1.5 equiv, 0.2 mL, 1.46×10^{-3} mol) was added to the mixture. The reaction mixture was stirred at room temperature for 3 weeks, poured into ice, and the precipitate formed was filtered and dried in a vacuum oven. Dipeptide **11** was obtained as a dark oil (0.150 g, 26 %); ^{1}H NMR (400 MHz, MeOH-d_4): 1.57 (3H, d, J 7.2 Hz, β-CH_3 Ala), 2.01 (3H, s, CH_3 Ac), 3.13–3.33 (2H, m, β-CH_2 Mca), 3.70 (3H, OCH_3), 4.07 (1H, m, α-H Ala), 4.40 (1H, m, α-H Mca), 4.95 (2H, s, CH_2), 6.63 (1H, s, H-3), 7.54 (1H, d, J 8.4 Hz, H-8), 7.58

(1H, dd, *J* 8.4 and 2.0 Hz, H-7), 7.75 (1H, d, *J* 2.0 Hz, H-5); ^{13}C NMR (100.6 MHz, MeOH-d_4): 16.24 (β-CH_3 Ala), 21.95 (CH_3 Ac), 37.62 (β-CH_2 Mca), 42.08 (CH_2), 54.20 (OCH_3), 55.22 (α-C Mca), 55.96 (α-C Ala), 116.08 (C-3), 117.96 (C-8), 118.50 (C-4a), 126.50 (C-5), 134.50 (C-7), 152.44 (C-8a), 152.65 (C-4), 153.99 (C-6), 162.34 (C-2), 171.40 (C=O ester), 172.29 (C=O amide), 173.88 (C=O amide Ac); UV/Vis (ethanol, nm): λ_{max} (log ε) = 312 (3.28); MS: *m/z* (ESI) 411 (M^++1 ^{37}Cl, 7), 409 (M^++1 ^{35}Cl, 19); HRMS: *m/z* (ESI) calc. for $C_{19}H_{21}^{37}ClN_2O_6$ 411.11341, found 411.11384; calc. for $C_{19}H_{21}^{35}ClN_2O_6$ 409.11609, found 409.11700.

General photolysis procedure

A 1×10^{-4} M methanol/HEPES buffer (80:20) solution of conjugate **5** (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. HEPES buffer solution was prepared in distilled water with HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] (10 mM), sodium chloride (120 mM), potassium chloride (3 mM), calcium chloride (1 mM) and magnesium chloride (1 mM) and the 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 0.4 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 absorption (318 nm) and the retention time of conjugate **5** was 6.2 min.

