Journal of Peptide Science
J. Peptide Sci. **11**: 153–160 (2005)
Published online 11 October 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.608

JOURNAL OF PEPTIDE SCIENCE

Synthesis of N^{ε}-(7-diethylaminocoumarin-3-carboxyl)- and N^{ε}-(7-methoxycoumarin-3-carboxyl)-L-Fmoc lysine as tools for protease cleavage detection by fluorescence

THOMAS BERTHELOT,[a] JEAN-CLAUDE TALBOT,[b] GEORGES LAÏN,[a] GÉRARD DÉLERIS,[a]* and LAURENT LATXAGUE[a]

[a] Groupe de Chimie Bio-Organique, Université Victor Segalen Bordeaux 2, France
[b] IBGC-CNRS, Université Victor Segalen Bordeaux 2, France

Received 27 May 2004; *Revised 29 July 2004*; *Accepted 8 August 2004*

Abstract: Two coumarin-labelled lysines were conveniently prepared as a fluorescence resonance energy transfer (FRET) pair for peptide cleavage detection. 7-Methoxy and 7-diethylamino coumarin-3-carboxylic acids were synthesized according to a modification of known procedures. Labelling 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. Subsequently, these modified amino acids were used in solid phase peptide synthesis (SPPS), and their potential utility in an extracellular matrix metalloprotease (MMP-1) activity measurement via FRET and/or quenching studies was demonstrated.

Keywords: fluorogenic substrates; fluorescent amino acids; coumarins; matrix metalloprotease activity

INTRODUCTION

Proteases have been implicated in a variety of cancers; extracellular matrix metalloproteases (MMPs), for example, are key proteins secreted during cancer growth [1–3]. Therefore, the influences of specific MMPs on tumour growth, invasion, migration and identification of new synthetic inhibitors drive the development of new anticancer therapeutics [4]. To facilitate the identification of inhibitor effects on MMP activity, fluorescence resonance energy transfer (FRET) or donor dequenching may be used as an easy to run test in high throughput screening [5]. It involves transfer between a donor probe and an acceptor probe linked at the *N*- and *C*-termini of a short sequence of amino acids containing the MMP cleavable specific site. A number of intramolecularly quenched fluorogenic peptide substrates have been designed and reported [6–10]. Their design generally embodies F-X-Q structure, in which F is the fluorescent (or donor) group and Q is the quenching (or acceptor) group, separated by a peptide sequence containing the protease cleavage site. Examples of such substrates include the use of 4-methyl-7-coumarylamide (MCA) as the donor and 2,4-dinitrophenyl (Dnp) as the acceptor [11–13]; *o*-aminobenzoic acid (Abz) as the donor and Dnp, nitrotyrosine ($Tyr(NO_2)$) or nitrophenylalanine ($Phe(NO_2)$) as the acceptor [14]; and 5-(2′ aminomethyl)naphtalenesulfonic acid (EDANS) as the donor and 4-(4′-dimethylaminophenylaza) benzoic acid (DABCYL) as the acceptor [15–17]. In most of them, donor fluorescence is quenched by the acceptor without subsequent fluorescence emission. FRET is observed when two fluorescent dyes are in close proximity and one fluorophore's emission overlaps the other's excitation spectrum [18–20]. In FRET, energy is transferred from the donor to acceptor via a non-radiative dipole–dipole interaction. The energy transfer efficiency depends on the inverse sixth power of the distance between the donor and acceptor. Dyes attached to amino acids within a peptide undergo FRET (fluorescence of the donor is quenched), producing a unique fluorescence spectrum. If proteolysis occurs, separation of the donor and the acceptor results in a loss of energy transfer and subsequent enhancement of donor fluorescence. A trivial fluorescence quenching between the two fluorophores may also take place [21] *via* stacking or collision effects. The enzymatic digestion of the scissile bond of a double-labelled peptide might result in fluorescence dequenching, enabling direct activity measurements via fluorescence enhancement of the 'donor' part, similarly to FRET events.

As a part of our work dealing with design, synthesis and *in vitro* evaluation of novel MMP inhibitors, we

Abbreviations: ACN, acetonitrile; DAC, 7-diethylamino coumarin-3-carboxylic acid; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; ESMS, electrospray mass spectroscopy; HBTU *N*-[1*H*-benzotriazol-1-yl)dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, in full HOBt: 1-hydroxy-benzotriazole; MC, 7-methoxy coumarin-3-carboxylic acid; MMP, extra cellular matrix metalloprotease; NMP, 1-methyl-2-pyrrolidone; pAMPA, 4-aminophenylmercuric acetate; PyBOP, benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TRIS, tris(hydroxymethyl)aminomethane.

*Correspondence to: Gérard Déleris, Groupe de Chimie Bioorganique, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, F-33076 Bordeaux Cedex, France;
e-mail: gerard.deleris@u-bordeaux2.fr

have synthesized two original fluorescent modified amino as FRET substrates directly usable within solid phase peptide synthesis. A suitable donor-acceptor pair composed of two coumarin derivatives was chosen. The rationale of our approach is that they exhibit a wide absorption range, a high emission quantum yield, a fluorescence emission (402 and 473 nm in this case) outside of the intrinsic protein fluorescence range, allowing work with complex biological media, and a good spectral overlap; moreover, their low steric hindrance should limit disturbance of substrate recognition.

Several coumarin-containing amino acid 'building blocks' and their utility in FRET substrates have already been described, including the synthesis of L-(6,7-dimethoxy-4-coumaryl) alanine [22] or N^{ε}-(7-methoxycoumarin-3-acetyl)-L-Fmoc-lysine [23] for example. In this case, the straightforward synthesis of two novel fluorescent lysine derivatives, N^{ε}-(7-methoxycoumarin-3-carboxyl)-L-Fmoc-lysine (Fmoc-Lys(MC)) and N^{ε}-(7-diethylaminocoumarin-3-carboxyl)-L-Fmoc-lysine (Fmoc-Lys(DAC)) was considered, since one of the starting compounds (7-methoxycoumarin-3-carboxylic acid) was commercially available. In order to provide a pharmacological tool to monitor the proteolytic activity (or inhibition) of an MMP, two model peptides were synthesized, one incorporating one fluorescent moiety and the other incorporating two while embodying a sequence substrate of this MMP. The results dealing with synthetic, biophysical and preliminary biochemical work are presented.

