Synthesis and Chemistry of 7-Amino-4-(trifluoromethyl)coumarin and Its Amino Acid and Peptide Derivatives[1]

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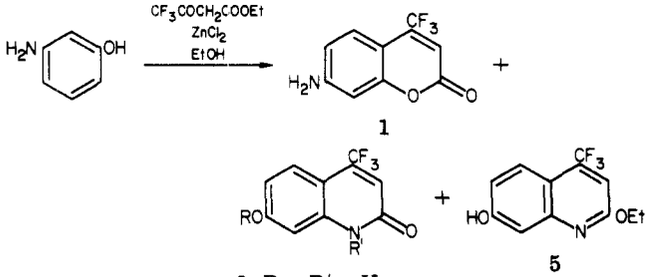
The synthesis and purification of 7-amino-4-(trifluoromethyl)courmarin, a novel fluorescent marker for the sensitive detection of proteinases, were investigated. Two byproducts, 7-hydroxy-4-(trifluoromethyl-2-quinolone and 2-ethoxy-7-hydroxy-4-(trifluoromethyl)quinoline, were isolated and identified. 7-Methoxy-4-(trifluoromethyl)-2-quinolone was also prepared. Amino acid and peptide derivatives prepared by solution methods using the stepwise approach included 7-(L-leucinamido)-, 7-(D-alanyl-L-leucyl-L-lysinamido)-, 7-(D-valyl-L-leucyl-L-lysinamido)-, and 7-(N^{α}-Z-glycylglycyl-L-argininamido)-4-(trifluoromethyl)coumarins.

The use of synthetic peptides as substrates for proteinases is well-known through the work of Bergmann and Fruton.[2] An increased awareness of the importance of proteinases in biological control and as possible indicators of various disease states, including neoplasia,[3] leads to increased interest in such substrates. We report here our work on the synthesis of novel peptide derivatives designed to be substrates for proteinases in single cells as well as in body fluids.[4,5]

In our search for improved fluorescent markers for these substrates we found that *N*-acyl derivatives of certain 7-aminocoumarins can serve admirably.[5,6] This article describes the synthesis and characterization of 7-amino-4-(trifluoromethyl)coumarin (1), the isolation and identification of two byproducts, and the preparation of amino acid and peptide derivatives of 1.

Synthesis of 1 from 3-aminophenol by the von Pechmann reaction was first reported in 1977,[7] but it was not characterized. It was claimed[8] that the quinolone byproduct (2) normally produced by this reaction[9] could be avoided if the reaction was carried out in refluxing ethanol in the presence of anhydrous zinc chloride. In our hands, however, not only 1 and 2 but also a third condensation product (5) were produced under all conditions tried (see Table I).



2, R = R′ = H
3, R = H; R′ = CH_3
4, R = CH_3; R′ = H

Table I. Preparation of 7-Amino-4-(Trifluoromethyl)coumarin (1)[a]

$ZnCl_2$[b]	solvent[c]	reflux time, h	total yield, %[d]	analysis,[e] % 1	2	5
1.24[f]	abs EtOH (0.6)	12	75.1	58.6	20.3	12.8
1.24[g]	abs EtOH (0.6)	12	74.7	45.8	23.8	17.5
1.24	abs EtOH (0.6)	20	82.8	60.3	20.3	12.3
1.24[g,h]	abs EtOH (0.6)	22		50.0	25.8	17.6
1.50	abs EtOH (0.6)	12	77.5	72.4	14.7	10.0
1.71	abs EtOH (0.6)	12	78.7	65.9	16.3	10.3
1.00	abs EtOH (0.6)	12	67.3	52.6	19.9	17.4
1.24	abs EtOH (0.6)	12[i]	74.0	68.6	13.9	10.0
1.24[j]	abs EtOH (0.6)	12	75.9	61.5	16.2	10.8
1.24	abs EtOH (1.2)	12	43.4	62.8	16.5	14.3
1.24	95% EtOH (0.6)	12	75.1	59.3	21.4	13.8
1.24[k]	95% EtOH (1.2)	12	61.1	58.4	23.3	11.2
1.24	MeOH (0.6)	20	51.3	61.4	14.8	14.8
1.24	*i*-PrOH (0.6)	12	80.3	60.4	21.5	8.7
1.24	THF (0.6)	12	68.2	60.3	19.6	11.1

[a] 50 mmol of 3-aminophenol except as noted; the molar ratio of ethyl trifluoroacetoacetate to 3-aminophenol was 1.05. [b] mmol per mmol of 3-aminophenol. [c] mL per mmol of 3-aminophenol. [d] Calculated as 1. [e] High-performance LC, Waters Associates μ-Bondapak C_{18} column (0.4 × 30 cm), acetonitrile/0.01 N aqueous HCl (1:1) at 2 mL/min, UV detection at 340 nm; retention times: 1, 3.4 ± 0.1 min; 2, 2.08 ± 0.04 min; 5, 7.3 ± 0.3 min. [f] 10 mmol of 3-aminophenol. [g] 500 mmol of 3-aminophenol. [h] Dried overnight at 60 °C (3 Pa). Another experiment in which the $ZnCl_2$ was dried by fusing and cooling under vacuum and the EtOH was distilled from sodium gave similar results. [i] Light excluded. [j] I_2 (0.05 mmol/mmol of 3-aminophenol) was added. [k] The ethyl trifluoroacetoacetate was added last, after refluxing started.

2 was identified from its elemental analysis, NMR spectrum, characteristic blue fluorescence, and solubility

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in alkali, and **5** was identified from its elemental analysis, NMR spectrum, red fluorescence, and its solubility in alkali plus its behavior on methylation with trimethyl phosphate.

