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Synthesis of Novel Fluorogenic L-Fmoc Lysine Derivatives as Potential Tools for Imaging Cells

Thomas Berthelot,[1] Georges Laïn,[1] Laurent Latxague,[1] and Gérard Déleris[1,2]

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Two coumarin-labeled lysines were conveniently prepared as fluorescent probes. 7-Methoxy and 7-diethylamino coumarin-3-carboxylic acids were synthesized according to a modification of known procedures. Labeling at lysine was achieved in solution via the active *N*-hydroxysuccinimide ester of the carboxylic acid coumarin derivatives to give the target compounds in good yield. Spectroscopic data (UV-vis and fluorescence) were recorded for all compounds.

KEY WORDS: Fluorescent probes; fluorescent amino acids; lysine; coumarins; imaging cell.

INTRODUCTION

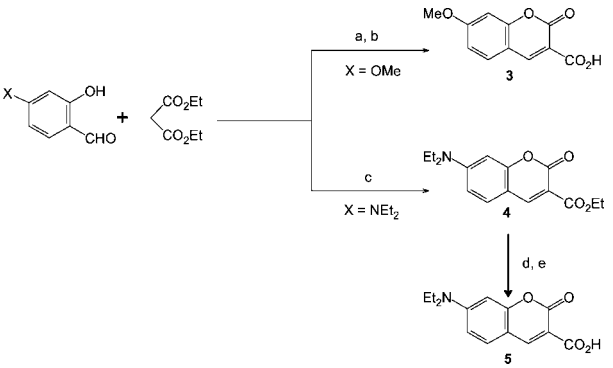
The development of new technologies to enable higher sensitivity biological imaging in living cells has been identified as one of the key scientific challenges. Fluorescent probes can detect particular components of complex biomolecular assemblies, imaging dynamic processes, etc. with exquisite sensitivity and selectivity. An other application is labelling substrates as synthetic peptides for monitoring enzyme activity [1]. Enzyme assays using labelled synthetic substrates are advantageous in that they usually provide a very direct connection between enzymatic activity and the signal. Furthermore labelling by fluorescent probes is an alternative to the use radioactive compounds [2]. In this view, we chose to synthesize modified fluorogenic amino acid with coumarin derivatives [3]. They appear to be attractive molecules due to their extended spectral range, high emission quantum yields, photostability, and good solubility in the safest solvents [4]. In this paper, we here report the synthesis of two novel coumarin-labelled lysines (Fig. 1) as fluorescent building blocks for solid phase peptide synthesis (SPPS).

[1] Groupe de Chimie Bio-Organique, INSERM U577, Université Victor Segalen Bordeaux 2, France.

[2] To whom correspondence should be addressed. E-mail: gerard.deleris@u-bordeaux2.fr

EXPERIMENTAL

Target compounds are N^{ε}-(7-methoxycoumarin-3-carboxyl)-L-Fmoc lysine (Fmoc-Lys(MC)-OH) **1** and N^{ε}-(7-diethylamninocoumarin-3-carboxyl)-L-Fmoc lysine (Fmoc-Lys(DAC)-OH) **2** (Fig. 1). The first step to obtain compounds **1** and **2** was the synthesis of the two coumarin derivatives.



Scheme 1. (a) Piperidine cat., EtOH, reflux 12 hr. (b) NaOH 20%, reflux 2 hr, HCl 12 N, 0°C. (c) Piperidine (3 eq.), MeCN/Toluene, reflux 12 hr. (d) NaOH 0.5 M, MeOH, reflux. (e) HCl 2 N.