MATERIALS AND METHODS

All standard chemicals and solvents were of analytical grade and purchased from Sigma Aldrich (St Quentin Fallavier, France). 7-Methoxycoumarin-3-carboxylic acid (MC) was purchased from Fluka (St Quentin Fallavier, France). N^{α}-9-fluorenylmethoxycarbonyl L-lysine hydrochloride (Fmoc-Lys-OH.HCl), Fmoc-Ala-Wang resin and Fmoc-amino acids were purchased from Advanced ChemTech (Cambridge, UK). DCC, DIEA and HOBt were purchased from Avocado (La Tour du Pin, France). 1-Methyl-2-pyrrolidone (NMP) was purchased from Scharlau Chemie S.A. (Barcelona, Spain), piperidine, HBTU, 0.6 M HOBt/DMF, 2.0 M DIEA/NMP were purchased from applied Biosystems (Warnington, UK) and were of peptide synthesis grade. Triisopropylsilane (TIS) and 4-aminophenylmercuric acetate (pAPMA) were purchased from Sigma Aldrich (St Quentin Fallavier, France). Human MMP-1 (90% 53 kDa zymogen and 10% activated enzyme) was purchased from Oncogene Research Products (San Diego, USA).

All chemical reactions were carried out under nitrogen with dry solvents under anhydrous conditions unless otherwise stated. DMF was refluxed over CaH_2 overnight and distilled, and methylene chloride was distilled before use. Thin layer chromatography (TLC) was performed on aluminium plates precoated with silica gel $60F_{254}$ (Merck, Germany) with detection by UV light or by staining with $KMnO_4$ (1% aqueous solution), and column chromatography was performed on silica gel 60, 70–230 mesh (SDS, France).

The ^1H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively, on a Bruker Avance 300 spectrometer at 298 K for solutions in $CDCl_3$ or DMSO-d_6. Electrospray mass spectra (ESMS) were recorded on LCQ Advantage Thermo Finnigan and LSMS-HRMS spectra were recorded on AutoSpec-Q Fisons-Instruments spectrometers. Peptides were synthesized on Applied Biosystems 433A Peptide Synthesizer (Foster City, USA) using Fmoc protection. High-performance liquid chromatography (HPLC) was carried out on a Shimadzu instrument equipped with a SPD-10 AVP UV-Vis detector, a SCL-10 AVP system controller and LC8A HPLC pumps (Shimadzu Scientific Instruments Inc., Columbia, USA). Preparative HPLC was carried out using a Hibar® pre-packed RP-18 column (5 μm, 250 × 25 mm). The following solvent systems were used for the elution in a linear gradient mode, at a flow rate of 12 ml/min: (A) 0.1% aqueous trifluoroacetic acid (TFA) and (B) 0.1% TFA in 70% aqueous acetonitrile (ACN). Purity of the products was determined on a Hibar® pre-packed RP-18 column (5 μm, 250 × 4.6 mm) and gradient elution with the same solvent system as described above was used at a flow rate of 1 ml/min. Both columns were purchased from Merck (Darmstadt, Germany). Absorption spectra were recorded on a Uvikon 933 spectrophotometer (Bio-Tek Kontron Instruments). Fluorescence spectra were recorded over a range of 280 to 520 nm on a PTI spectrofluorometer (Photon Technology Instrument, Canada), or a Jobin-Yvon JY3 spectrofluorometer (Longjumeau, France). The fluorometric assay was performed for both peptides in 0.1 M TRIS-HCl buffer pH 7.6 with 150 mM NaCl and 10 mM $CaCl_2$.

7-Diethylaminocoumarin-3-carboxylic Acid (DAC) (2)

7-Diethylamino salicylaldehyde (2.66 g, 13.8 mmol) and diethyl malonate (3.31 g, 20.7 mmol) were dissolved in (1 : 2) toluene-acetonitrile (150 ml). Piperidine (3.53 g, 41.5 mmol) was added and the mixture was refluxed for 12 h. After cooling, the solvent was evaporated and the residue was purified by silica gel chromatography (methylene chloride-ethyl acetate, 8 : 2) to give product **1** as a yellow oil (2.39 g) (60%). To a refluxed solution of compound **1** (1.07 g, 3.70 mmol) in methanol (9.4 ml), 0.5 M NaOH (9.4 ml) was added dropwise. A yellow solid appeared. After cooling, a solution of 2 M HCl was added and an orange solid appeared. It was filtered off, washed twice with 2 M HCl, then with H_2O and methanol to yield product **2** (870 mg) (90%). (**1**) ^1H NMR (DMSO-d_6) δ 8.38 (1H, s), 7.31 (1H, d), 6.61 (1H, d, $J = 9.0$ 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). (**2**) ^1H NMR (DMSO-d_6) δ 12.51 (1H, s), 8.58 (1H, s), 7.63 (1H, d), 6.81 (1H, d, $J = 9.0$ 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; UV ($c = 5 \times 10^{-5}$ M, 0.1 M Tris) $\lambda_{max} = 407$ nm, $\varepsilon = 32\,000$ M^{-1} cm^{-1}.

Succinimidyl Ester of 7-Methoxycoumarin-3-carboxylic Acid (6)

To a solution of 7-methoxycoumarin-3-carboxylic acid **5** (440.36 mg, 2 mmol) in DMF (5 ml) *N*-hydroxysuccinimide

(230.18 mg, 2 mmol) was added. After dissolution, the reaction mixture was cooled at 0 °C for 1 h. DCC (453.92 mg, 2.2 mmol) was added. After stirring for 30 min at this temperature, the reaction mixture was stirred at room temperature for 2 h. The solution was filtered off to remove dicyclohexylurea. An isopropanol–hexane solution (1 : 20) was added to the filtrate to give product **6** as a white solid (539 mg) (85%). ^{1}H NMR($CDCl_3$) δ 8.76 (1H, s), 7.57 (1H, d), 6.95 (1H, d, $J = 9.0$ 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

Succinimidyl Ester of 7-Diethylaminocoumarin-3-carboxylic Acid (3)

To a solution of 7-diethylaminocoumarin-3-carboxylic acid **2** (289.31 mg, 1 mmol) in DMF (5 ml), *N*-hydroxysuccinimide (115 mg, 1 mmol) was added. After dissolution, the reaction was cooled at −10 °C. After stirring for 1 h at this temperature, *N*,*N*′-dicyclohexylcarbodiimide (226.96 mg, 1.1 mmol) was added. The solution was stirred at −10 °C for 12 h. The solution was filtered off to remove DCU. An isopropanol–hexane mixture (1 : 20) was added to the filtrate to give product **3** as a yellow solid (315 mg) (88%). ^{1}H NMR($CDCl_3$) δ 8.58 (1H, s), 7.38 (1H, d, $J = 9.0$ 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.