Treatment of **5** with trimethyl phosphate under conditions suitable for methylation of aromatic amines[10] produced a new alkali-soluble compound analyzed as $C_{11}H_8F_3NO_2$; but **2** was recovered unchanged under these conditions. Two structural assignments, **3** and **4**, were consistent with the analysis of the new compound and its NMR and IR spectra. **4** was eliminated as the correct assignment by synthesizing it independently from 3-methoxyaniline and ethyl trifluoroacetoacetate and showing that its chemical and spectral properties were different from those of the methylation product, which must therefore be **3**.

Various amine-protecting groups were investigated in the synthesis of **1** in hopes of eliminating **2** and **5** as byproducts. Acetyl and phthalyl groups prevented the von Pechmann reaction, using $ZnCl_2$ catalysis. 3-Acetamidophenol (**6**) yielded 4% of crude material that on high-performance LC analyzed as 62% **1**, 11% **2**, and 7% **5**; no acylated coumarins were formed. 3-Phthalimidophenol (**7**) was recovered 99% unchanged. Urethane-blocking groups gave varying results, depending on the nature of the alkyl group. 3-Hydroxycarbanilic acid isobutyl ester (**8**) yielded 77% of a crude product that contained 53% **1** and 23% **2**; no **5** or urethane derivatives of **1** were found. 3-Hydroxycarbanilic acid benzyl ester (**9**), prepared by treating an ether solution of 3-aminophenol with 0.5 equiv of carbobenzoxy chloride, under similar conditions gave some unreacted starting material plus very small amounts of **1**, **2**, and **5**; again, no urethane derivatives were found. However, 3-hydroxycarbanilic acid ethyl ester (**10**)[8] did give the expected urethane of **1** free of either **2** or **5**, but the yield (37%) was not high enough to use this reaction sequence as an alternate route to **1**. With **10** the von Pechmann reaction could also be carried out with 70% sulfuric acid (the condensing agent of choice for the preparation of 7-amino-4-methylcourmarin).[9,11] The yield was higher, but the purity was considerably lower than with zinc chloride.

The spectral properties of **1** enable it to be detected either colorimetrically or fluorometrically. Thus, **1** with λ_{max} 365 nm can be employed as 4-nitroaniline is in proteinase assays.[12] More importantly, small amounts of **1** (<0.1%) can be determined fluorometrically in the presence of large amounts of *N*-acyl derivatives of **1** because the former fluoresces in the yellow–green region (λ_{max} 495 nm) whereas the latter fluoresces in the blue region (λ_{max} 430 nm).[5] These spectral properties also facilitate the purification of derivatives of **1** because the products, as well as most byproducts, fluoresce under long-wavelength ultraviolet light (366 nm); this allows us to detect very small quantities on thin-layer chromatograms and chromatographic columns.

Another remarkable feature of **1** is its complete stability under conditions of acid hydrolysis (6 N HCl, 2 h, 140 °C) that quantitatively cleave the amide bonds of peptides;[13] thus **1** may be determined fluorometrically in the same acid hydrolysate used for amino acid analysis of its peptide derivatives.

Amino acid and peptide derivatives of **1** were prepared in solution by conventional techniques for peptide synthesis.[14] Optically active amino acids were coupled as urethane-protected derivatives, using the stepwise strategy of Bodanszky.[15] Many derivatives were not crystalline and required repeated purification by preparative layer chromatography (PLC) and/or high-performance LC. The mixed anhydride method[16] gave amino acid derivatives in modest yields, with lower yields realized from water-soluble carbodiimide/hydroxybenzotriazole[17] or EEDQ.[18]

The mixed anhydride of Boc-L-leucine was reacted with **1** to yield 7-(N^{α}-Boc-L-leucinamido)-4-(trifluoromethyl)-coumarin (**11**) which was treated with hydrogen bromide in acetic acid to yield 7-(L-leucinamido)-4-(trifluoromethyl)coumarin hydrobromide (**12**).

7-(D-Alanyl-L-leucyl-L-lysinamido)-4-(trifluoromethyl)-coumarin dihydrobromide (**13**) was prepared stepwise as follows. The mixed anhydride of N^{α}-Boc-N^{ϵ}-Z-L-lysine was reacted with **1** to give 7-(N^{α}-Boc-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (**14**). The Boc group was removed with trifluoroacetic acid, and the salt was acylated with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide/1-hydroxybenzotriazole[17] to give 7-(N^{α}-Boc-L-leucyl-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (**15**). Deprotection and coupling with Z-D-alanine gave 7-(N^{α}-Z-D-alanyl-L-leucyl-N^{ϵ}-L-lysinamido)-4-(trifluoromethyl)coumarin (**16**), which was treated with hydrogen bromide in acetic acid to yield **13**. 7-(D-Valyl-L-leucyl-L-lysinamido)-4-(trifluoromethyl)coumarin (**17**) was prepared similarly. The alternate approach to these compounds via 7-(N^{α}-Z-N^{ϵ}-Boc-L-lysinamido)-4-(trifluoromethyl)coumarin (**18**), using hydrogenation to remove Z groups, was found to be less satisfactory.

7-(N^{α}-Z-L-Argininamido)-4-(trifluoromethyl)coumarin hydrochloride (**19**) was obtained by acylation of **1** with N^{α}-Z-L-arginine using the mixed anhydride method.[16] Acylation of **1** with N^{α}-Z-L-arginine hydrobromide[19] by the mixed anhydride method, by water-soluble carbodiimide/1-hydroxybenzotriazole,[17] or by EEDQ[18] gave the hydrobromide of **19**. Removal of the Z group by catalytic hydrogenation or by treatment with hydrogen bromide in acetic acid and acylation with Z-glycylglycine using the mixed anhydride or *p*-nitrophenyl ester method gave 7-(N^{α}-Z-glycylglycyl-L-argininamido)-4-(trifluoromethyl)-coumarin (**20**).