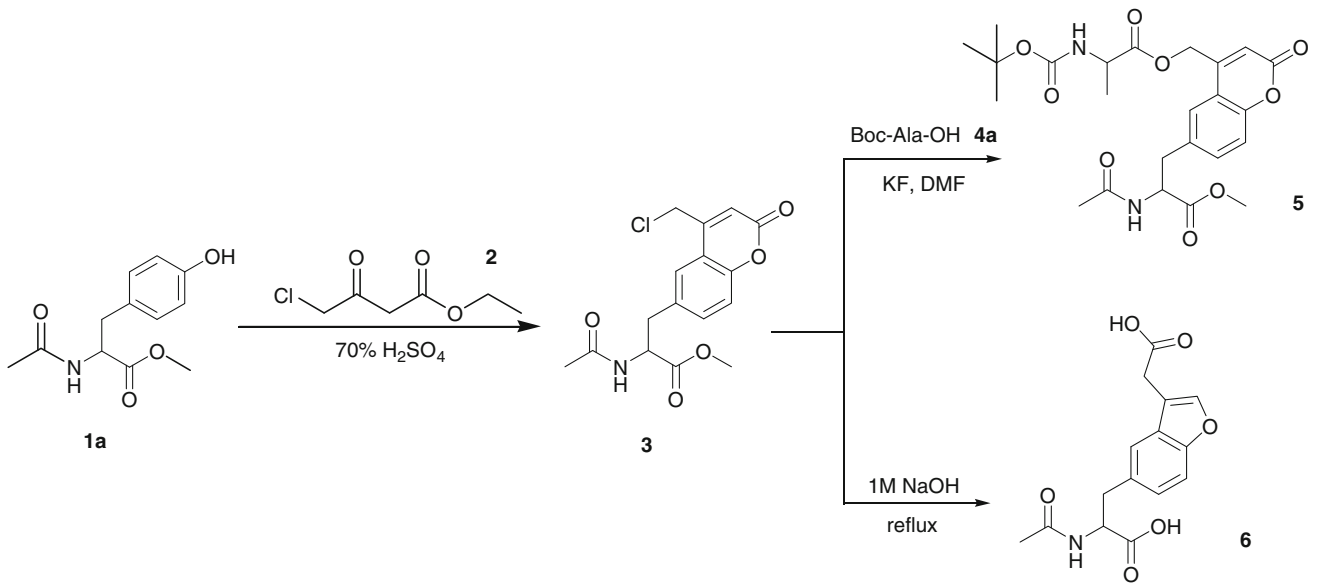
Results and discussion

Synthesis

N-acetyl-L-(4-chloromethyl)coumarin-6-yl-alanine methyl ester **3** was prepared in moderate yield by reaction of

Table 1 Synthesis, UV/Vis absorption and emission data for coumarin-6-yl-alanine **3**, its derivatives **5**, **8**, **9**, **11**, and for benzofuran-5-yl-alanine **6**, in absolute ethanol

Compound	Yield (%)	Absorption		Emission		
		λ_{max} (nm)	log ε	λ_{em} (nm)	ϕ_F	Stokes' shift (nm)
3	55	320	3.67	378	0.05	58
5	95	318	3.58	417	0.08	99
6	83	314	3.72	386	0.08	72
8	16	320	3.62	389	0.06	69
9	63	319	3.51	432	0.07	113
11	26	312	3.28	372	0.06	60