General Procedure to Obtain Fmoc-Lys(DAC) (7) and Fmoc-Lys(MC) (4)

To a solution of Fmoc-L-Lys-OH.HCl (408 mg, 1 mmol) in DMF (5 ml) diisopropylethylamine (388 mg, 3 mmol) was added and the resulting solution was stirred at 0 °C for 30 min. Compound **3** or **6** (0.5 mmol) was added and the solution was stirred at room temperature for 2 h. After evaporation of the solvent under reduced pressure, the mixture was treated with a 2 M HCl solution and stored at +4 °C overnight. A precipitate appeared which was filtered off and washed twice with 2 M HCl then with H_2O, to give the crude product. It was purified on a silica gel column with toluene–methyl alcohol (8 : 2) for **4** and methylene chloride–methyl alcohol (9 : 1) for **7** to give **4** (158 mg) (50%) as a yellow solid, or **7** (182 mg) (64%) as a white solid. (**4**) ^{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; UV (c = 5×10^{-5} M, 0.1 M Tris) $\lambda_{max} = 350$ nm, $\varepsilon = 17\,500$ M^{-1} cm^{-1}; HRMS m/e (m + 1) calcd for $C_{35}H_{37}N_3O_7$634.2529, found 634.2532. (**7**) ^{1}H NMR (DMSO-d_6) δ 8.73 (1H, s), 8.60 (1H, t), 7.94–6.81 (10H, m), 4.33–4.10 (3H, m), 3.85 (3H, m), 3.36–3.35 (4H, m), 2.72 (1H, s), 1.74–1.02 (6H, m); ^{13}C NMR (DMSO-d_6) δ 166.10, 164.13, 163.01, 162.67, 157.85, 157.29, 149.42, 145.74, 142.49, 133.25, 129.35, 128.84, 127.05, 121.87, 115.40, 113.89, 111.64, 101.97, 67.08, 58.02, 57.09, 48.60, 33.96, 30.90, 24.67; UV ($c = 5 \times 10^{-5}$ M, 0.1 M Tris) $\lambda_{max} = 431$ nm, $\varepsilon = 42\,000$ M^{-1} cm^{-1}; HRMS m/e (m + 1) calcd for $C_{32}H_{30}N_2O_8$ 571.2080, found 571.2092.

Peptide Synthesis

Two model peptides **pDACMC**: (Fmoc)Lys(DAC).GPQGLLGA.Lys(MC).A (**8**) and **pMC**: KGPQGLLGA.Lys(MC).A (**9**) were synthesized on a 0.1 mmol scale using Fmoc strategy. For **pDACMC** (**8**), standard removal of the Fmoc protecting group of Fmoc-Ala-Wang resin (0.5 g, 0.8 mmol/g) was done manually to give the unprotected resin which was reacted with Fmoc-Lys(MC) (**7**) (228 mg, 0.4 mmol) in the presence of PyBOP (173 mg, 0.392 mmol), HOBt (54 mg, 0.4 mmol) and DIEA (103 mg, 0.8 mmol) in DMF (5 ml) for 5 h at room temperature. The mixture was filtered and washed with 5 × 10 ml each of DMF, dichloromethane and methyl alcohol to give 0.14 g of Fmoc-Lys(MC)-Ala-Wang resin. Substitution level of 0.425 mmol/g was estimated for this new-preloaded resin by UV determination of the concentration of liberated dibenzofulvene, after cleavage of the Fmoc group with piperidine [24]. Unreacted amino functions were acylated to prevent any side reactions on resin. This capping step was performed with acetic anhydride (16 mg, 0.16 mmol) and piperidine (12 mg, 0.16 mmol) in DMF (2 ml) for 40 min at room temperature. After the usual washings, 0.14 g of the methoxy-coumarin functionalized resin was isolated, and the capping efficiency was checked with a ninhydrin test. After several runs, 0.238 g of the Fmoc-Lys(MC)-Ala-Wang resin was packed in the reaction column of the automatic peptide synthesizer. N^{α}-Fmoc amino acids (Ala, Gly, Leu, Gln(Trt) and Pro) were used in a ten-fold excess using HBTU in the presence of HOBt and DIEA for 18 h, giving 0.314 g of peptide-bound resin. After Fmoc deprotection of the last amino acid, Fmoc-Lys(DAC) (**4**) (305.6 mg, 0.5 mmol) was coupled manually to the resin-bound peptide in the presence of PyBOP (216.7 mg, 0.49 mmol), HOBt (67.5 mg, 0.5 mmol) and DIEA (129.2 mg, 1 mmol) in DMF for 5 h at room temperature. The mixture was filtered and washed to give 0.335 g of resin. After treatment with 20% piperidine in DMF at room temperature, **pDACMC** (**8**) was cleaved from the resin, and the trityl side chain protection of Gln was simultaneously removed by treatment with TFA/H_2O/TIS (95 : 2,5 : 25) mixture for 2.5 h at room temperature. The resin was removed by filtration and washed with TFA (2 × 1 ml) and dichloromethane (10 ml). The filtrate and washings were combined and evaporated under reduced pressure and the remaining residue was triturated with cold diethyl ether. After standing at −20 °C overnight, the precipitated peptide (140 mg) was collected by filtration and purified by preparative HPLC to yield 32 mg of pure Fmoc protected **pDACMC** (**8**). ESMS m/e (m + 1) 1706.5. It must be noted that the Fmoc protecting group could not be removed under the conditions used as evidenced by the mass spectrometry analysis.

pMC (**9**) was similarly synthesized using the same Fmoc-Ala-Wang resin with the following N^{α}-Fmoc amino acids: Ala, Gly, Leu, Gln(Trt), Pro, Lys(Boc) and compound **7**. After deprotection, resin cleavage and purification by preparative HPLC of the crude peptide, 50 mg of **pMC** (**9**) was obtained. ESMS m/e (m + 1) 1241.7. This peptide was further dissolved in 3 ml 0.1 M TRIS-HCl buffer previously mentioned, to a final concentration of 10 μM. A solution of **pMC** (**9**) (10 μl) was used for calibration purpose to assess the maximum donor fluorescence intensity.