Compounds **12**, **13**, **17**, and **20** have been successfully used as proteinase substrates in preliminary studies.[5] The preparation of more complex peptide derivatives of **1** is in progress, and early studies of *N*-acyl derivatives of other aminocoumarins indicate that they too may be useful as substrates for proteinases.

Experimental Section

General Methods. Melting points, taken on a Mettler Model FP1 apparatus at a heating rate of 2 °C/min, are corrected; boiling points are uncorrected. Rotations were determined with a Rudolph Model 52 polarimeter. NMR spectra, in Me_2SO-d_6 solvent except as noted, were taken on a Varian EM-360 spectrometer; shifts (δ) are given in parts per million relative to internal Me_4Si;

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s = singlet, d = doublet, t = triplet, q = quartet, m = complex multipet; coupling constants (J) are given in hertz. Infrared absorption spectra (in cm^{-1}; KBr pellets) were taken on a Perkin-Elmer Model 457 or Model 597 spectrometer. Elemental analyses were performed by Midwest Microlab Ltd., Indianapolis, IN. Amino acid determinations were obtained with a Beckman 120 B amino acid analyzer after hydrolysis of the peptides in 6 N aqueous hydrochloric acid at elevated temperature (140 °C) for 2–3 h.[13] Two types of analytical thin-layer chromatography (TLC) plates were used: (A) 0.25-mm silica gel GF precoated on glass (Merck, Inc.) and (B) 0.2-mm silica gel N-HR/UV_{254} precoated on plastic sheets (Macherey-Nagel and Co.). Solvent systems used were the following: (I) chloroform/acetic acid 8:2; (II) chloroform/acetic acid 99:1; (III) methylene chloride/tetrahydrofuran 95:5; (IV) methylene chloride/methanol 99:1; (V) methylene chloride/methanol 8:2; and (VI) ethyl acetate/methyl ethyl ketone/formic acid/water 5:3:1:1. Spots were visualized with ultraviolet light at 254 or 366 nm and by spraying with ninhydrin (0.2% in 1-butanol). Chromatograms containing protected amino acids or peptides were heated for 20 min at 200 °C prior to spraying. Preparative layer chromatography (PLC) was performed either on commercial plates (20 × 20 × 0.2 cm (Merck, Inc.)) or on plates (20 × 40 × 0.5 cm) prepared from silica gel PF-254 containing calcium sulfate binder (Brinkmann Instruments). Except as noted, column chromatography was performed on silica or C18 columns on a Waters Associates PrepLC/Systems 500 chromatograph. All solvents and bulk chemicals were reagent grade except dimethylformamide (DMF) (Matheson Coleman and Bell, spectroquality, stored over Linde 4A molecular sieves) and acetonitrile and methylene chloride (Burdick and Jackson, chromatography grade). Amino acid derivatives were obtained from Bachem Inc. or Peninsula Laboratories and were used without further purification. All solid products were vacuum dried for 18–24 h at room temperature and 5 Pa or less. Solvent ratios are given as v/v; percents are given as weight percents. Nonaqueous solutions of organic compounds were routinely dried over anhydrous $MgSO_4$.

7-Amino-4-(trifluoromethyl)coumarin (1). 3-Aminophenol (5.45 g, 50 mmol), ethyl 4,4,4-trifluoroacetoacetate (9.70 g, 55 mmol), and anhydrous $ZnCl_2$ (8.50 g, 62 mmol) were refluxed in 30 mL of absolute ethanol for 12 h. The cooled reaction mixture was poured with stirring into 500 mL of 0.1 N aqueous HCl, and the precipitated crude product was collected by filtration, washed with water, and dried to give 8.6 g (75%) of solid, mp 227 °C. Results from variations on these conditions are listed in Table I.

Purified **1** with 0.2% or less of **2** and no detectable **5** could be obtained by column chromatography on a C18 column eluted with acetonitrile/0.01 N aqueous HCl (1:1), by column chromatography on a silica gel column eluted with methylene chloride, by recrystallization from i-PrOH (EtOH or EtOH/H_2O were less satisfactory), or by extracting a CH_2Cl_2 solution (4 g/L) with 1 N aqueous NaOH (3 × 0.1 L) followed by drying ($MgSO_4$), filtration, and evaporation: mp 222 °C; NMR δ 6.48 (s, 1, H_3), 7.0 (m, 5, $H_{5,6,8}$ + NH_2); (CF_3COOD added) δ 6.48 (s, 1, H_3), 6.68 (s, 1, H_5), 5.10 (m, 2, $H_{6,8}$); IR 3460 and 3360 (NH_2), 3250, 1710 (lactone), 1630, 1600 (NH def.), 1540, 1450, 1350 (CF_3 sym. def.), 1290 (CN str.), 1200, 1195, and 1160 (CF_3 asym. def.), 1140, 855, 815, 720, 655, 495 cm^{-1}.

Anal. Calcd for $C_{10}H_6F_3NO_2$: C, 52.41; H, 2.64; N, 6.11. Found: C, 52.17; H, 2.52; N, 6.05.

7-Hydroxy-4-(trifluoromethyl)-2-quinolone (2). **2** was isolated from the crude preparations of **1** either directly by preparative chromatography on a C18 column eluted with acetonitrile/0.01 N aqueous HCl or, better, by acidifying the NaOH extracts of a methylene chloride solution of crude **1**, filtering, drying, and chromatographing: mp >300 °C; NMR δ 3.3 (m, 2, NH + OH), 7.2 (m, 4, Ar H); IR 1665 (lactam) cm^{-1}.

Anal. Calcd for $C_{10}H_6F_3NO_2$: C, 52.41; H, 2.64; N, 6.11. Found: C, 52.41; H, 2.75; N, 6.36.