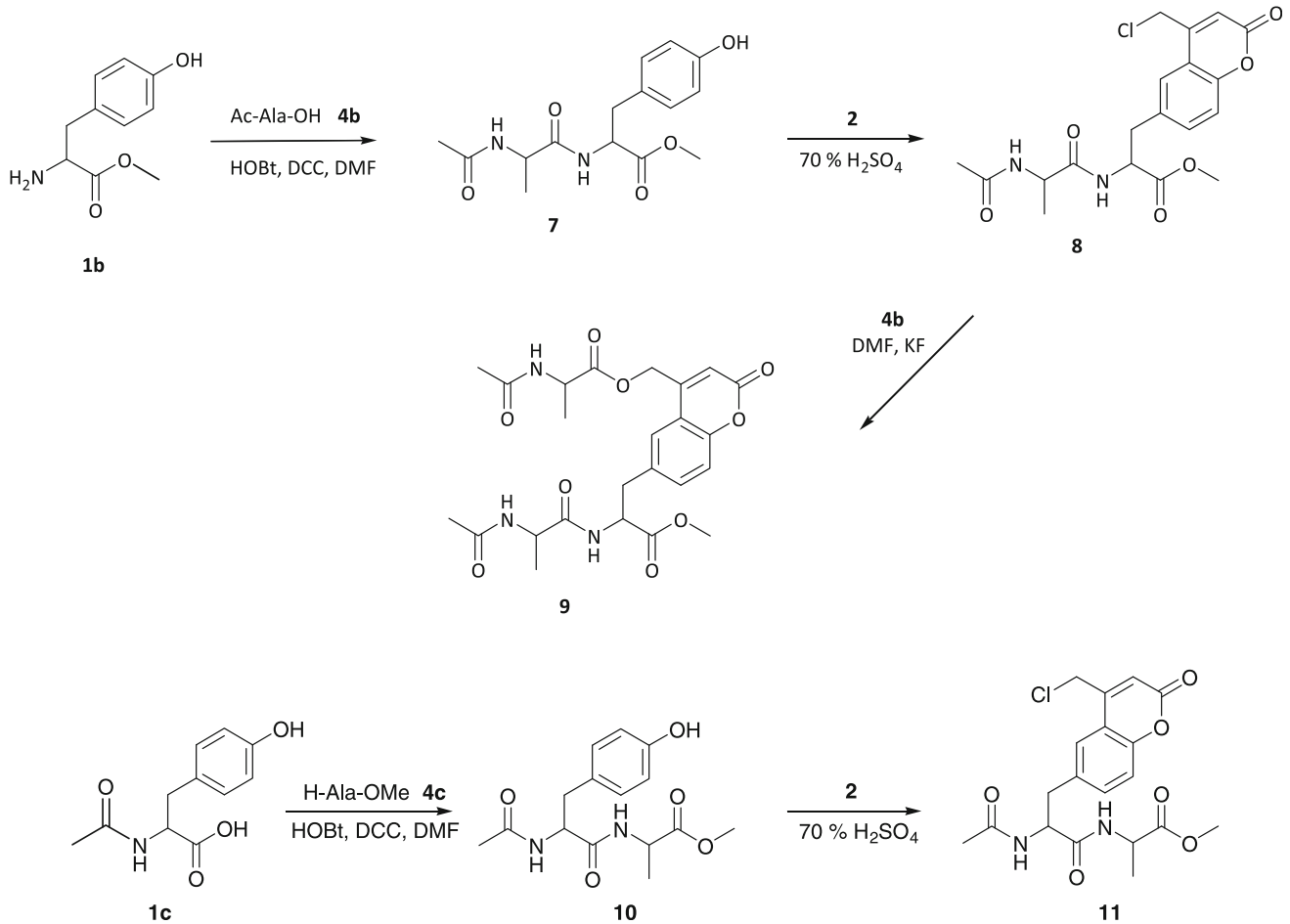


Scheme 1 Synthesis of coumarin-6-yl-alanine **3**, its ester conjugate **5** and benzofuran-5-yl-alanine derivative **6**

N-acetyl-L-tyrosine methyl ester **1a** and ethyl 4-chloro-3-oxobutanoate **2**, through a Pechmann reaction, in the presence of aqueous 70 % sulphuric acid (Fonseca et al. 2010). No evidence was found for the loss of the integrity of the chiral centre in these reaction conditions. The structure of the novel amino acid **3** was confirmed by the usual spectroscopic techniques, with the ^{1}H NMR signals of the coumarin aromatic protons at δ 6.67 ppm (singlet for H-3), δ 7.72 ppm (doublet for H-5), δ 7.51 ppm (double doublet for H-7) and δ 7.37 ppm (doublet for H-8), as well as its methylene group at position 4 (singlet at δ 4.99 ppm). The confirmation of the assembly of the coumarin ring at the amino acid side chain was also possible by the ^{13}C NMR spectrum signal at δ 159.62 ppm corresponding to the carbonyl group at position 2.

Derivatization at the C-terminus of *N*-*t*-butyloxycarbonyl-L-alanine **4a** with the chloromethyl group of coumarin-6-yl-alanine **3** was carried out in *N*,*N*-dimethylformamide, at room temperature, in the presence of potassium fluoride (Fonseca et al. 2010), yielding fluorescent ester conjugate **5** in excellent yield (Table 1; Scheme 1). The ^{1}H NMR spectra of conjugate **5** showed signals as multiplets for α-H at δ 4.48–4.53 (coumarin-6-yl-alanine) and at 4.15–4.22 ppm (for the alanine residue), as well as the characteristic protons of the coumarin ring, namely H-3 (δ 6.48 ppm), H-5 (δ 7.62 ppm), H-7 (δ 7.50 ppm) and H-8 (δ 7.36 ppm), as and its methylene group at position 4 (δ 5.42 ppm). The confirmation of the presence of the newly formed ester bond was also supported by the ^{13}C NMR spectrum signal of the carbonyl group, which was found at δ 172.64 ppm.

Treatment of coumarin-6-yl-alanine **3** with base yielded benzofuran-5-yl-alanine derivative **6**, accordingly to a previously reported alkaline ring contraction of a coumarin bearing a chloromethyl group (Piloto et al. 2006). Its structure was confirmed by the usual spectroscopic techniques, such as the ^{1}H NMR signals of the benzofuran aromatic protons that appeared at δ 7.17 ppm (double doublet for H-6), δ 7.42 ppm (doublet for H-4), δ 7.44 ppm (doublet for H-7) and δ 7.83 ppm (singlet for H-2). It was possible to react both carboxylic acid groups present in the molecule with L-alanine methyl ester, but the lack of selectivity in this reaction prevented further application of the novel benzofuranyl amino acid **6**.