Peptide Cleavage Assay

Commercial human MMP-1 (8.8 µl) under 90% zymogen form was activated with 100 µl of a solution of 4-aminophenylmercuric acetate [25] (3.52 mg in 1 ml buffer and 818 µl glycerol) to a final concentration of 74 nM, at 37 °C for 45 min. **pDACMC** (**8**) was dissolved in 3 ml TRIS-HCl buffer, to a final concentration of 10 µM. Fluorescence spectra were measured at 30 °C with $\lambda_{excitation} = 340$ nm over a 350–600 nm range. A solution of 10 µM **pDACMC** (**8**) was mixed with the activated MMP-1 solution to a final protein concentration of 1.6 nM and the evolution of fluorescence was monitored over approximately 1100 min.

RESULTS AND DISCUSSION

In the first instance, the preparation of DAC (**2**) was revealed to be more difficult than described in the literature. The synthesis of DAC (**2**) was first performed by way of a Knoevenagel condensation of 4-diethylamino salicylaldehyde to give the corresponding ester **1** [26–34] in poor yield (10%). Furthermore, its subsequent hydrolysis to DAC proved to be difficult. The use of a three-fold excess of piperidine in a toluene–acetonitrile (1 : 2) mixture [35] largely improved the yield of crude **1**. This was purified on silica gel with dichloromethane–ethylacetate (8 : 2) as eluent (yield: 60%) and was further hydrolysed to give DAC (**2**) (90%) (Scheme 1). More recently, Song *et al.* described the synthesis of coumarin-3-carboxylic acids via Knoevenagel condensation of Meldrum's acid with ortho-hydroxyaryl aldehydes or ketones [36].

To label Fmoc-L-lysine, an *in situ* activation of the carboxylic function of either DAC or the commercially available 7-methoxycoumarin-3-carboxylic acid (MC) **5** with DCC and *N*-hydroxysuccinimide was considered. In this approach, the key step was protection of the lysine α-carboxylic function as its lysine-diisopropylethylammonium salt. Unfortunately the resulting compounds were difficult to purify and several side reactions were noted involving the lysine carboxylic acid. As an alternative, the carboxylic function of DAC and MC were separately activated to their succinimidyl esters **3** and **6** which were obtained in good yields. For the labelling step, the commercial hydrochloride salt of L-Fmoc-Lysine was first neutralized by means of DIEA which presents the best compromise between nucleophilicity and Fmoc conservation [37]. Subsequent reaction with succinimidyl esters **3** or **6** yielded the corresponding labelled lysines Fmoc-Lys(DAC) (**4**) (50%) or Fmoc-Lys(MC) (**7**) (64%) (Scheme 2).

The UV spectra of DAC and Fmoc-Lys(DAC) then MC and Fmoc-Lys(MC) (Figure 1) were recorded. Binding of the coumarin derivatives to lysines induced a red shift of the absorption band of 24 nm for DAC and 15 nm for MC, respectively.

The purity of each fluorogenic derivative Fmoc-Lys(DAC) and Fmoc-Lys(MC) was assessed by high resolution mass spectrometry (HRMS) and analytical HPLC (Figure 2) with detection at different wavelengths: 214 nm (peptidic bond), 267 nm (Fmoc group), 335 nm (methoxycoumarin) and 407 nm (diethylaminocoumarin).

As a model matrix metalloprotease, interstitial collagenase (MMP-1) was selected as it is known that it cleaves sequence GPQGLLGA within type I human collagen [38]. Furthermore, it could be inferred that eight amino acids (substrate sequence and the two lysines) between the chromophores should be an adequate length for fluorescence energy transfer to occur. Therefore the substrate peptide, GPQGLLGA, was synthesized incorporating K(DAC) at the *N*-terminus and K(MC).Ala at the *C*-terminus of the peptide, to give K(DAC).GPQGLLGA.K(MC).A.

For synthetic improvements, a Wang-resin was used preloaded with alanine which was first deprotected with piperidine and coupled on the bench

