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A photolabile amino acid building block based on a new fluorescent functionalised coumarin-6-yl-alanine

Andrea S. C. Fonseca, M. Sameiro T. Gonçalves and Susana P. G. Costa*

Centro de Química, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

email: spc@quimica.uminho.pt

Abstract: A new fluorescent functionalized amino acid, 4-chloromethylcoumarin-6-yl-alanine, was used in the synthesis of an amino acid conjugate by coupling with a model *N*-protected alanine, through an ester linkage. The photophysical properties of the fluorescent conjugate are presented, as well as its behaviour towards photocleavage by irradiation in methanol/HEPES buffer (80:20) solution, in a photochemical reactor at different wavelengths (254, 300, 350 and 419 nm), followed by HPLC/UV and ^{1}H NMR monitoring.

Keywords*:* Coumarin-6-yl-alanine; Amino acid conjugates; Photochemical cleavage

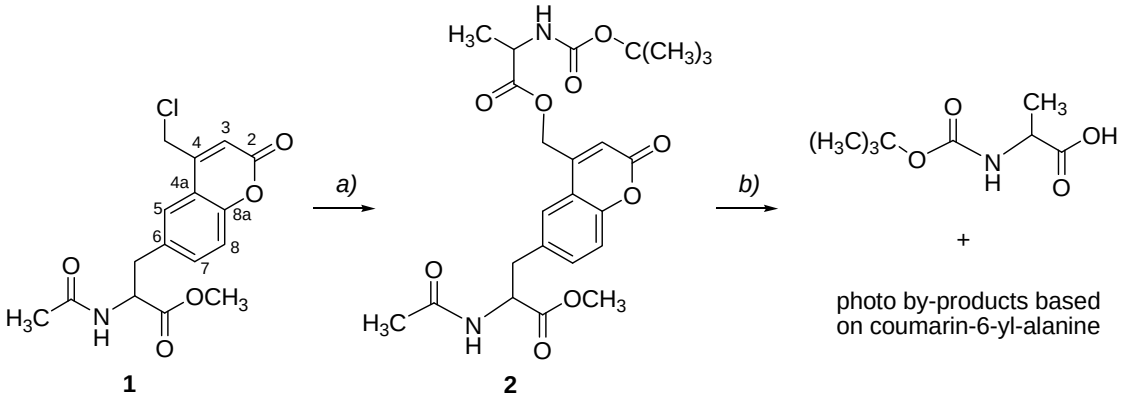
1. Introduction

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.[1] Moreover, if an extra functional group is introduced in 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. By using certain photoactive groups that are cleaved by the action of light,[2] the ability to remove the constriction by photolysis may be an interesting goal. 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 example 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. Coumarin

derivatives have been reported as photocleavable protecting groups for polyfunctional molecules bearing different functional groups. Therefore, in connection with our current research interests in the development of new fluorescent heterocyclic compounds based on coumarins and their applications as fluorescent labels and photoreleasable protecting groups,[3] we now report the synthesis and application of a functionalised alanine derivative bearing a fluorescent coumarin nucleus at its side chain, namely *N*-acetyl-4-chloromethylcoumarin-6-yl-alanine methyl ester. Upon 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. Thus, in order to evaluate the potential application of this new amino acid in photocleavage processes, we decided to synthesize a model ester conjugate 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.

2. Results and discussion

N-Acetyl-4-chloromethylcoumarin-6-yl-alanine methyl ester **1** was prepared in moderate yield (54%) by reaction of *N*-acetyltyrosine methyl ester and ethyl 4-chloro-3-oxobutanoate, through a Pechmann reaction, in the presence of aqueous 70% H_2SO_4.[4] Derivatisation at the C-*terminus* of *N*-butyloxycarbonyl-alanine with coumarin-6-yl-alanine **1** was carried out in DMF, at room temperature, in the presence of potassium fluoride,[5] yielding the fluorescent conjugate **2** in 92% yield (Scheme). The synthesized compound was fully characterized by the usual spectroscopic techniques.



Scheme. Synthesis and photolysis of ester conjugate **2**. Reagents and conditions: *a*) *N*-butyloxycarbonylalanine, KF, DMF, rt; *b*) hυ, methanol/HEPES buffer (80:20) solution.

The ^{1}H NMR spectra of conjugate **2** showed signals as multiplets for the α-CH at δ 4.48-4.53 (coumarin-6-yl-alanine) and at 4.15-422 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) 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 spectra signal of the carbonyl group, which was found at δ 172.64 ppm.

The photophysical properties of compounds **1** and **2** were evaluated and the UV/Vis absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol of those compounds were measured (Table 1). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard (Φ_F = 0.95 in ethanol).[6] Both coumarin-6-yl-alanine **1** and the ester conjugate **2** showed fair fluorescence quantum yields, whereas compound **2** displayed a large Stokes' shift. The fact that the absorption and emission maxima for coumarin-6-yl-alanine **1** occur at longer wavelengths than those of the natural amino acids that exhibit fluorescence, namely tryptophan (λ_{abs}= 278 nm, λ_{em}= 352 nm), tyrosine (λ_{abs}= 274 nm, λ_{em}= 303 nm) and phenylalanine (λ_{abs}= 257 nm, λ_{em}= 282 nm), suggests that it could be used as a fluorescent label when incorporated into biologically active peptides and proteins.