Scheme 2 Synthesis of model dipeptides **8**, **9** and **11**, bearing a coumarin-6-yl-alanine by post-coupling assembly of the coumarin ring

Having proven the possibility of assembling the coumarin ring by modification at the side chain of free tyrosine, the same idea was envisaged for tyrosine in a dipeptide, through a post-coupling assembly of the coumarin ring. Thus, L-tyrosine methyl ester **1b** or *N*-acetyl-L-tyrosine **1c** was coupled to *N*-acetyl-L-alanine **4b** or L-alanine methyl ester **4c**, by a standard coupling protocol with *N*,*N*′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in *N*,*N*-dimethylformamide, yielding the corresponding model dipeptides **7** and **10**. These compounds, in the presence of ethyl 4-chloro-3-oxobutanoate **2**, in the same reaction conditions as mentioned earlier, afforded the dipeptides bearing a chloromethylated coumarin-6-yl-alanine at the C- and N-terminus, dipeptides **8** and **11**, respectively (Scheme 2). The low isolated yields of these compounds can be explained by the relatively harsh reaction conditions that cause loss of the N-and C-termini blocking groups, leading to deprotected by-products. For the alaninyl–coumarinyl–alaninyl dipeptide **8**, further reaction at the chloromethyl group was carried out with *N*-acetyl-L-alanine **4b** and potassium fluoride in *N*,*N*-dimethylformamide, as previously mentioned. The dipeptide ester conjugate **9** was obtained in good yield (63 %) (Scheme 2). All the dipeptides were fully characterised by the usual spectroscopic techniques and the obtained data were in accordance with that obtained for the free coumarin-6-yl-alanine.

Coumarin and benzofuran derivatives are known for their fluorescence properties and so the photophysical properties of all synthesised compounds bearing a coumarin (**3**, **5**, **8**, **9** and **11**) or a benzofuran (**6**) were evaluated and the UV/Vis absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol of these compounds were measured (Table 1). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard ($\phi_F = 0.95$ in ethanol) (Morris et al. 1976).

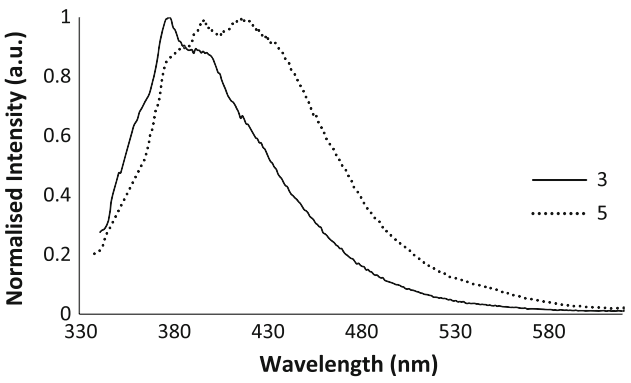


Fig. 1 Normalised fluorescence spectra of coumarin-6-yl-alanine **3** and its ester conjugate **5** in absolute ethanol (**3**, $\lambda_{exc} = 320$ nm; **5**, $\lambda_{exc} = 318$ nm)

Coumarin-6-yl-alanine **3**, its ester conjugates **5** and **9** and the dipeptides **8** and **11** displayed fair fluorescence quantum yields. The fact that the absorption and emission maxima for coumarin-6-yl-alanine **3** and its derivatives **5**, **8**, **9** and **11** occurs at longer wavelengths than those of the natural amino acids that exhibit fluorescence, namely tryptophan ($\lambda_{max} = 278$ nm, $\lambda_{em} = 352$ nm), tyrosine ($\lambda_{max} = 274$ nm, $\lambda_{em} = 303$ nm) and phenylalanine ($\lambda_{max} = 257$ nm, $\lambda_{em} = 282$ nm), confirms that coumarin-6-yl-alanine **3** could be used as an intrinsic fluorescent label when incorporated into biologically active peptides and proteins. All compounds displayed large Stokes' shifts (58–113 nm), being dipeptide ester conjugate **9** associated with the largest shift (113 nm), which is an advantageous property in fluorescence techniques as it will minimise self-quenching phenomena. From the data in Table 1, it can be seen that upon reaction of the chloromethyl group to yield the ester linkage to the amino acid in conjugates **5** and **9**, a 40 nm bathochromic shift of the emission band occurred, when compared to **3** and **8**, respectively (Fig. 1).

Photolysis studies of coumarin-6-yl-alanine ester conjugate **5**

One of the goals of this work was to assess the photostability of the linkage between coumarin-6-yl-alanine and alanine, as a model amino acid, in ester conjugates, having in mind a possible application of the novel amino acid in photoactivable processes for biological studies. Therefore, photolysis studies were carried out by irradiating solutions of conjugate **5** in methanol/HEPES buffer (80:20) in a Rayonet RPR-100 reactor, at 254, 300, 350 and 419 nm. The course of the photocleavage reaction was followed by