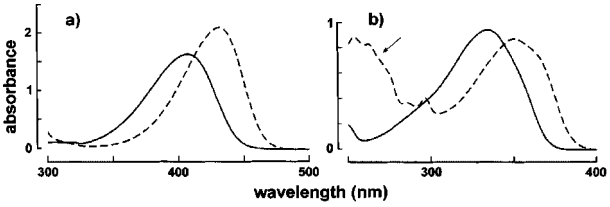
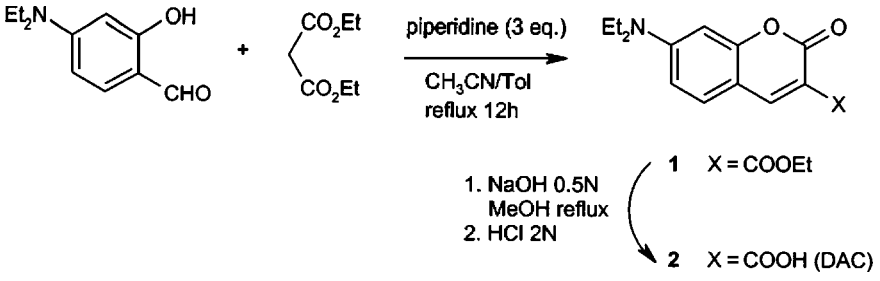
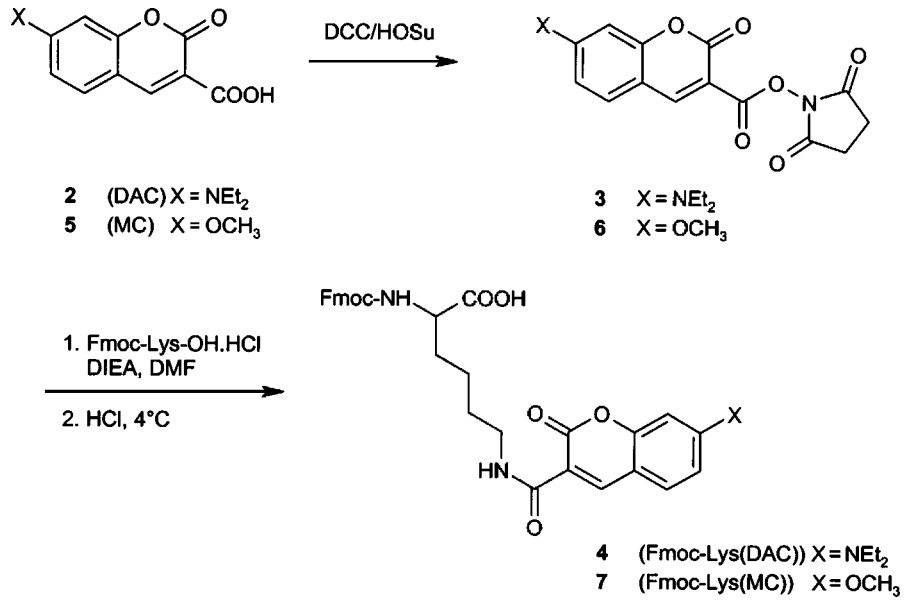


Figure 1 UV spectrum of (a) DAC (———): $\lambda_{max} = 407$ nm, $\varepsilon = 32\,000$ M^{-1}cm^{-1} and Fmoc-Lys(DAC) (- - - -): $\lambda_{max} = 431$ nm, $\varepsilon = 42\,000$ M^{-1} cm^{-1}; (b) MC (———): $\lambda_{max} = 335$ nm, $\varepsilon = 19\,000$ M^{-1}cm^{-1} and Fmoc-Lys(MC) (- - - -): $\lambda_{max} = 350$ nm, $\varepsilon = 17\,500$ 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.



Scheme 1



Scheme 2

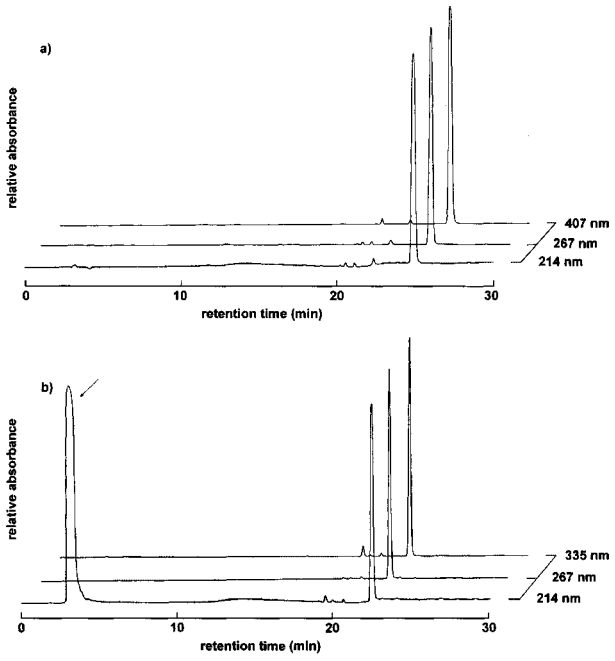


Figure 2 HPLC profile of (a) Fmoc-Lys(DAC) **4** (retention time 24.9 min) and (b) Fmoc-Lys(MC) **7** (retention time 22.4 min) with a linear gradient from 40% to 100% B over 30 min. The arrow in chromatogram (b) indicates DMF.

with Fmoc-Lys(MC) (**7**) to give the functionalized Fmoc-Lys(MC)-Ala-Wang resin which was used afterwards in automatic solid phase peptide synthesis. Finally, Fmoc-Lys(DAC) (**4**) was incorporated, on the bench again, further cleaved from the resin to yield the 11-residue peptide **pDACMC**: (Fmoc)Lys(DAC).Gly.Pro.Gln.Gly.Leu.Leu.Gly.Ala.Lys (MC).Ala (**8**). Mass spectrometry analysis showed that the Fmoc protecting group could not be removed from **pDACMC** (**8**). Whilst deprotection with piperidine is effective in most cases, incomplete Fmoc deprotection has been observed even under high piperidine concentrations, mainly when peptide chains form secondary structures or aggregates either with other peptide chains or with the polymer support [39]. Similarly, a second peptide (pMC), was synthesized as a reference tool identical to pDACMC except that the fluorescent amino acid Lys(DAC) was replaced by an unmodified lysyl residue. Both peptides were purified by HPLC. Peptide **pDACMC** (**8**) exhibited a 23 min retention time (gradient from 40% to 100% B over 30 min) while **pMC** (**9**) exhibited a 20.7 min retention time (linear gradient from 20% to 100% B over 25 min) (Figure 3).

Figure 4 displays the emission spectrum of the lysine derivative Fmoc-Lys(MC) (**7**) and the absorption spectrum of Lys(DAC) (**4**). The overlap of these spectra shows that FRET could occur between these two probes if present within the same peptide (e.g. **pDACMC** (**8**)). Fmoc-Lys(MC) would act as the donor with an emission maximum at 404 nm ($\lambda_{excitation}$ = 335 nm), and Fmoc-Lys(DAC) as the acceptor with an absorption maximum at 431 nm ($\lambda_{emission}$ = 480 nm).

As expected, the incorporation of the fluorophore pair Fmoc-Lys(MC) and Fmoc-Lys(DAC) into the model peptide **pDACMC** (**8**), resulted in a wide emission peak at ca 476 nm characteristic of the acceptor emission on excitation at ca 340 nm in the absorption band of the donor (MC). The emission peak from the donor moiety (the only fluorophore present in **pMC** (**9**)) at ca 403 nm was markedly reduced (Figure 5). It can be noticed that excitation at 340 nm of the acceptor dye at the same concentration resulted in negligible signal at 476 nm.