2-Ethoxy-7-hydroxy-4-(trifluoromethyl)quinoline (5). **5** was obtained from the later fractions of the chromatography of **1** and **2**: mp 198 °C; NMR δ 1.39 (t, 3, J = 6.7, CH_3), 4.50 (q, 2, J = 6.7, CH_2), 7.5 (m, 4, Ar H); IR 1625 (lactam) cm^{-1}.

Anal. Calcd for $C_{12}H_{10}F_3NO_2$: C, 56.04; H, 3.92; N, 5.45. Found: C, 56.38; H, 3.73; N, 5.65.

7-Hydroxy-1-methyl-4-(trifluoromethyl)-2-quinolone (3). **5** (217.8 mg, 0.44 mmol) was refluxed for 2.5 h with 1.0 mL (1.21 g, 8.7 mmol) of trimethyl phosphate. The bulk of the excess trimethyl phosphate was removed by distillation at room temperature (3 Pa), and the residue was recrystallized twice from aqueous ethanol: mp 276 °C; R_f (B, 5% MeOH/CH_2Cl_2) 0.37; R_f (C18, 1:1 CH_3CN/0.01 N aqueous HCl) 0.48; NMR δ 3.61 (s, 3, NCH_3), 7.3 (m, 4, Ar H); **3** is soluble in dilute aqueous alkali.

Anal. Calcd for $C_{11}H_8F_3NO_2$: C, 54.33; H, 3.32; N, 5.76; F, 23.44. Found: C, 54.54; H, 3.22; N, 5.24; F, 23.24.

7-Methoxy-4-(trifluoromethyl)-2-quinolone (4). 3-Methoxyaniline (6.16 g, 50 mmol), ethyl trifluoroacetoacetate (9.7 g, 55 mmol), anhydrous $ZnCl_2$ (8.5 g, 62 mmol), and absolute EtOH (30 mL) were refluxed together for 12 h. The cooled reaction mixture was poured with stirring into 500 mL of 0.1 N aqueous HCl, and the precipitated product was filtered off, washed with water, and dried to give 7.35 g (61%) of product, mp 187 °C. After three recrystallizations from EtOH, the melting point was constant at 252 °C: R_f (B, 5% MeOH/CH_2Cl_2) 0.54; NMR δ 3.90 (s, 3, OCH_3), 7.2 (m, 5, Ar H + NH); **4** is insoluble in dilute aqueous alkali.

Anal. Calcd for $C_{11}H_8F_3NO_2$: C, 54.33; H, 3.32; N, 5.76; F, 23.44. Found: C, 54.25; H, 3.11; N, 5.52; F, 23.27.

3-Hydroxycarbanilic Acid Isobutyl Ester (8) and *N,O*-Bis(isobutyloxycarbonyl)-3-aminophenol. To a stirred solution of 7.65 g (70 mmol) of 3-aminophenol in 450 mL of dry ether was added 10 mL (7.28 g, 72 mmol) of triethylamine followed by 10 mL (10.43 g, 76 mmol) of isobutyl chloroformate. After being stirred at room temperature for 17 h, the mixture was filtered and the filtrate was reduced on a rotary evaporator to an amber-colored oil that was separated on a silica gel column by elution with methylene chloride to give two liquid fractions. The first fraction, 5.62 g (26%), R_f 0.78, was *N,O*-bis(isobutyloxycarbonyl)-3-aminophenol: n^{29}_D 1.4976; NMR (CCl_4) δ 0.92 and 1.00 (two d, 12, J = 6, CH_3), 2.00 (m, 2 CH), 3.90 (t, 4, J = 6, CH_2), 7.0 (m, 5, Ar H + NH).

Anal. Calcd for $C_{16}H_{23}NO_5$: C, 62.12; H, 7.49; N, 4.53. Found: C, 62.41; H, 7.44; N, 4.87.

The second fraction, 2.05 g (14%), R_f 0.69, was **8**: bp 120 °C (1 Pa); n^{27}_D = 1.5165; NMR (CCl_4) δ 1.00 (d, 6, J = 6, CH_3), 2.00 (m, 1, CH), 3.51 (s, 2, OH + NH), 3.89 (d, 2, J = 6, CH_2), 6.7 (m, 4, Ar H).

Anal. Calcd for $C_{11}H_{15}NO_3$: C, 63.19; H, 7.23; N, 6.69. Found: C, 62.90; H, 7.12; N, 6.65.

3-Hydroxycarbanilic Acid Benzyl Ester (9). To a solution of 7.65 g (70 mmol) of 3-aminophenol in 450 mL of dry ether was added a solution of 5.94 g (35 mmol) of carbobenzoxy chloride in 50 mL of ether dropwise with stirring over 30 min. After being stirred at room temperature for 18 h, the mixture was filtered; the 3-aminophenol hydrochloride was then washed with ether. The combined filtrates were evaporated and the residue was dried to give 8.78 g (100%) of product, mp 123 °C. After three crystallizations from toluene it melted at 127 °C; NMR δ 5.17 (s, 2, CH_2), 6.9 (m, 9, Ar H), 9.40 (s, 1, NH), 9.70 (s, 1, OH); IR 1675 (carbonyl) cm^{-1}.

Anal. Calcd for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76. Found: C, 69.35; H, 5.49; N, 5.57.

Reaction of N-Blocked 3-Aminophenols under von Pechmann Conditions. The blocked 3-aminophenol, ethyl trifluoroacetoacetate (1.1 mmol/mmol), anhydrous $ZnCl_2$ (1.2 mmol/mmol), and absolute EtOH (0.6 mL/mmol) were refluxed for 12 h and worked up as described above. **6**[20] yielded 4% of crude product that analyzed by high-performance LC for 62% **1**, 11% **2**, and 7% **5**. **7**[21] yielded starting material nearly quantitatively. **8** yielded a crude product containing 53% **1** and 23% **2**. No **5** or urethane derivatives of **1** or **2** were present. **9** yielded a crude product containing 5% **1**, 2% **2**, 1% **5**, and starting material.

