Bifunctional Coumarin Derivatives in Solution and Solid Phase Synthesis of Fluorogenic Enzyme Substrates

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The use of the fluorescent bifunctional compounds 7-amino-4-coumarinyl-acetic acid **1**, 7-hydroxy-4-coumarinyl-acetic acid **2** and ethyl 7-amino-4-coumarinyl-acetate **3** in solution and solid phase synthesis of fluorogenic enzyme substrates was examined. The intramolecularly quenched fluorogenic substrate *N*-(7-amino-4-coumarinyl-acetyl)-L-phenylalanyl-*p*-nitroanilide **5**, and the fluorogenic one ethyl 7-(glutaryl-L-phenylalanilamido)-4-coumarinyl-acetate **8**, both suitable for chymotrypsin and/or chymotrypsin like enzymes determination, were prepared in solution. The substrates 7-oleyloxy-4-coumarinyl-acetic acid **13** and 7-palmitoyloxy-4-coumarinyl-acetic acid **14**, suitable for the enzymatic study of lipases, were prepared by solid phase technique using 2-chloro-chlorotrityl-resin. The study of the fluorescence properties of the fluorophores **1, 2, 3,** and substrates **5, 8, 13, 14** showed that the examined bifunctional coumarin derivatives are suitable markers for solution and solid phase synthesis of fluorogenic enzyme substrates.

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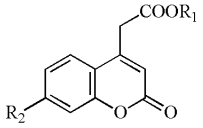
Introduction.

Coumarin derivatives continue to be an interesting area of research, because of their various and diverse properties. They exhibit important biological activities, such as antibiotic [1-3], anticoagulant [4-6], anticancer [7-11], antiinflammatory [12,13], *etc*. A considerable number of natural or synthetic derivatives have pharmaceutical application [14-16]. In the last years their strong inhibitory activity against several enzymes, such as integrase [17,18], monoaminooxidase (MAO) [19], human leucocyte elastase [12], and HIV protease [17,20-22] has been reported.

Coumarins, however, are also interesting because of their fluorescence properties and have been shown to be important in fluorimetry applications. Some very well known fluorescent markers, such as the 7-amino-4-methyl-coumarin (Amec) [23], have been widely used in synthetic substrates, fluorogenic or intramolecularly quenched fluorogenic ones. Both types of substrates have been successfully used for the sensitive determination of several proteases and the study of their active site and secondary specificity as well.

During the last ten years we have developed a number of fluorescent markers and fluorogenic substrates for various proteases [24,25]. We have also prepared platinum and copper complexes of some coumarins and tested them for anticancer activity [26,27]. Continuing our research in this area, we have examined the use of the compounds 7-amino-4-coumarinyl-acetic acid (Aca, **1**), 7-hydroxy-4-coumarinyl-acetic acid (Hca, **2**) and the ethyl ester of **1** (Aca-OEt, **3**) (Scheme 1) as markers in synthetic substrates suitable for fluorometric enzymatic analysis. These compounds were chosen because, in addition to their excellent fluorescence properties, they allow selective chain elongation through the two different functional groups they bear. They also can be used for the preparation of either classical or intramolecularly quenched fluorogenic substrates. Moreover, they can be connected through their carboxyl group to a solid support and serve as fluorescent linkers in the solid phase synthesis of substrates.

Scheme 1



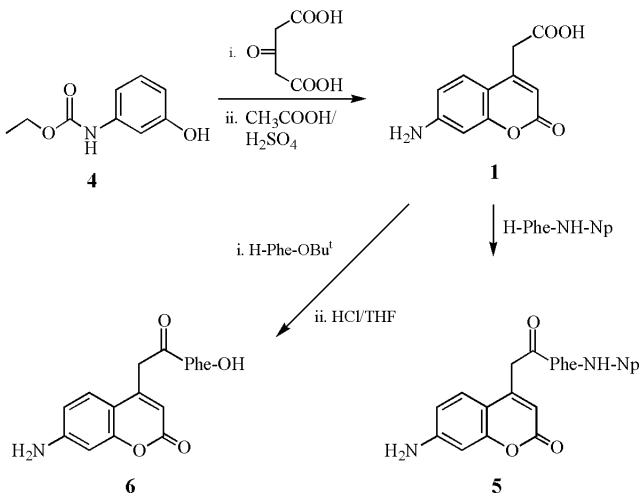
1 R_1 = H, R_2 = NH_2
2 R_1 = H, R_2 = OH
3 R_1 = Et, R_2 = NH_2