This preliminary result suggested the existence of a fluorescence energy transfer between the two dyes. In order to unambiguously validate this proposed

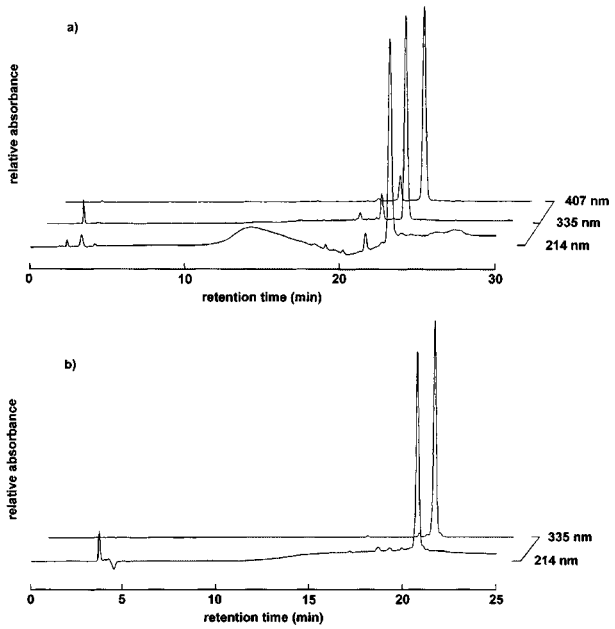


Figure 3 HPLC profiles of (a) pDACMC (retention time 23 min) with a linear gradient from 40% to 100% B over 30 min and (b) pMC (retention time 20.7 min) with a linear gradient from 20% to 100% B over 25 min.

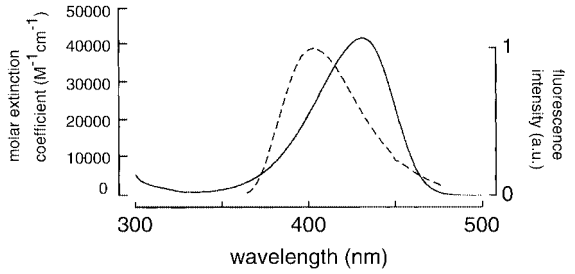


Figure 4 Overlapping of the emission spectrum of Fmoc-Lys (MC) **7** (- - - -) and the absorption spectrum of Fmoc-Lys(DAC) **4** (———).

FRET pair for proteolysis detection, 10 μM of peptide **pDACMC** (**8**) was treated with 1.6 nM human MMP-1 and fluorescence spectra were recorded versus time (Figure 6).

With an increased incubation time due to the proteolytic activity of the enzyme, separation between the donor and the acceptor was achieved, resulting in an increase of the emission peak of the donor at ca 400 nm as expected in the case of separation between donor and acceptor pair. In the range of the acceptor emission spectrum, a first decrease of the intensity occurred, cogent with a decrease of FRET, followed with an emission enhancement. Direct excitation of the acceptor part, in the double-labelled peptide, and in the acceptor lysine (Lys(DAC)) proved that quenching occurred in the double-labelled peptide concomitantly with FRET (data not shown). This can explain the subsequent enhancement of the fluorescence acceptor

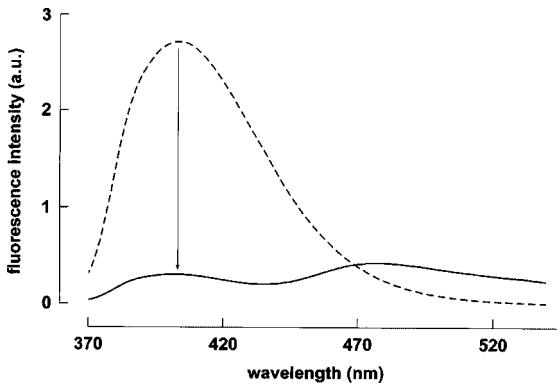


Figure 5 Emission spectra of pDACMC (———) and pMC (- - - -): the excitation wavelength was 340 nm. The arrow indicates the decrease of the donor fluorescence. Spectra correspond to 0.1 μM of each peptide in 0.1 M Tris-HCl at pH 7.6, 150 mM NaCl and 10 mM $CaCl_2$.

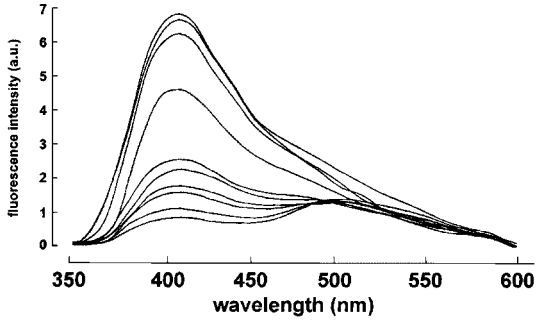


Figure 6 Continuous fluorometric assay of MMP-1 with pDACMC. Around 400 nm, from bottom to top, fluorescence spectra were recorded after addition of the enzyme at t_0, then $t_0 + 15$ min, $t_0 + 38$ min, $t_0 + 55$ min, $t_0 + 72$ min, $t_0 + 99$ min, $t_0 + 267$ min, $t_0 + 974$ min and $t_0 + 1098$ min ($\lambda_{excitation} = 340$ nm).

observed during peptide digestion by MMP1. It can be noticed that this fluorescence quenching may be the consequence of peptide flexibility. Therefore, it was demonstrated that a radiation-less transfer of excitation energy from the donor Lys(MC) to the acceptor Lys(DAC) and fluorescence quenching occurred prior to enzymatic cleavage. The change observed in the emission of the donor can be used to characterize enzyme activity as shown in Figure 7. Assuming the reaction to be complete, the initial velocity derived from the slope of the quasi-linear part of the curve allows the specific activity to be measured: 1 mg of activated MMP-1 (MW 42 570 g.mol^{-1}) [40] hydrolyses 0.4 μmol of peptide per minute, under these experimental conditions.