Thus, 7-methoxycoumarin-3-carboxylic acid **3** was synthesized in a good yield (70%) by Knoevenagel condensation of 2-hydroxy-4-methoxybenzaldehyde with

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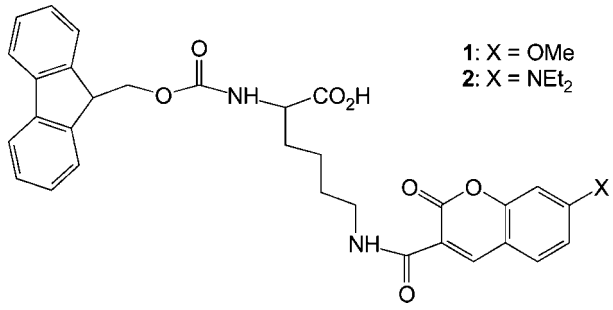
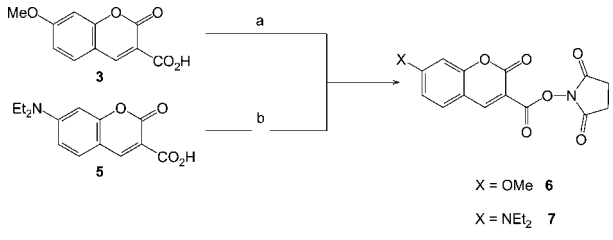


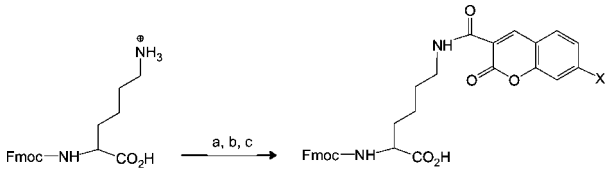
Fig. 1. Structure formulas of the two coumarin-labelled lysines.

diethylmalonate, and piperidine in catalytic amount [5]. The synthesis of 7-diethylaminocoumarin-3-carboxylic acid **5** appeared more difficult. In a first attempt, Knoevenagel condensation of 4-diethylamino salicylaldehyde gave the corresponding ester **4** [6] in poor yield (10%) and its susequent hydrolysis to **5** proved to be difficult. Use of a three fold excess of piperidine, in a toluene–acetonitrile mixture (1:2) [7] largely improved the yield of crude **4**. This latter was purified on silica gel with dichloromethane–ethylacetate (8:2) as eluent (yield: 60%) and further hydrolized to give **5** (90%) [**3** : ^{1}H NMR (DMSO-d_6) δ 12.99 (1H, s), 8.72 (1H, s), 7.82 (1H, d), 7.00 (1H, d, $J = 8.5$ Hz), 6.97 (1H, s), 3.88 (3H, s); ^{13}C NMR (DMSO-d_6) δ 164.65, 164.17, 157, 156.89, 149.14, 131.58, 113.76, 113.31, 111.60, 100.25, 56.26. **4** : ^{1}H NMR (DMSO-d_6) δ 8.38 (1H, s), 7.31 (1H, d), 6.61 (1H, d, J = 9 Hz), 6.31 (1H, d, J = 2.5 Hz), 4.39 (2H, q), 3.49 (4H, q), 1.27 (6H, t), 1.39 (3H, t). **5** : ^{1} H NMR (DMSO-d_6) δ 12.51 (1H, s), 8.58 (1H, s), 7.63 (1H, d), 6.81 (1H, d, J = 9 Hz), 6.56 (1H, s), 3.47 (4H, q), 1.16 (6H, t); ^{13}C NMR (DMSO-d_6) δ 164.21, 157.71, 153.14, 149.61, 131.58, 110.26, 107.75, 105.74, 96.02, 44.74, 12.04.] (Scheme 1).



Scheme 2. (a) DCC, HOSu, DMF, 0°C, 2 hr. (b) DCC, HOSu, DMF, −10°C, 20 hr.