7-(Carbethoxyamido)-4-(trifluoromethylcoumarin). **10**[8] (1.82 g, 10 mmol), ethyl trifluoroacetoacetate (1.94 g, 11 mmol), anhydrous $ZnCl_2$ (1.70 g, 12 mmol), and absolute EtOH (6 mL) were refluxed for 12 h. The previously described workup gave 1.10 g (36.5%) of crude urethane melting at 90 °C. After four

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crystallizations the melting point was 172 °C: NMR δ 1.32 (t, 3, CH_3), 4.28 (q, 2, CH_2O), 3.32 (s, 1, NH), 6.97 (s, 1, Ar H_3), 7.7 (m, 3, Ar $H_{5,6,8}$); IR 1745, 1712 (carbonyls) cm^{-1}.

Anal. Calcd for $C_{13}H_{10}F_3NO_4$: C, 51.84; H, 3.35; N, 4.65. Found: C, 51.59; H, 3.21; N, 4.65.

7-(L-Leucinamido)-4-(trifluoromethyl)coumarin Hydrobromide (12). To a solution of 1.16 g (5 mmol) of Boc-L-leucine and 0.70 mL (5 mmol) of triethylamine in 12 mL of THF at −15 °C was added 0.66 mL (5 mmol) of isobutyl chloroformate. After 10 min, 1.15 g (5 mmol) of **1** in 6 mL of THF was added. The mixture was held at −15 °C for 30 min and then at room temperature overnight. The solution was evaporated in vacuo and the resulting residue was dissolved in 200 mL of EtOAc. This solution was washed with 1 M $NaHCO_3$ and saturated NaCl, dried, filtered, and evaporated to dryness to yield a yellow oil (2.20 g). This material was partially purified by PLC in solvent I and further by repeated PLC in solvent II, yielding 0.44 g (20%) of **11** as an essentially homogeneous oil: R_f (A, I) 0.82; R_f (A, II) 0.30; $[\alpha]^{22}_D$ −51.5° (*c* 2, $CHCl_3$).

11 (0.383 g, 0.866 mmol) was treated with 2 mL of 32% HBr/HOAc for 10 min. The crude hydrobromide salt was precipitated by the addition of Et_2O/petroleum ether (2/1). Two precipitations of the crude salt from 1:30 MeOH/Et_2O afforded analytically pure material (0.155 g, 42%) that was slightly hygroscopic: mp 249 °C; R_f (A, VI) 0.27; $[\alpha]^{24}_D$ +56° (*c* 1, CH_3OH).

Anal. Calcd for $C_{16}H_{18}BrF_3N_2O_3$: C, 45.40; H, 4.29; N, 6.62. Found: C, 45.24; H, 4.26; N, 6.47.

7-(N^{α}-Boc-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (14). N^{α}-Boc-N^{ϵ}-Z-L-Lysine (5.00 g, 13.1 mmol) was dissolved in 25 mL of EtOAc (dried over NaPb alloy) and cooled to −15 °C. *N*-Methylmorpholine (1.60 mL, 14.3 mmol) and isobutyl chloroformate (1.75 mL, 13.4 mmol) were added, and the mixture was stirred at −15 °C for 30 min. **1** (1.60 g, 7.0 mmol) was added and stirring continued at −15 °C for 22 h. EtOAc (25 mL) and saturated aqueous $NaHCO_3$ (25 mL) were then added and the mixture was stirred at −15 °C for 15 min. After the mixture warmed to room temperature, the layers were separated. The EtOAc layer was washed twice with 25-mL portions of 1 N H_2SO_4, once with 25 mL of saturated $NaHCO_3$, and twice with saturated NaCl, dried, filtered, and evaporated in vacuo. The residue was dissolved in 20 mL of 3% THF in CH_2Cl_2 and separated by column chromatography on silica gel using the same solvent. Fractions shown by TLC to contain **14** were combined and further purified by PLC on a 20 × 40 × 0.5 cm silica gel plate with solvent II. The major zone was eluted with EtOAc which was evaporated in vacuo. The yield of pure **14** was 0.93 g (23%): mp 171 °C; R_f (A, I) 0.83; R_f (A, II) 0.68; R_f (A, III) 0.50; R_f (B, III) 0.31; R_f (B, VI) 0.23; $[\alpha]^{22}_D$ −37° (*c* 2, $CHCl_3$); NMR (CD_3OD) δ 1.42 (s, Boc + Lys CH_2), 5.03 (s, Z CH_2), 6.83 (s, coumarin H_3), 7.30 (s, Z, Ar H), 7.8 (M, coumarin Ar H).

Anal. Calcd for $C_{29}H_{32}F_3N_3O_7$: C, 58.88; H, 5.45; N, 7.10. Found: C, 58.75; H, 5.34; N, 6.92.