CONCLUSION

It has been shown that two novel N^{ε}-coumarin-labelled-L-Fmoc lysines could be readily synthesized as a potential fluorescent resonance energy transfer (FRET)

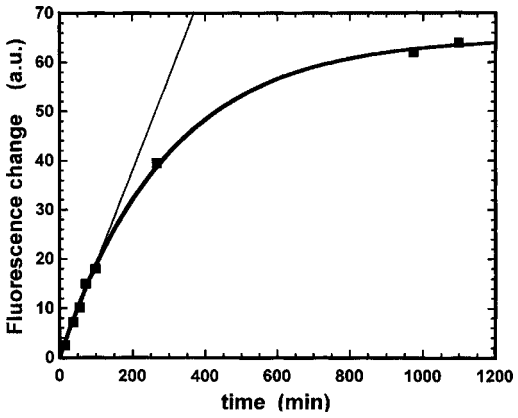


Figure 7 Kinetics of MMP-1 activity. Change in fluorescence intensity of the donor (measured at 403 nm) from the spectra of Figure 6. Assuming a first order kinetics, the fit gives an extrapolated value of 65.1 ± 0.7 a.u. for the maximum fluorescence change. The slope of the straight line (0.190 ± 0.003 a.u./min) represents the initial velocity characteristic of enzyme activity.

pair. Once incorporated in a peptide substrate for an extracellular matrix metalloprotease, they behave as a powerful tool allowing monitoring of proteolysis. Work is in progress in our group to optimize this system for other MMPs and towards their application in high throughput screening systems.

Acknowledgements

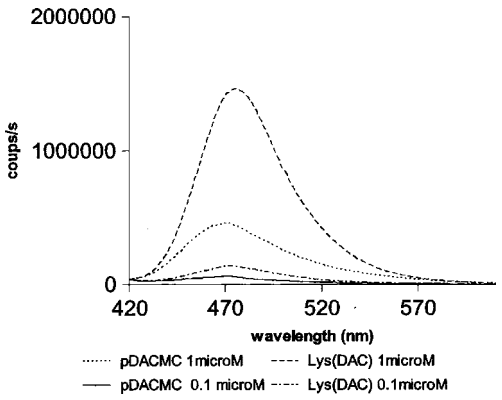
Financial support is acknowledged from 'Conseil Régional d'Aquitaine' and 'Ligue Nationale contre le cancer, Comité de la Gironde'. Moreover we thank Katell Bathany, CNRS UMR 5144 MOBIOS, Université Bordeaux 1 for electrospray mass spectra acquisitions.

ANNEXE 1

As reported by the referee, in Figure 6 emission at ca 480 nm increased. The fluorescence characteristics for Lys(DAC) compound and doubly labelled peptide were compared Lys(DAC) and pDACMC were excited at 407 nm and fluorescence data were recorded for each species at 1 μM and 0.1 μM, respectively.

The fluorescence intensity of the acceptor within the double-labelled peptide after direct excitation was lower than for Lys(DAC). This shows that quenching occurred within pDACMC, which was possibly related to the peptide flexibility.

Nevertheless, occurrence of an excitation-energy transfer from the donor Lys(MC) to the acceptor Lys(DAC) was demonstrated prior to the enzymatic cleavage. At 340 nm (excitation wavelength), fluorescence was not observed for Lys(DAC). When pDACMC was excited at 340 nm, fluorescence appeared at ca 480 nm. Quenching of fluorescence alone would have



led to a decreased fluorescence intensity for the donor and without acceptor fluorescence. This result shows that two physical phenomena occurred: FRET and fluorescence quenching. In a more constrained structure, this fluorescent pair could not have formed a non-covalent dimer, which should have resulted in only a FRET phenomenon.

REFERENCES

1. Karameris A, Panagou P. Association of expression of metalloproteinases and their inhibitors with the metastasis potential of squamous cell using carcinoma. A molecular and immnohistochemical study. *Am. J. Resp. Crit. Care Med.* 1997; **156**: 1930–1936.
2. Chambers AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. *J. Natl. Cancer Inst.* 1997; **89**: 1260–1270.
3. Khokha R, Denhardt DT. Matrix metalloproteinases and tissue inhibitor of metalloproteinases: a review of their role in tumorigenesis and tissue invasion. *Invas. Metastasis* 1989; **9**: 391–405.
4. Hidalgo M, Eckhardt SG. Development of matrix metalloproteinase inhibitors in cancer therapy. *J. Natl Cancer Inst.* 2001; **93**: 178–193.
5. Stack MS, Gray RD. Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. *J. Biol. Chem.* 1989; **264**: 4277–4281.
6. Yarron A, Carmel A, Katchalski-Katzir E. Intramolecularly quenched fluorogenic substrates for hydrolytic enzymes. *Anal. Biochem.* 1979; **95**: 228–235.
7. Meldal M, Breddam K. Anthranilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases: multicolumn peptide synthesis of enzyme substrates for subtilisin carlsberg and pepsin. *Anal. Biochem.* 1991; **195**: 141–147.
8. Wang GT, Krafft GA. Automated synthesis of fluorogenic protease substrates: design of probes for Alzhemer's desease-associated proteases. *Bioorg. Biomed. Chem. Lett.* 1992; **2**: 1665–1668.
9. Hirata IY, Cezari MHS, Nakaie CR, Boschcov P, Ito AS, Juliano MA, Juliano L. Internally quenched fluoroge, ie protease substrates: solid-phase synthesis and fluorescence spectroscopy of peptides contaning ortho-amino benzoyl dinitrophenyl groups as donor-acceptor pairs. *Lett. Pept. Sci.* 1994; **1**: 299–308.
10. Lauer-Fields JL, Kele P, Sui G, Nagase H, Leblanc RM, Fields GB. Analysis of matrix metalloproteinase triple-helical peptidase activity with substrates incorporating fluorogenic L- or D-amino acids *Anal. Biochem.* 2003; **321**: 105–115.