To label Fmoc-L-Lysine, we considered an *in situ* activation of the carboxylic function of eitheir **3** or **5** with dicyclohexylcarbodiimide (DCC) and *N*-hydroxy succinimide (HOSu). In this approach, the key step was to protect the Lysine α-carboxylic function as its lysine-diisopropylethylamonium salt. Unfortunately the resulting compounds were difficult to purify and several side reactions involving the lysine carboxylic acid occured. As an alternative, the carboxylic acid function of **3** and **5** were separately actived to their succinimid esters **6** and **7** which were obtained in good yields [**6** : ^{1}H NMR ($CDCl_3$) δ 8.76 (1H, s), 7.57 (1H, d), 6.95 (1H, d, $J = 9$ Hz), 6.85 (1H, s), 3.95 (3H, t), 2.90 (4H, s); ^{13}C NMR ($CDCl_3$) δ 175.60, 172.82, 164.42, 161.92, 158.51, 138.31, 120.46, 117.45, 114.33, 106.66, 62.51, 31.88. **7** : ^{1}H NMR ($CDCl_3$) δ 8.58 (1H, s), 7.38 (1H, d, J = 9 Hz), 6.64 (1H, d), 6.45 (1H, s), 3.48 (4H, q), 2.90 (4H, s), 1.26 (6H, t); ^{13}C NMR ($CDCl_3$) δ 170.85, 160.57, 160.26, 158.45, 155.53, 152.49, 133.39, 111.58, 109.03, 103.81, 98.09, 46.72, 27.04, 13.81.] (Scheme 2).



1 X = OMe (64%)

2 X = NEt_2 (50%)

Scheme 3. (a) DIEA, DMF, 0°C, 30 min. (b) **6** or **7**, r.t. 2 hr. (c) HCl 2 M, 4°C, 12 hr.

For the labeling step, the commercial hydrochloride salt of L-Fmoc-Lysine was first neutralized by means of diisopropylethlamine (DIEA) which presents the best ratio between nucleophily and Fmoc conservation [8]. Subsequent reaction with succinimid esters **6** or **7** yielded the corresponding labelled lysines 1 (64%) or 2 (50%) [**1** : ^{1}H NMR (DMSO-d_6) δ 8.73 (1H, s), 8.60 (1H, t), 7.94-6.81 (10H, m), 4.33–4.10 (3H, m), 3.85 (3H, m), 3.36–3.35 (4H, m), 2.72 (1H, s), 1.74–1.02 (6H, m); ^{13}C NMR (DMSO-d_6) δ 166.10, 164.13, 163.01, 162.67, 157.85, 157.29, 149.42, 145.74, 142.49, 133.25, 129.35, 128.84, 127.05, 121.87, 115.40, 113.89, 111.64, 101.97, 67.08, 58.02, 57.09, 48.60, 33.96, 30.90, 24.67. **2** : ^{1}H NMR ($CDCl_3$) δ 9.01 (1H, s), 8.68 (1H, s), 7.72–7.22 (8H, m), 6.48 (1H, d, $J = 8.5$ Hz), 6.37 (1H, s), 6.05–5.96 (2H, m), 4.40–4.11 (4H, m), 3.52–3.44 (2H, m), 3.36 (4H, q), 1.95–1.45 (6H, m), 1.16 (6H, t); ^{13}C NMR ($CDCl_3$) δ 164.98, 163.90, 158.78, 157.47, 153.81, 145.46, 142.41, 139.26, 132.77, 130.43, 129.62, 128.75, 128.34, 126.71, 120.97, 111.28, 109.80, 97.58, 68.00, 56.47, 48.53, 46.27, 40.59, 30, 44, 23.65, 22.86, 13.75.] (Scheme 3).

The purity of each fluorogenic derivative Fmoc-Lys(DAC) **2** and Fmoc-Lys(MC) **1** was assessed by high resolution mass spectrometry (HRMS) [**1** : HRMS m/e