7-(N^{α}-Z-N^{ϵ}-Boc-L-Lysinamido)-4-(trifluoromethyl)coumarin (18). The dicyclohexylamine salt of N^{α}-Z-N^{ϵ}-Boc-L-lysine (4.11 g, 7.3 mmol) and *p*-toluenesulfonic acid (1.39 g, 7.3 mmol) were dissolved in 20 mL of DMF. Dicyclohexylamine *p*-toluenesulfonate crystallized at −15 °C and was removed by centrifugation. To the clear DMF solution of free N^{α}-Z-N^{ϵ}-Boc-L-lysine, at −15 °C, was added 1.0 mL (7.6 mmol) of isobutyl chloroformate and 0.80 mL (7.2 mmol) of *N*-methylmorpholine. The mixture was stirred at −15 °C for 2 h. Then 1.60 g (7 mmol) of **1** was added and stirring continued overnight while the cooling bath gradually warmed to room temperature. The reaction mixture was evaporated to dryness at 35 °C (500 Pa) and the residue dissolved in 50 mL of EtOAc. This solution was washed twice with 25-mL portions of 1 N H_2SO_4, twice with 25-mL portions of saturated $NaHCO_3$, and twice with 25-mL portions of saturated NaCl, dried, filtered, and evaporated. The residue was chromatographed on a silica gel column with solvent IV to yield 1.35 g (33%) of **18**: mp 69 °C; R_f (B, IV) 0.40; NMR (CD_3OD) δ 1.42 (s, 15, Boc + Lys CH_2), 4.3 (m, 1, Lys CH), 5.18 (s, 2, Z CH_2), 6.80 (s, 1, coumarin H_3), 7.5 (m, 8, Ar H).

Anal. Calcd for $C_{29}H_{32}F_3N_3O_7$: C, 58.88; H, 5.45; N, 7.10. Found: C, 58.84; H, 5.61; N, 6.86.

7-(N^{α}-Boc-L-Leucyl-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (15). **14** (4.07 g, 6.88 mmol) was dissolved in 50 mL of trifluoroacetic acid (TFA). After 10 min at room temperature, excess TFA was removed in vacuo at 25 °C by repeatedly evaporating it in the presence of toluene. The resulting salt was neutralized with 0.962 mL (6.88 mmol) of triethylamine and treated with 1.59 g (6.88 mmol) of Boc-L-leucine, 1.05 g (6.88 mmol) of 1-hydroxybenzotriazole (HBt), and 1.32 g (6.88 mmol) of 1-ethyl-3-(3-(dimethylamino)propyl)carbodimide hydrochloride (EDCI) in 35 mL of DMF for 1 h at 4 °C and then at room temperature for 18 h. The DMF was removed at 45 °C in vacuo, yielding an oil that was taken up in 600 mL of Et_2O and washed with 5% $KHSO_4$, saturated NaCl, saturated $NaHCO_3$, and again with saturated NaCl. It was then dried, filtered, and evaporated to yield a yellow foam (3.89 g, 80%). This material was purified by PLC with solvent II and yielded **15** (63%) as a pale yellow glass: mp 65 °C; R_f (A, I) 0.84; $[\alpha]^{22}_D$ = −33° (*c* 2, $CHCl_3$); NMR ($CDCl_3$) δ 0.93 (d, Leu CH_3), 1.40 (s, Boc + Lys CH_2), 5.10 (s, Z CH_2), 6.68 (s, coumarin H_3), 7.35 (s, Z Ar H), 7.6 (m, coumarin Ar H).

Anal. Calcd for $C_{35}H_{43}F_3N_4O_8$: C, 59.65; H, 6.15; N, 7.95. Found: C, 60.00; H, 6.24; N, 7.72.

7-(N^{α}-Z-D-Alanyl-L-leucyl-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (16). **15** (1.20 g, 1.70 mmol) was deprotected in 20 mL of TFA as described above. The salt was neutralized with 0.24 mL (1.70 mmol) of triethylamine and treated with 0.379 g (1.70 mmol) of Z-D-alanine, 0.260 g (1.70 mmol) of HBt, and 0.326 g (1.70 mmol) of EDCI in 12 mL of DMF for 1 h at 4 °C and 12 h at room temperature. The DMF was removed in vacuo, and the resulting oil was taken up in Et_2O and worked up as described above to yield 1.01 g (73%) of pale yellow **16**. The pale yellow material was purified by PLC with solvent II to yield 0.77 g (56%) of **16**: mp 143 °C; R_f (A, I) 0.73; $[\alpha]^{21}_D$ −16° (*c* 2, $CHCl_3$); NMR ($CDCl_3$) δ 0.98 (s, Leu + Ala CH_3), 1.7 (m, CH_2), 4.88 (d, Z CH_2), 5.12 (s, Z CH_2), 6.70 (s, coumarin H_3), 7.2 (s, Z Ar H), 7.4 (s, Z Ar H), 7.7 (m, coumarin Ar H); amino acid analysis: Ala 1.00, Leu 1.06, Lys 1.02.

Anal. Calcd for $C_{41}H_{46}F_3N_5O_9$: C, 60.80; H, 5.73; N, 8.65. Found: C, 61.10; H, 6.17; N, 8.20.

7-(D-Alanyl-L-leucyl-L-lysinamido)-4-(trifluoromethyl)coumarin Dihydrobromide (13). **16** (0.744 g, 0.92 mmol) was deprotected by treatment with 10 mL of 32% HBr/HOAc for 15 min at room temperature. Excess HBr/HOAc was removed in vacuo, yielding 0.608 g (92%) of crude **13**. Six precipitations from 1/9 MeOH/Et_2O afforded 0.413 g (62%) of pure **13** as a light tan slightly hygroscopic powder: mp 213 °C; R_f (A, VI) 0.37; $[\alpha]^{21}_D$ −54° (*c* 2, CH_3OH); NMR (CD_3OD) δ 1.00 (m, Leu CH_3), 1.28 (d, Ala CH_3), 1.6 (m, CH_2), 6.83 (s, coumarin H_3), 8.1 (m, coumarin Ar H); amino acid analysis: Ala 1.00, Leu 1.03, Lys 1.03.

Anal. Calcd for $C_{25}H_{36}Br_2F_3N_5O_5 \cdot H_2O$: C, 41.62; H, 5.31; N, 9.71. Found: C, 41.39; H, 5.31; N, 9.70.