Table 1. UV/Vis and fluorescence data for coumarin-6-yl-alanine **1** and conjugate **2** in absolute ethanol.

Compound	UV/Vis		Fluorescence		
	λ_{max} (nm)	log ε	λ_{max} (nm)	Stokes' shift (nm)	Φ_F
1	320	3.67	378	58	0.04
2	318	3.58	417	99	0.03

Considering that the main goal was to compare the photostability of the ester linkage between the coumarin-6-yl-alanine and alanine in conjugate **2**, photolysis studies were carried out. In order to determine the best cleavage conditions, solutions of conjugate **2** in methanol/HEPES buffer (80:20) solution were irradiated in a Rayonet RPR-100 reactor, at 254, 300 and 350

nm,. The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection and ^{1}H NMR.

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 3 runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). Photochemical quantum yields (Φ_{phot}) of the photocleavage reaction of conjugate **2** were calculated as previously described[7] and short irradiation times were obtained for irradiation at 254 and 300 nm. Irradiation times at 350 and 419 nm are too long to be useful for pratical applications. As reported before,[3e] the *N*-butyloxy group was stable in the tested conditions, no cleavage being detected.

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

		Conjugate 2
254 nm	Irr time	69
	k	4.2×10^{-3}
	Φ_{phot}	0.023
300 nm	Irr time	79
	k	3.7×10^{-3}
	Φ_{phot}	0.012
350 nm	Irr time	2910
	k	1.0×10^{-3}
	Φ_{phot}	0.002
419 nm	Irr time	8542
	k	4×10^{-4}
	Φ_{phot}	*

* not determined

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 correlation coefficients varying from 0.9912 to 0.9978.

Additionally to monitoring of the photolysis process through HPLC/UV detection, the release of Boc-protected alanine, as the expected product of the photolysis of conjugate **2**, was also followed by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solution. Upon irradiation, the signals due to the alanine residue in the conjugate form disappeared completely and were replaced by the corresponding set of signals of Boc-Ala-OH, thus confirming the release of the amino acid. Moreover, signals due to by-products related to the coumanin-6yl-alanine **1** were also detected in the ^{1}H NMR spectra.

3. Conclusions

By using a simple potassium fluoride mediated coupling, a model ester conjugate **2** was prepared through reaction of *N*-acetyl-4-chloromethylcoumarin-6-yl-alanine methyl ester **1** and the C-*terminus* of *N*-butyloxycarbonylalanine. The photocleavage study of the fluorescent conjugate **2** in methanol/HEPES buffer (80:20) solution at 254, 300, 350 and 419 nm, revealed that the ester linkage in conjugate **2** cleaved readily with short irradiation times at 254 and 300 nm, releasing Boc-Ala-OH quantitatively, as confirmed by the ^{1}H NMR spectra. The obtained synthesis, photophysical and photocleavage results suggest that the new 4-chloromethylcoumarin-6-yl-alanine derivative **1** could act as a fluorescent label, due to its fluorescence properties, and more importantly as a photocleavable 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.

4. Experimental

4.1. Synthesis of alanine conjugate 2: Compound **1** (0.050g, 1.48×10^{-4} mol) was dissolved in DMF (5 mL) and *N*-butyloxycarbonyl-L-alanine (1 equiv, 0.028g, 1.48×10^{-4} mol) and KF (3 equiv, 0.025g, 4.45×10^{-4} mol) were added. The mixture was stirred at room temperature for 3 days. The solvent was removed in a rotary evaporator and a yellow solid was obtained. The crude solid was recrystallized from methanol/diethyl ether and conjugate **2** was obtained as a light yellow solid (0.073 g, 92%); mp = 174.2-175.0 ºC; ^{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), 2.90-3.16 (2H, m, β-CH_2), 3.61 (3H, s, OCH_3), 4.15-4.22 (1H, m, α-CH Ala), 4.48-4.53 (1H, m, α-CH), 5.42 (2H, s, CH_2), 6.48 (1H, s, H-3), 7.35 (1H, d, *J*= 8.7 Hz, H-8), 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) ppm; ^{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), 49.15 (α-CH Ala), 51.87 (OCH_3), 53.39 (α-CH), 61.39 (CH_2), 78.41 (C(CH_3)$_3$), 111.75 (C-3), 116.31 (C-4a), 116.43 (C-8), 125.58 (C-5), 133.33 (C-7), 133.67 (C-6), 150.49 (C-4), 152.15 (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) ppm; IR (KBr 1%, cm^{-1}): ν = 3315, 3295, 3084, 2996, 2985, 2938, 1755, 1731, 1688, 1666, 1575, 1551, 1534, 1436, 1372, 1350, 1316, 1287, 1235, 1174, 1160, 1133, 1070, 1013, 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.

4.2 General photolysis procedure: A 1×10^{-4} M methanol/HEPES buffer (80:20) solution of conjugate **2** (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), NaCl (120 mM), KCl (3 mM), $CaCl_2$ (1 mM) and $MgCl_2$ (1mM) and pH adjusted to 7.2.

Aliquots of 100 μL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 3:1, at a flow rate of 0.8 mL/min, 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 (318 nm) and the retention time was 6.2 min.

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