We report here the synthesis and the study of the fluorescence properties of the above markers **1**, **2**, **3** and their derivatives *N*-(7-amino-4-coumarinyl-acetyl)-L-phenylalanyl-*p*-nitroanilide (Aca-Phe-NH-Np, **5**), *N*-(7-amino-4-coumarinyl-acetyl)-L-phenylalanine (Aca-Phe-OH, **6**), ethyl 7-(glutaryl-L-phenylalanilamido)-4-coumarinyl-acetate (Glt-Phe-NH-ca-OEt, **8**), 7-oleyloxy-4-coumarinyl-acetic acid (Ole-Oca, **13**) and 7-palmitoyloxy-4-coumarinyl-acetic acid (Pal-Oca, **14**). The last two compounds were prepared by solid phase synthesis technique.

Results and Discussion.

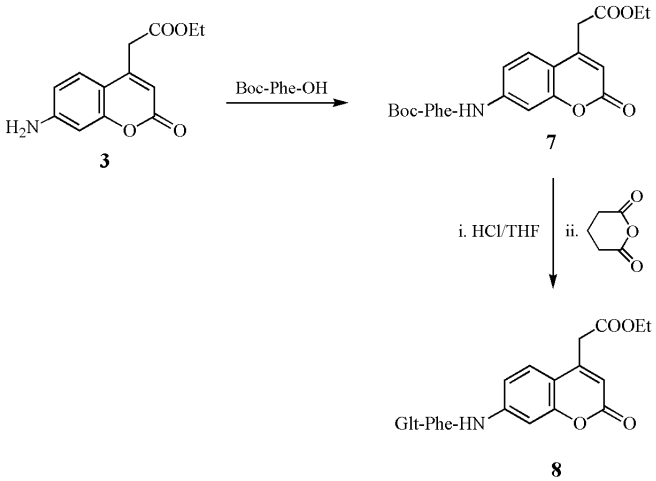
The synthesis of Aca (**1**) and the intramolecularly quenched fluorogenic substrate **5** is outlined in Scheme 2. The preparation of **1** was achieved by the condensation of carbethoxyaminophenol (**4**) [28] with acetone dicarboxylic acid and acidolysis of the intermediate derivative. Compound **1** was converted to its ethyl ester by treatment with thionylchloride in anhydrous ethanol. The substrate **5** was prepared by the condensation of Aca with phenylalanyl-*p*-nitroanilide (H-Phe-NH-Np) [29] using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCI) as the coupling agent in the presence of 1-hydroxy-benzotriazole (HOBt) [30] with a 65% yield. The *p*-nitroanilide group, widely used in chromogenic substrates, was chosen as the quencher. The same coupling method was applied for the preparation of Aca-Phe-OBu^t, acidolysis of which afforded the compound Aca-Phe-OH (**6**) (Scheme 2). This compound was synthesized for comparison reasons. Both couplings took place without previous