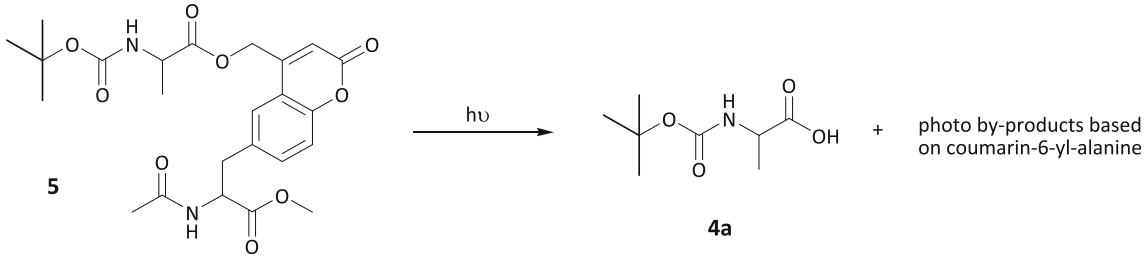
Table 2 Irradiation times (t_{irr}, in min), rate constant (k, in min^{-1}) and photochemical quantum yield (ϕ_{phot}) for the photolysis of conjugate **5** at different wavelengths in MeOH/HEPES buffer (80:20) solution

Wavelength of irradiation (nm)		
254	t_{irr}	69
	k	4.2×10^{-3}
	ϕ_{phot}	2.3×10^{-2}
300	t_{irr}	79
	k	3.7×10^{-3}
	ϕ_{phot}	1.2×10^{-2}
350	t_{irr}	2,910
	k	1.0×10^{-3}
	ϕ_{phot}	0.2×10^{-2}
419	t_{irr}	8,542
	k	0.4×10^{-3}
	ϕ_{phot}	*

* Not determined

reverse phase HPLC with UV detection and ^{1}H NMR. The determined irradiation time represents the time necessary for the consumption of the starting materials until <5 % of the initial peak area (*A*) was detected (Table 2). 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, with high correlation coefficients, and rate constants were calculated.

Photochemical quantum yields (ϕ_{phot}) of the photocleavage reaction of conjugate **5** were calculated as previously described (Muller et al. 2001) and short irradiation times were obtained for irradiation at 254 and 300 nm. Irradiation times at 350 and 419 nm were too long to be useful for practical applications (Table 2). In the tested conditions, no cleavage of the *N*-*t*-butyloxycarbonyl and *N*-acetyl groups was detected. In addition to monitoring the photolysis process by HPLC/UV detection, the release of Boc-protected alanine, as the expected product of the