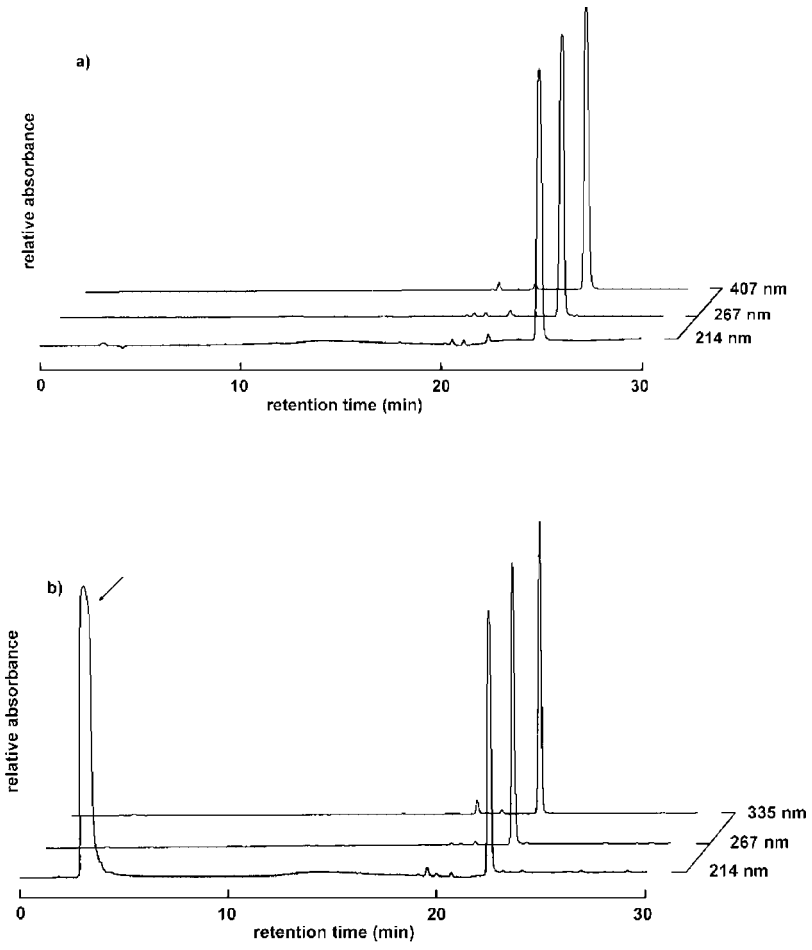


Fig. 2. HPLC profile of (a) Fmoc-Lys(DAC) **2** (retention time 24.9 min) and (b) Fmoc-Lys(MC) **1** (retention time 22.4 min) with a linear gradient from 40% to 100% B over 30 min. The arrow in chromatogram b) indicates DMF.

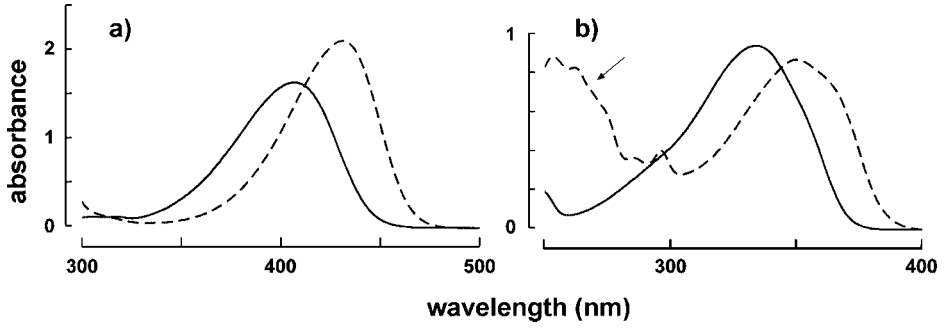


Fig. 3. UV spectrum of (a) **5** (—): $\lambda_{max} = 407$ nm, $\varepsilon = 32000$ M^{-1} cm^{-1} and **2** (- - -): $\lambda_{max} = 431$ nm, $\varepsilon = 42000$ M^{-1} cm^{-1}; (b) **3** (—): $\lambda_{max} = 335$ nm, $\varepsilon = 19000$ M^{-1} cm^{-1} and **1** (—): $\lambda_{max} = 350$ nm, $\varepsilon = 17500$ M^{-1} cm^{-1}. Spectra correspond to 50 μM of each compound in 0.1 M TRIS-HCl at pH = 9. The arrow in spectrum b) indicates the Fmoc absorption.