Scheme 2



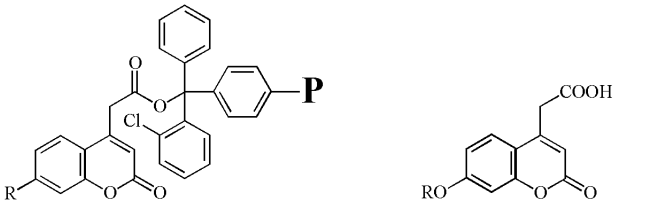
protection of the 7-amino group of Aca, due to its low nucleophilicity, as is also mentioned in the literature [31]. Indeed, efforts to protect the 7-amino group with *t*-butyloxycarbonyl (Boc) or benzyloxycarbonyl (Z) group resulted in very low yields, while reaction with 9-fluorenyl-methyloxycarbonyl chloride (Fmoc-Cl) produced the dimer Fmoc-NH-ca-NH-ca. Finally, Fmoc-NH-ca was obtained by acidolysis of Fmoc-Aca-OEt, prepared from Aca-OEt (**3**) by treatment with Fmoc-Cl. It seems that esterification of the carboxyl group increases the nucleophilicity of the aromatic 7-amino group, permitting its acylation in solution. Thus, for the synthesis of the fluorogenic substrate Glt-Phe-NH-ca-OEt (**8**), outlined in Scheme 3, compound **3** was coupled with Boc-Phe-OH by the mixed anhydride method [32], to produce compound **7**. After removal of the Boc group, the free intermediate reacted with glutaric anhydride to give the substrate in good yield. Both substrates, **5** and **8**, are suitable for the fluorometric determination of chymotrypsin or chymotrypsin-like enzymes. The substrate **5**, which is a *p*-nitroanilide derivative, can be used in colorimetry as well.

Scheme 3



In order to examine the use of the bifunctional compounds **1** and **2** in the solid phase synthesis of fluorogenic substrates, they were successfully linked through their carboxyl group with 2-chloro-chlorotrityl-resin [33] to produce the resin derivatives **9** and **10** (Scheme 4). However, efforts for acylation of the amino group of **9** with Fmoc-protected amino acids, using several coupling methods, resulted in very low yields. On the contrary the resin derivative **10** was successfully acylated with oleic or palmitic acid using dicyclohexylcarbodiimide (DCC) as a coupling agent in the presence of a catalytic amount of dimethylaminopyridine (DMAP) [34]. The substrates Ole-Oca (**13**) and Pal-Oca (**14**) (Scheme 4), suitable for the enzymatic study of lipases, were obtained after cleavage from the resin with a mixture of trifluoroethanol (TFE), dichloromethane (DCM), acetic acid (AcOH) (TFE:DCM:AcOH 2:7:1).

Scheme 4



9 R = NH_2
10 R = OH
11 R = $OCO(CH_2)_7CH{=}CH(CH_2)_7CH_3$
12 R = $OCO(CH_2)_{14}CH_3$

13 R = $CO(CH_2)_7CH{=}CH(CH_2)_7(CH_3)$
14 R = $CO(CH_2)_{14}CH_3$

The fluorescence properties of the intramolecularly quenched fluorogenic substrate **5** and the fluorescent compound **6** are shown in Table 1, while their fluorescence spectra are shown in Figure 1. Efficient quenching (88.9%) of fluorescence was observed at the excitation and emission maxima of compound **6**.

Table 1

Fluorescence Properties of Compounds **6** and **5**

Compound [a]	λ_{ex}/nm	λ_{em}/nm	F/a.u.[b]	q.e.[c] %
6	364	458	1674	
5	364	458	186	88.9

[a] 1 μ*M* solution in 0.05 *M* Tris-HCl pH 7.8, containing 1% DMSO. [b] Arbitrary units. [c] q.e. = $(F_0\text{-}F)/F_0$; F_0 and F are the fluorescence intensities of the donor in the absence and presence of the acceptor.

Table 2 lists the fluorescence properties of markers **2** and **3** and substrates **8**, **13** and **14**. The emission maxima and fluorescence intensities of compounds **3** and **8** are distinctly different (Figure 2). Their fluorescence intensities at various λ_{ex}