11. Knight CG, Willenbrock F, Murphy G. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS* 1992; **296**: 263–266.
12. Nagase H, Fields CG, Fields GB. Design and characterization of a fluorogenic substrate selectively hydrolyzed by Stromelysin 1 (matrix metalloproteinase-3). *J. Biol. Chem.* 1994; **269**: 20 952–20 957.
13. Lauer-Fields JL, Broder T, Sritharan T, Chung L, Nagase H, Fields GB. Kinetic analysis of matrix metalloproteinase activity using fluorogenic triple-helical substrates *Biochemistry* 2001; **40**: 5795–5803.
14. Yan Z-H, Ren K-J, Wang Y, Chen S, Brock TA, Rege AA. Development of intramolecularly quenched fluorescent peptides as substrates of angiotensin-converting enzyme 2. *Anal. Biochem.* 2003; **312**: 141–147.
15. Matayoshi ED, Wang GT, Krafft GA, Erickson J. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* 1990; **247**: 954–958.
16. Maggiora LL, Smith CW, Zhang Z-Y. A general method for the preparation of internally quenched fluorogenic protease substrates using solid-phase peptide synthesis. *J. Med. Chem.* 1992; **35**: 3727–3730.
17. Gulnik SV, Suvorov LI, Majer P, Collins J, Kane BP, Johnson DG, Erickson JW. Design of sensitive fluorogenic substrates for human cathepsin D. *FEBS Lett.* 1997; **413**: 379–384.
18. Förster T. Intermolecular energy migration and fluorescence. *Anal. Phys.* 1948; **6**: 55–75.
19. Förster T. Action of light and organic crystals. *Modern Quantum Chemistry, Istanbul Lectures*, Part III, Academic Press: New York, 1965; 93–137.
20. Lakowicz JR. *Energy Transfer: Principles of Fluorescence Spectroscopy*. Kluwer Academic; Plenum Publishers: New York, 1999; 305–341.
21. Wei A-P, Blumenthal DK, Herron JN. Antibody-mediated fluorescence enhancement based on shifting the intramolecular dimer < — > monomer equilibrium of fluorescent dyes. *Anal. Chem.* 1994; **66**: 1500–1506.
22. Bennett FA, Barlow DJ, Dodoo ANO, Hider RC, Lansley AB, Lawrence MJ, Marriott C, Bansal SS. L-(6,7-dimethoxy-4-comaryl) alanine: an intrinsic probe for labelling of peptides. *Tetrahedron Lett.* 1997; **38**: 7449–7452.
23. Malkar NB, Fields GB. Synthesis of N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}[(7-methoxycoumarin-4-yl)acetyl]-L-lysine for use in solid-phase synthesis of fluorogenic substrates. *Lett. Pept. Sci.* 2001; **7**: 263–267.
24. Meienhofer J, Waki M, Heimer EP, Lambros TJ, Makofske RC, Chang CD. Solid phase synthesis without repetitive acidolysis. Preparation of leucyl-alanyl-glycyl-valine using 9-fluorenylmethoxycarbonyl amino acids *Int. J. Pept. Protein Res.* 1979; **13**: 35–42.
25. DeClerck YA, Yean TD, Lu HS, Ting J, Langley KE. Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-Z *J. Biol. Chem.* 1991; **266**: 3893–3899.
26. Luan XH, Cerqueira NMFSA, Oliveira AMAG, Raposo MMM, Coelho P, Oliveira-Campos AMF. Synthesis of fluorescent 3-benzoxazol-2-yl-coumarins. *Adv. Colour Sci. Technol.* 2002; **5**: 18–23.
27. Rodrigues LM, Muller CEP, Viriot ML, Carré MC. Protection and labelling of thymidine by a fluorescent photolabile group. *Helv. Chim. Acta* 2001; **84**: 3735–3741.
28. Corrie JET, Munasinghe VRN, Rettig W. Synthesis and fluorescence properties of substituted 7-aminocoumarin-3-carboxylate derivatives. *J. Heterocyclic Chem.* 2000; **37**: 1447–1455.
29. Takechi H, Oda Y, Nishizono N, Oda K, Machida M. Screening search for organic fluorophones: syntheses and fluorescence properties of 3-azolyl-7-diethylaminocoumarin derivatives *Chem. Pharm. Bull.* 2000; **48**: 1702–1710.
30. Bonsignore L, Cottiglia F, Lavagna SM, Loy G, Secci D. Synthesis of coumarin-3-*O*-acylisoureas by different carbodiimides. *Heterocycles* 1999; **50**: 469–478.
31. Takechi HK, Kamada S, Machida M. Fluorescent labelling reagents. 1. 3-[4-(bromomethyl)phenyl]-7-(diethylamino) 2*H*-l-benzopyran-2-one (MPAC-Br): a highly sensitive fluorescent derivatization reagent for carboxylic acids and high-performance liquid chromatography. *Chem. Pharm. Bull.* 1996; **44**: 793–799.
32. Ayyangar NR, Srinivasan KV, Daniel T. Polycyclic compounds. Part VI. Structural features of C:1. Disperse Yellow 232. *Dyes Pigments* 1990; **13**: 301–310.
33. Takadate AYI, Irikura M, Fujino H, Goya S. 3-(-methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolones as new fluorescent labelling reagents for high-performance liquid chromatography. *Chem. Pharm. Bull.* 1989; **37**: 373–376.
34. Schwander H. Coumarin dyestuffs their manufacture and use. *European Patent* 1973; 2 319 230.
35. Van Gompel J, Schuster GB. Chemiluminescence of organic peroxides: intramolecular electron-exchange liminescence from a secondary perester. *J. Org. Chem.* 1987; **52**: 1465–1468.
36. Song A, Wang X, Lam KT. A convenient synthesis of coumarin-3-carboxylic acids via Knoevenagel condensation of Meldrum's acid with *ortho*-hydroxyaryl aldehydes or ketones. *Tetrahedron Lett.* 2003; **44**: 1755–1758.
37. Atherton E, Sheppard RC. In *The Peptides. Analysis, Synthesis, Biology*, vol. 9. Underfriend S, Meienhofer J. (eds) Academic Press: New York, 1987; 1–38.
38. Nagase H, Fields GB. Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. *Biopolymers* 1996; **40**: 399–416.
39. Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*. Oxford University Press: 2000; 27.
40. Barrett AJ, Rawlings ND, Woessner JF (eds). *Handbook of Proteolytic Enzymes*. Academic Press: 1998; 1157.