Scheme 3 Irradiation of ester conjugate **5** and the corresponding products

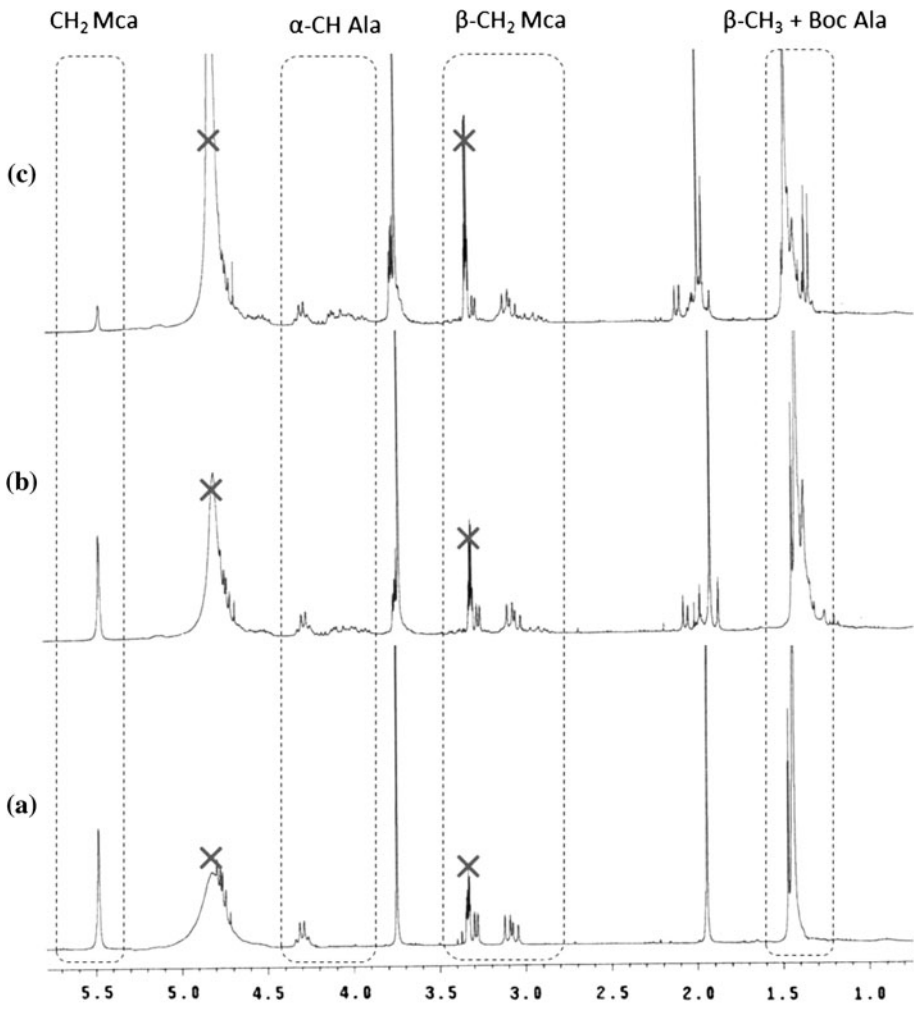


Fig. 2 ^{1}H NMR spectra in methanol-d_4/D_2O (80:20) of the photolysis of conjugate **5** ($C = 7.0 \times 10^{-3}$ M) at 300 nm: **a** before irradiation; **b** after irradiation for 60 min; **c** after irradiation for 120 min

photolysis of conjugate **5**, was also followed by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solution (Scheme 3).

Upon irradiation at 300 nm, the signals due to the alanine residue in the conjugate form disappeared progressively and were replaced by the corresponding set of signals of Boc-Ala-OH **4a**, with the α-CH and β-CH_3 appearing at δ 4.10 and 1.40 ppm, respectively. The decrease of the benzylic-type CH_2 at position 4 of the coumarin ring was visible at δ 5.50 ppm, thus confirming the release of the amino acid. The coumarin-6-yl-alanine β-CH_2 showed a small shift to lower δ. Moreover, signals due to by-products related to the coumarin-6-yl-alanine were also detected in the ^{1}H NMR spectra (Fig. 2).

The mechanism of cleavage is believed to follow the previously reported mechanism for this type of compounds: after homolytical (followed by electron transfer) or heterolytical cleavage of the ester O–CH_2 bond, the methylene carbocation can be attacked by a nucleophile (in this case, water or methanol) yielding by-products related to coumarinyl-alanine and the free amino acid, by hydrogen abstraction from the solvent by the amino acid anionic intermediate (Yamaji et al. 2009; Schmidt et al. 2007).

Conclusions

A novel unnatural amino acid, *N*-acetyl-L-(4-chloromethyl)coumarin-6-yl-alanine methyl ester **3**, was synthesised by modification at the tyrosine side chain through a Pechmann reaction with a β-ketoester. This amino acid had a functional chloromethyl group at the coumarin ring that allowed further reaction using a simple potassium fluoride mediated coupling. A model ester conjugate **5** was prepared by reaction of **3** and the C-terminus of *N*-*t*-butyloxycarbonyl-L-alanine. In addition, the assembly of the coumarin ring was achieved at the tyrosine side chain in a dipeptide, both at the N- and C-terminal position, yielding fluorescent dipeptides. The reactivity of the chloromethyl group in the dipeptides was also confirmed. The photocleavage study of the fluorescent amino acid ester conjugate **5** in methanol/HEPES buffer (80:20) solution by irradiation at 254, 300, 350 and 419 nm revealed that the ester linkage in conjugate **5** cleaved readily with short irradiation times at 254 and 300 nm, releasing the model amino acid quantitatively, as confirmed by the ^{1}H NMR spectra. The obtained synthesis, photophysical and photocleavage results suggest that the new L-(4-chloromethyl)coumarin-6-yl-alanine derivative **3** could act as a fluorescent label, due to its fluorescence properties, and more importantly as a photoactivable unit, due to the short irradiation times necessary to cleave the newly formed ester bond between the model amino acid and the coumarin-6-yl-alanine.

The incorporation of this amino acid into biologically active peptides is currently being addressed with the preparation of cyclic peptides by taking advantage of its interesting features, namely the possibility of intramolecular crosslinking/cyclization for replacement of disulphide bonds, the photosensitivity for the preparation of open-chain derivatives of these peptides, and the intrinsic labelling with an UV-active moiety, allowing the use of fluorescence-based techniques.

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