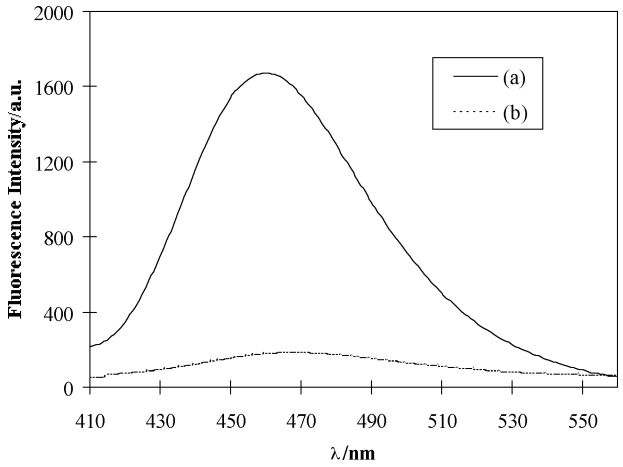


Figure 1. Fluorescence spectra of (a) Aca-Phe-OH **(6)**, (b) Aca-Phe-NH-Np **(5)**; λ_{ex}=364 nm. 1 μ*M* solutions in 0.05 *M* Tris-HCl, pH 7.8, containing 1% DMSO.

Table 2

Fluorescence Properties of Compounds **2**, **3, 8, 13, 14**

Compound [a]	λ_{ex}/nm	λ_{em}/nm	F/a.u.[b]
3	366	460	665
8	341	408	453
2	350	453	360
13	368	458	15
14	368	458	13

[a] 1 μ*M* solution in 0.05 *M* Tris-HCl pH 7.8, containing 1% DMSO. [b] Arbitrary units.

and λ_{em} were determined. At chosen λ_{ex}=380 nm and λ_{em}=490 nm the fluorescence intensity of the marker **3** was approximately 350-fold greater than that of an equimolar solution of the substrate **8**, while retaining 40% of its maximum fluorescence. At these wavelengths the presence of substrate **8** does not interfere with the fluorescence of marker **3**. As shown in Table 2 compound **2** has λ_{em}-max=453 nm at λ_{ex}max=350 nm, while compounds **13** and **14** have λ_{em}max=458 nm at λ_{ex}max=368 nm (Figure 3). The fluorescence intensity of marker **2** at its maxima wavelengths was 24-fold and 28-fold greater than that of equimolar solutions of substrates **13** and **14**, respectively.

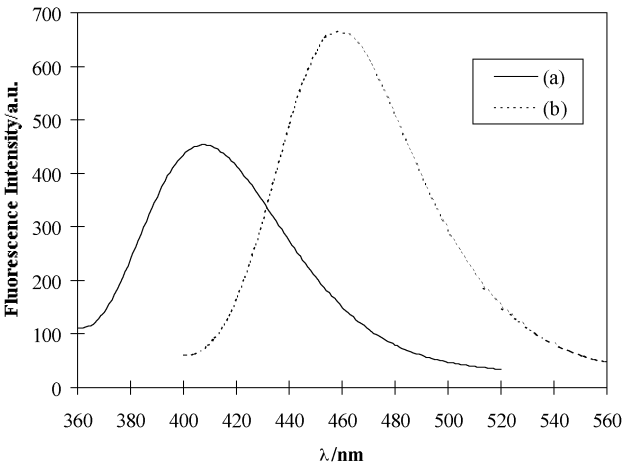


Figure 2. Fluorescence spectra of (a) Glt-Phe-NH-ca-OEt (**8**); λ_{ex}=341 nm, (b) Aca-OEt (**3**); λ_{ex}=366 nm. 1 μ*M* solutions in 0.05 *M* Tris-HCl, pH 7.8, containing 1% DMSO.

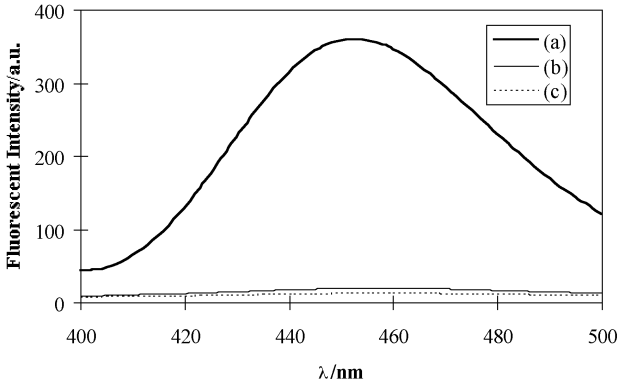


Figure 3. Fluorescence spectra of (a) Hca (**2**); λ_{ex}=350 nm, (b) Ole-Oca (**13**), (c) Pal-Oca (**14**); λ_{ex}=368 nm. 1 μ*M* solutions in 0.05 *M* Tris-HCl, pH 7.8, containing 1% DMSO.