7-(N^{α}-Boc-D-Valyl-L-leucyl-N^{ϵ}-Z-L-lysinamido)-4-(trifluoromethyl)coumarin (21). **15** (0.656 g, 0.932 mmol) was treated with 10 mL of TFA to yield a salt that was neutralized with 0.13 mL (0.931 mmol) of triethylamine and allowed to react with 0.202 g (0.931 mmol) of Boc-L-valine, 0.143 g (0.931 mmol) of HBt, and 0.178 g (0.931 mmol) of EDCI in 10 mL of DMF for 1 h at 4 °C and 18 h at room temperature. The DMF was removed in vacuo and the resulting oil taken up in EtOAc and worked up as described for **15**. The oil was purified by PLC with solvent II and yielded 0.229 g (31%) of a white solid: mp 171 °C; R_f (A, I) 0.86; $[\alpha]^{22}_D$ −38° (*c* 2, $CHCl_3$); NMR ($CDCl_3$) δ 1.0 (m, Leu + Val CH_3), 1.18 (s, Boc), 1.7 (m, CH_2), 5.10 (s, Z CH_2), 6.70 (s, coumarin H_3), 7.4 (s, Z Ar H), 7.7 (m, coumarin Ar H); amino acid analysis: Val 0.97, Leu 1.03, Lys, 0.97.

Anal. Calcd for $C_{40}H_{52}F_3N_5O_9$: C, 59.76; H, 6.52; N, 8.71. Found: C, 59.58; H, 6.39; N, 8.94.

7-(D-Valyl-L-leucyl-L-lysinamido)-4-(trifluoromethyl)coumarin Dihydrobromide (17). **21** (0.206 g, 0.256 mmol) was deprotected in 4 mL of 32% HBr/HOAc for 15 min. Workup and purification as described for **13** yielded **17** as a light tan slightly hygroscopic powder (0.060 g, 32%): mp 240 °C; R_f (A, VI) 0.38; $[\alpha]^{22}_D$ −61° (*c* 0.74, CH_3OH); amino acid analysis: Val 0.89, Leu 1.04, Lys 1.07.

Anal. Calcd for $C_{27}H_{40}Br_2F_3N_5O_5$: C, 44.33; H, 5.51; N, 9.58. Found: C, 44.20; H, 5.73; N, 9.71.

7-(N^{α}-Z-L-Argininamido)-4-(trifluoromethyl)coumarin Hydrochloride (19). N^{α}-Z-L-Arginine (2.27 g, 7.36 mmol) was suspended in 20 mL of DMF and cooled to −15 °C. Isobutyl

chloroformate (1.0 mL, 7.63 mmol) was added and the mixture stirred at −15 °C until solution was complete (about 2 h). To this solution was added 1.6 g (6.98 mmol) of **1**. Stirring was continued for 18–20 h while the cooling bath was allowed to warm to room temperature. The solvent was removed in vacuo and the residue dried. The crude product was dissolved in 50–60 mL of 10% $MeOH/CH_2Cl_2$ and separated by column chromatography on silica gel with the same solvent. The fractions shown by TLC to contain **19** (usually column volumes 5 to 9) were combined and evaporated. The product, 1.38–1.77 g (36–46%), at this stage was still contaminated by minor amounts of unreacted **1** and other impurities. It was further purified by chromatography with a second silica gel column or by PLC. Once the impurities were reduced to a sufficiently low level, the product could also be dissolved in MeOH and precipitated by adding 10 volumes of Et_2O: mp 193 °C; $[\alpha]^{21}_D$ −21° (*c* 2, MeOH); R_f (A, V) 0.59; R_f (B, I) 0.28; R_f (A, I) 0.15; NMR (CD_3OD) δ 1.78 (m, Arg CH_2), 5.12 (s, Z CH_2), 6.80 (s, coumarin H_3), 7.39 (s, Z Ar H), 7.7 (m, coumarin Ar H).

Anal. Calcd for $C_{24}H_{25}ClF_3N_5O_5$: C, 51.85; H, 4.53; N, 12.60. Found: C, 52.00; H, 4.62; N, 12.77.

7-(N^{α}-Z-L-Argininamido)-4-(trifluoromethyl)coumarin Hydrobromide (19a). N^{α}-Z-L-Arginine hydrobromide[19] (4.08 g, 10.48 mmol) was dissolved in 25 mL of DMF and cooled to −15 °C. *N*-Methylmorpholine (1.17 mL, 10.48 mmol) and isobutyl chloroformate (1.31 mL, 9.96 mmol) were added and the resulting suspension was stirred for 20 min. **1** (1.50 g, 6.55 mmol) was then added and the suspension stirred at −15 °C for 3 h and at room temperature for 1.5 h. The DMF was removed in vacuo, yielding an oil that was taken up in 750 mL of Et_2O/H_2O (1:2) and shaken. The resulting emulsion was kept at 4 °C for 3 days. The insoluble material that separated in the water layer was collected by filtration, washed with water and ether, and dried to yield 1.44 g of a light yellow solid. The solid was **19a** slightly contaminated with **1**. Recrystallization from MeOH/EtOAc gave **19a** (1.18 g, 30%), mp 169 °C. Trituration with hot EtOAc and drying at 100 °C under high vacuum raised the melting point to 176 °C; $[\alpha]^{23}_D$ −23° (*c* 2, MeOH).

Anal. Calcd for $C_{24}H_{25}BrF_3N_5O_5$: C, 48.01; H, 4.20; N, 11.67; Br, 13.31. Found: C, 48.53; H, 4.36; N, 11.92; Br, 13.16.