(m + 1) calcd for $C_{32}H_{30}N_2O_8$ 571.2080 found 571.2092; **2** : HRMS m/e (m + 1) calcd for $C_{35}H_{37}N_3O_7$ 634.2529 found 634.2532.] and analytical HPLC with linear gradient H_2O/ACN (Fig. 2) with detection at different wavelengths: 214 nm (amide bond), 267 nm (Fmoc group), 335 nm (methoxycoumarin) and 407 nm (diethylaminocoumarin).

RESULTS

Spectroscopic data (UV and Fluorescence) were recorded for all compounds in 0.1 M TRIS-HCl buffer at pH = 9.

UV spectra of **5** and **2** then **3** and **1** (Fig. 3) were recorded. Binding of the coumarine derivatives to lysines induced a red shift of the absorption band of respectively 24 nm for **5** and 15 nm for the compound **3**.

Fluorescence spectra of compounds **5** and **2** (Fig. 4) then **3** and **1** (Fig. 5) were also recorded. These results show that compound 2 presents a maximum of absorption at 413 nm and a maximum of emission at 480 nm and compound 1 presents a maximum of absorption at 335 nm and a maximum of emission at 404 nm.

In conclusion, we have shown that two N^{ε}-coumarin-labelled-L-Fmoc lysines could be readily synthesized as a fluorescent probes. These novel compounds are currently studied in our group according to their introduction within automatic peptide solid phase synthesis and subsequent proteolysis assays.

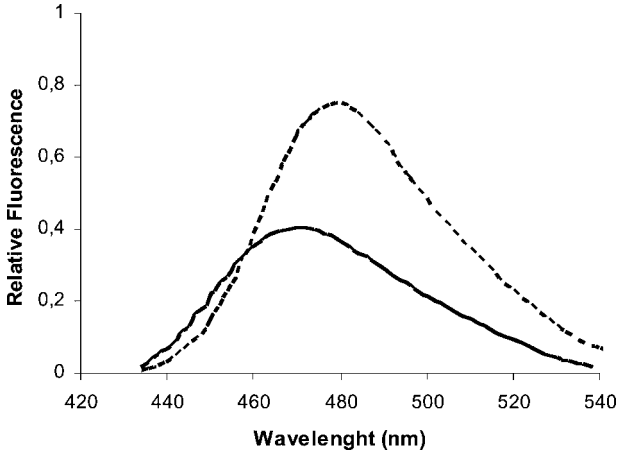


Fig. 4. Emission spectrum of compounds **2** (- - -) and **5** (—): the excitation wavelength was 407 nm. Spectra correspond to 1 μM of **2** or **5** in 0.1 M TRIS at pH = 9.

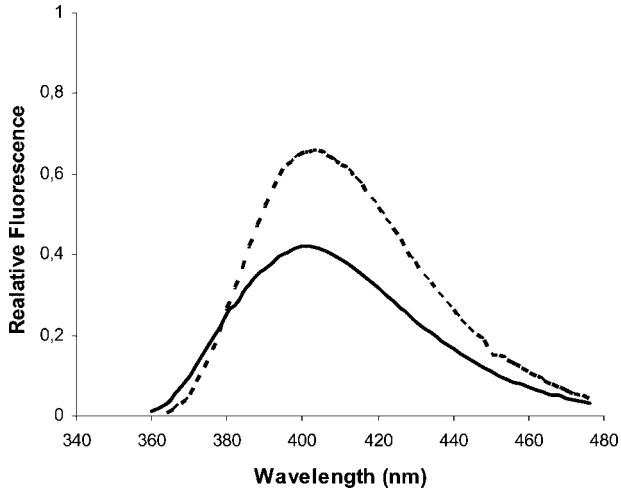


Fig. 5. Emission spectrum of compounds **1** (- - -) and **3** (—): the excitation wavelength was 335 nm. Spectra correspond to 1 μM of **1** or **3** in 0.1 M TRIS at pH = 9.

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