From the above results it is concluded that the proposed compounds **1** and **3** are suitable markers in the solution synthesis of fluorogenic or intramolecularly quenched fluorogenic substrates for proteases, whereas compound **2** can be used for the development of fluorogenic enzyme substrates on a solid support. It should also be mentioned that marker **1** can be used for the conversion of peptide nitroanilide substrates into intramolecularly quenched fluorogenic ones, which by retaining their chromogenic properties, may be applied in both colorimetric and fluorometric assays.

EXPERIMENTAL

Melting points are all uncorrected and were determined in capillary tubes using Buchi apparatus. Thin layer chromatography was performed on precoated plates of silica gel 60 F_{254} (Merck, Darmstand, Germany) using the following solvent systems: chloroform/methanol (5:1); chloroform/methanol (9:1); chloroform/methanol (95:5); chloroform/methanol/acetic acid (3:1:1); chloroform/methanol/acetic acid (90:10:0.3); chloroform/methanol/acetic acid (50:10:0.3); 1-butanol/acetic acid/water (4:1:1). The crude products were purified using column chromatography on silica gel 60 (70-230 mesh, Merck). Elemental analyses were carried out on a Perkin-Elmer CHN 2400 instrument. Fast Atom Bombardment (FAB) mass spectra were obtained on a VG Analytical ZAB-SE instrument using positive ion mode and *m*-nitrobenzyl alchohol as matrix. The ^{1}H nmr spectra were recorded on a Varian 200 spectrometer in dimethyl sulfoxide-d_6. The solvent signal at 2.54 ppm was used as reference.

The fluorescence spectra were recorded on a Perkin-Elmer 512 fluorescence spectrophotometer. Phenylalanine was of L-configuration and purchased from Fluka and 7-hydroxy-4-coumarinyl-acetic acid (Hca) from Aldrich.

7-Amino-4-coumarinyl-acetic Acid (**1**).

In a procedure similar to this of Benson *et al.* [31] a solution of citric acid (10.51 g, 50.05 mmol) in concentrated sulfuric acid (2.32 ml) was stirred at room temperature for one hour and at 70 °C for thirty minutes. The solution then was cooled to 0 °C and 3-carbethoxyaminophenol (**4**) (7.24 g, 40 mmol) and concentrated sulfuric acid (5.61 ml) were added in three equal portions. The mixture was stirred at 0 °C for one hour and at room temperature overnight. The reaction mixture was poured into ice (100 g) and the resulted solid was filtered and washed several times with water. The crude product, 7-carbethoxyamino-4-coumarinyl-acetic acid, was triturated with 50 ml of 1 *N* sodium carbonate solution and the resulting mixture was filtered. The filtrate was acidified with concentrated hydrochloric acid. The resulting white precipitate was collected by filtration and recrystallized from methanol. The obtained pure 7-carbethoxyamino-4-coumarinyl-acetic acid (2.86 g, 9.8 mmol) was dissolved in a mixture of glacial acetic acid (8.43 ml) and concentrated sulfuric acid (4.80 ml). The mixture was heated at 100 °C for one hour and then at 120 °C for four hours. The mixture was allowed to cool to room temperature and then water (40 ml) was added, followed by a 25% solution of ammonium hydroxide until pH 2. The yellow precipitate was filtered and dissolved in 5% sodium carbonate solution. The solution was extracted with chloroform and the aqueous phase was acidified with concentrated hydrochloric acid until pH 2. The resulting yellow solid was filtered. The crude product was purified by recrystallization from methanol, yield 0.94 g (44%), mp 225-227 °C; ^{1}H nmr: δ 3.8 (s, 2H, CH_2), 6.1 (s, 1H, H_3), 6.6 (d, $J_{8,6}$ = 1 Hz, 1H, H_8), 6.7 (dd, $J_{6,5}$ = 9 Hz, J = 2 Hz, 1H, H_6), 7.4 (d, J = 9 Hz, 1H, H_5); ms: m/z 242(M^++23), 220(M^++1).