7-(N^{α}-Z-Glycylglycyl-L-argininamido)-4-(trifluoromethyl)coumarin Dihydrochloride (20). Via Mixed Anhydride. **19** (1.41 g, 2.54 mmol) was dissolved in a mixture of 15 mL of DMF and 75 mL of absolute EtOH, and 0.45 g of 5% Pd/C was added. Hydrogen was passed through the solution at room temperature until TLC (B, V) of an aliquot showed that the reaction was complete (about 6 h). The catalyst was removed by filtration on a bed of Celite, the solvents were removed in vacuo, and the residue was dried. Deblocked **19** dissolved in 6 mL of DMF was added to the mixed anhydride prepared at −15 °C from 0.84 g (3.15 mmol) of Z-glycylglycine, 0.35 mL (3.13 mmol) of *N*-methylmorpholine, and 0.39 mL (2.98 mmol) of isobutyl chloroformate in 5 mL of DMF. The reaction mixture was stirred at −15 °C for 3 h. Water (0.15 mL) was added and stirring continued for 5 min. Solvents were removed in vacuo, and the residue was dried to yield 2.55 g of crude **20**. The crude product (1.85 g) was dissolved in 20 mL of solvent V and chromatographed on a silica gel column. Fractions shown by TLC to contain **20** (column volumes 4–8) were combined and evaporated. The residue was further purified by PLC with solvent V; the major zone was eluted from the plate with 1/1 $MeOH/CH_2Cl_2$. Evaporation of the eluant left a tan semisolid that was dissolved in 0.3 mL of MeOH and precipitated by addition of 10 mL of Et_2O. The precipitated material, which was still gummy, was dissolved in 8 mL of MeOH and freeze-dried to give 0.29 g (20%) of a friable slightly tan solid: mp 132 °C; $[\alpha]^{21}_D$ −17° (*c* 2.7, MeOH); R_f (A, I) 0.31; R_f (A, V) 0.25; NMR (CD_3OD) δ 1.85 (m, Arg CH_2), 3.9 (m, Gly CH_2), 4.25 (br s, Arg CH), 5.10 (s, Z CH_2), 6.80 (s, coumarin H_3), 7.30 (s, Z Ar H), 7.7 (m, coumarin Ar H); ($CDCl_3$ + Me_2SO-d_6) δ 3.3 (br s, CH_3OH); amino acid analysis: Arg 1.00, Gly 1.91.

Anal. Calcd for $C_{28}H_{32}Cl_2F_3N_7O_7 \cdot 2CH_3OH$: C, 46.77; H, 5.23; N, 12.72; Cl, 9.20. Found: C, 46.84; H, 4.85; N, 12.89; Cl, 9.33.

Via *p*-Nitrophenyl Ester. **19** (0.421 g, 0.757 mmol) was treated at room temperature for 30 min with 4 mL of 32% HBr/HOAc. Addition of 70 mL of Et_2O precipitated 7-(L-argininamido)-4-(trifluoromethyl)coumarin dihydrobromide that was washed several times with Et_2O and dried to yield 0.363 g (88%) of a white powder, R_f (A, VI) 0.45. This material, without further purification, was dissolved in 6 mL of dry pyridine containing 0.19 mL (1.33 mmol) of triethylamine and 0.514 g (1.33 mmol) of Z-glycylglycine *p*-nitrophenyl ester.[22] The reaction proceeded at room temperature for 3 days. The pyridine was removed in vacuo, yielding a yellow oil that was subjected to PLC in EtOAc/HCOOH/H_2O (16:1:1), then to column chromatography on Sephadex LH-20 with MeOH as eluant, and finally to PLC with solvent V. Treatment of the purified material in MeOH with HCl in Et_2O yielded 0.132 g (23%) of **20** as a white solid: mp 128 °C; R_f (A, VI) 0.70; amino acid analysis: Arg 1.00, Gly 2.10.

Anal. Calcd for $C_{28}H_{30}N_7O_7 \cdot 1.6HCl \cdot 3H_2O$: C, 45.08; H, 5.08; N, 13.15; Cl, 7.60. Found: C, 45.38; H, 4.58; N, 12.89; Cl, 7.55.

Registry No. **1**, 53518-15-3; **2**, 73496-29-4; **3**, 73496-30-7; **4**, 73496-31-8; **5**, 73496-32-9; **6**, 621-42-1; **8**, 54840-15-2; **9**, 7107-59-7; **10**, 63450-46-4; **11**, 73496-33-0; **12**, 73389-55-6; **13**, 73389-54-5; **14**, 73496-34-1; **15**, 73496-35-2; **16**, 73496-36-3; **17**, 73389-53-4; **18**, 73496-37-4; **19**, 73496-38-5; **19a**, 73496-39-6; **20**, 73389-51-2; **21**, 73506-19-1; 3-aminophenol, 591-27-5; ethyl 4,4,4-trifluoroacetoacetate, 372-31-6; 3-methoxyaniline, 536-90-3; *N*,*N*-bis[(isobutyloxy)carbonyl]-3-aminophenol, 543-27-1; *N*,*N*-bis[(isobutyloxy)carbonyl]-3-aminophenol, 73496-40-9; carbobenzoxy chloride, 501-53-1; Boc-L-leucine, 13139-15-6; N^{α}-Boc-N^{ϵ}-Z-L-lysine, 2389-45-9; N^{α}-Z-N^{ϵ}-Boc-L-lysine dicyclohexylamine salt, 2212-76-2; Z-D-alanine, 26607-51-2; Boc-D-valine, 22838-58-0; N^{α}-Z-L-arginine, 1234-35-1; N^{α}-Z-L-arginine hydrobromide, 73496-41-0; Z-glycylglycine, 2566-19-0; 7-(L-argininamido)-4-(trifluoromethyl)coumarin dihydrochloride, 73496-42-1; Z-glycylglycine *p*-nitrophenyl ester, 13574-81-7.

(22) M. Bodanszky, J. T. Sheehan, M. A. Ondetti, and S. Lande, *J. Am. Chem. Soc.*, **85**, 991 (1963).