Anal. Calcd. for $C_{11}H_9NO_4 \cdot 0.5H_2O$: C, 57.89; H, 4.42; N, 6.14. Found: C, 58.09; H, 4.26; N, 6.08.

Ethyl 7-Amino-4-coumarinyl-acetate (**3**), Hydrochloric Salt.

Thionylchloride (0.08 ml, 1.10 mmol) was added dropwise in absolute ethanol (0.5 ml) at -5 °C, under stirring. After the addition was completed, 7-amino-4-coumarinyl-acetic acid (**1**) (0.22 g, 1 mmol) was added. The reaction mixture was kept under stirring at 35-40 °C for two hours and then evaporated *in vacuo*. The residue was triturated with absolute ether and the resulting solid was filtered and purified by recrystallization from methanol/absolute ether, yield 0.20 g (71%); ^{1}H nmr: δ 1.3 (t, J = 7 Hz, 3H, CH_3), 4.0 (s, 2H, CH_2CO), 4.2 (q, J = 7 Hz, 2H, CH_3CH_2), 6.4 (s, 1H, H_3), 7.3 (m, 2H, H_6, H_8), 7.8 (d, $J_{5,6}$ = 9 Hz, 1H, H_5); ms: m/z 248(M^++1).

Anal. Calcd. for $C_{13}H_{13}NO_4 \cdot HCl \cdot 0.5H_2O$: C, 53.34; H, 4.82; N, 4.78. Found: C, 53.34; H, 5.00; N, 4.70.

N-(7-Amino-4-coumarinyl-acetyl)-L-phenylalanyl-*p*-nitroanilide (**5**).

To a stirred and cooled at 0 °C solution of phenylalanyl-*p*-nitroanilide in *N*,*N*-dimethylformamide (5 ml), 7-amino-4-coumarinyl-acetic acid (0.219 g, 1 mmol), triethylamine (0.15 ml, 1.1 mmol), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.21 g, 1.1 mmol) and 1-hydroxybenzotriazole aqueous (0.17 g, 1 mmol) were added consecutively. The reaction mixture was stirred for one hour at 0 °C and at room temperature overnight. After evaporation *in vacuo* the residue was dissolved in ethyl acetate and washed consecutively with water, a solution of 1 *M* potassium bicarbonate, brine, a solution of 5% sodium bicarbonate and brine. The organic phase was dried with sodium sulfate and evaporated *in vacuo* to yield a crude solid which was triturated with ether twice, yield 0.08 g (63%); ^{1}H nmr: δ 2.9 (dd, J_{gem} = 13 Hz, J = 14 Hz, 1H, C*H*HC$_6$H$_5$), 3.1 (dd, J_{gem} = 13 Hz, J = 5 Hz, 1H, CH*H*C$_6$H$_5$), 3.6 (s, 2H, CH_2), 4.7 (m, 1H, CH), 5.9 (s, 1H, H_3), 6.1 (s, 2H, NH_2), 6.4 (d, $J_{8,6}$ = 2 Hz, 1H, H_8), 6.5 (dd, $J_{6,5}$ = 9 Hz, J = 2 Hz, 1H, H_6), 7.3 (m, 6H, C_6H_5, H_5), 7.8 (d, J = 10 Hz, 2H, H_2, H_6 Np), 8.2 (d, J = 10 Hz, 2H, H_3, H_5 Np), 8.8 (d, J = 8 Hz, 1H, NH), 10.8 (s, 1H, NH); ms: m/z 509(M^++23), 487(M^++1), 349, 321, 202.

Anal. Calcd. for $C_{26}H_{22}N_4O_6 \cdot 0.25H_2O$: C, 63.60; H, 4.62; N, 11.41. Found: C, 63.72; H, 4.65; N, 11.21.

7-Amino-4-coumarinyl-acetyl-2-chlorotrityl-resin Ester (**9**).

2-Chloro-chlorotrityl-resin (1 g) was triturated with 2 ml of dichloromethane for 5 minutes. Then, diisopropylethylamine (0.69 ml, 4 mmol) was added followed by the addition of a solution of 7-amino-4-coumarinyl-acetic acid (0.263 g, 1.2 mmol) in *N*,*N*-dimethylformamide (3 ml). The mixture was stirred for thirty minutes and then was filtered. A solution of dichloromethane: methanol:diisopropylethylamine 85:10:5 was added to the resin and the mixture was stirred for ten minutes. The mixture was then filtered and the resin was washed first three times with the previous solution and then with *N*,*N*-dimethylformamide, 2-propanol and ether (four times each). Finally the resin was dried *in vacuo* over phosphorous pentoxide.

7-Hydroxy-4-coumarinyl-acetyl-2-chlorotrityl-resin Ester (**10**).

Resin derivative **10** was prepared in a similar manner to that described for the preparation of **9** starting with 7-hydroxy-4-coumarinyl-acetic acid (0.264 g, 1.2 mmol).

7-Oleyloxy-4-coumarinyl-acetic Acid (**13**).

Resin derivative **10** (0.12 g, equivalent to 0.12 mmol of Hca) was triturated with *N*,*N*-dimethylformamide (0.4 ml) for 5 minutes. Then, dimethylaminopyridine (2.5 mg, 0.024 mmol) and a solution of oleic acid (0.102 g, 0.36 mmol) and dicyclohexylcarbodiimide (0.082 g, 0.4 mmol) in *N*,*N*-dimethylformamide (0.76 ml) were added. The reaction mixture was stirred for three hours. The esterification was repeated one more time using oleic acid (0.034g, 0.12 mmol). The mixture was filtered and the resin was washed with *N*,*N*-dimethylformamide, 2-propanol and ether (four times each) and dried *in vacuo* over phosphorous pentoxide. A solution of trifluoroethanol:dichloromethane:acetic acid 2:7:1 (10 ml) was added to the dry resin and the mixture was stirred for one hour. The reaction mixture was filtered and the resin was washed with dichloromethane. The filtrate was evaporated *in vacuo* to give the crude product, which was purified by column chromatography using chloroform:methanol (9:1) containing 4 drops of acetic acid as the eluent, yield 0.025 g (45%); ^{1}H nmr: δ 0.8 (t, J = 7 Hz, 3H, CH_3), 1.3 (m, 20H, $CH_3(CH_2)_6$, $(CH_2)_4CH_2CH_2CO$), 1.7 (m, 2H, CH_2CH_2CO), 2.0 (m, 4H, $CH_2CH{=}CH{=}CH_2$), 2.6 (m, 2H, CH_2CH_2CO), 5.3 (m, 2H, CH=CH), 6.4 (s, 1H, H_3), 7.2 (dd, $J_{6,5}$ = 9 Hz, $J_{6,8}$ = 2 Hz, 1H, H_6), 7.3 (d, J = 2 Hz, 1H, H_8), 7.9 (d, J = 9 Hz, 1H, H_5); ms: m/z 508(M^++23), 486(M^++1), 266, 221.

Anal. Calcd. for $C_{29}H_{40}O_6 \cdot 0.25H_2O$: C, 71.21; H, 8.34. Found: C, 71.37; H, 8.42.

7-Palmitoyloxy-4-coumarinyl-acetic Acid (**14**).

Compound **14** was prepared and purified in a similar manner to that described for the preparation of **13** starting with resin derivative **10** (0.12 g) and palmitic acid (0.092 g, 0.36 mmol), yield 0.025 g (47%); ^{1}H nmr: δ 0.8 (t, J = 7 Hz, 3H, CH_3), 1.2 (m, 24H, $CH_3(CH_2)_{12}CH_2$), 1.6 (m, 2H, CH_2CH_2CO), 2.6 (m, 2H,CH_2CH_2CO), 6.4 (s, 1H, H_3), 7.2 (dd, $J_{6,5}$ = 9 Hz, $J_{6,8}$ = 2 Hz, 1H, H_6), 7.3 (d, J = 2 Hz, 1H, H_8), 7.9 (d, J = 9 Hz, 1H, H^5); ms: m/z 482(M^++23), 460(M^++1), 239, 221.

Anal. Calcd. for $C_{27}H_{38}O_6 \cdot 0.25H_2O$: C, 69.95; H, 8.37. Found: C, 70.06; H, 8.42.

Ethyl *N*-(7-*tert*-butyloxycarbonyl-L-phenylalanylamido)-4-coumarinyl-acetate (**7**).

A solution of *tert*-butyloxycarbonyl-phenylalanine (0.08 g, 0.3 mmol) in tetrahydrofuran (1.5 ml) was cooled to -10 °C and *N*-methylmorpholine (0.033 m1, 0.3 mmol) was added. Subsequently isobutyl chloroformate (0.039 ml, 0.3 mmol) was added dropwise. After stirring for 5 minutes at -10 °C, an ice cooled solution of **3** (0.07 g , 0.3 mmol) in tetrahydrofuran (2.5 ml) was added dropwise, so that the temperature did not exceed -5 °C. The reaction mixture was stirred at -10 °C for one hour and at room temperature overnight. After evaporation of the solvent, the residue was dissolved in ethyl acetate and washed consecutively with water, a 0.5 *N* solution of hydrochloric acid, water, a 5% solution of sodium bicarbonate, water, brine and dried with sodium sulfate. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography using petroleum ether (40-60 °C):ethyl acetate 1:1 as the eluent.

Ethyl 7-(glutaryl-L-phenylalanilamido)-4-coumarinyl-acetate (**8**).

The *N*-protected compound **7** (0.032 g, 0.064 mmol) was treated with a solution of 4 *N* hydrochloric acid in tetrahydrofuran (1.06 ml, 4.25 mmol) for one hour. The reaction mixture was evaporated *in vacuo* and the residue was triturated with ether to give a white solid, that was filtered and dried *in vacuo*. This solid was dissolved in ethyl acetate and washed three times with a 5% solution of sodium bicarbonate. The organic phase was evaporated until a small volume remained (approximately 2 ml). Then, glutaric anhydride (0.011g, 0.096 mmol) was added and the mixture was heated at 45 °C for two hours. The reaction mixture was allowed to cool to room temperature and then cooled to 0 °C. The precipitated solid was filtered and washed with cool ethyl acetate, yield 0.022 g (66%); ^{1}H nmr : δ 1.2 (t, J = 7 Hz, 3H, CH_3), 1.6 (m, 2H, $CH_2CH_2CH_2$), 1.9-2.2 (m, 4H, $CH_2CH_2CH_2$), 2.8 (dd, J_{gem} = 13 Hz, J = 14 Hz, 1H, CHH), 3.0 (dd, J_{gem} = 13 Hz, J = 5 Hz, 1H, CH*H*), 3.9 (s, 2H, CH_2CO), 4.1 (q, J = 7 Hz, 2H, CH_2O), 4.6 (m, 1H, CH), 6.4 (s, 1H, H_3), 7.2 (m, 5H, C_6H_5), 7.4 (dd, $J_{6,5}$ = 9 Hz, $J_{6,8}$ = 2 Hz, 1H, H_6), 7.6 (d, J = 9 Hz, 1H, H_5), 7.8 (d, J = 2 Hz, 1H, H_8), 8.4 (d, J = 8 Hz, 1H, NH), 10.6 (s, 1H, NH); ms: m/z 531(M^++23), 509(M^++1), 395, 377, 248.

Anal. Calcd. for $C_{27}H_{28}N_2O_8 \cdot 0.5H_2O$: C, 62.66; H, 5.64; N, 5.41. Found: C, 62.94; H, 5.72; N, 5.31.

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