[Chem. Pharm. Bull. 36(9)3496—3502(1988)]

New Water-Soluble Fluorogenic Amine. 7-Aminocoumarin-4-methanesulfonic Acid (ACMS) and Related Substrates for Proteinases[1)]

EISUKE SATO, AKIRA MATSUHISA, MARI SAKASHITA, and YUICHI KANAOKA*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

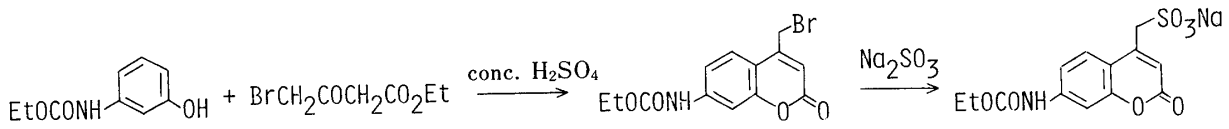
(Received January 25, 1988)

The synthesis and properties of a water-soluble fluorogenic amine, 7-aminocoumarin-4-methanesulfonic acid (ACMS, **5**) are described. Several peptide amides of **5** were synthesized and examined as new fluorogenic substrates for chymotrypsin, trypsin and related enzymes.

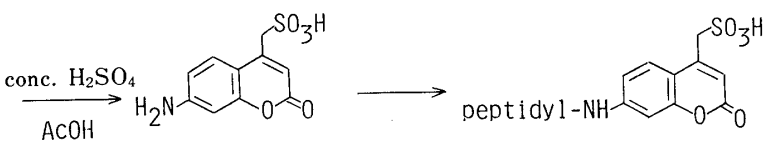
Keywords——water-soluble fluorogenic amine; 7-aminocoumarin-4-methanesulfonic acid; peptidylcoumarylamido-4-methanesulfonic acid; fluorometric enzyme assay; proteinase; kinetic study

Since we reported the usefulness of 7-amino-4-methylcoumarin (AMC) as a fluorogenic amine,[2)] peptide amides of AMC (MCA) have been widely used as fluorogenic substrates in the assay of proteinases. Among many synthetic substrates employed for such enzyme assays so far, the MCA substrates appear to be among the most sensitive and convenient. However, an organic solvent such as dimethyl sulfoxide is needed as a cosolvent due to the low solubility of the AMC moiety in water. In order to measure enzyme activities without any organic solvent, we designed a new water-soluble fluorogenic amine, 7-aminocoumarin-4-methanesulfonic acid (ACMS, **5**).

In this paper we wish to report the synthesis and spectroscopic properties of **5**, and an application of **5** to fluorogenic peptidyl substrates (coumarylamido-4-methanesulfonic acid: CAMS) for the assay of chymotrypsin (Tos–L-Phe–CAMS, **6**), trypsin (Bz–L-Arg–CAMS, **9**; Z–L-Arg–CAMS, **10**), thrombin (Z–Gly–Gly–L-Arg–CAMS, **11**), plasmin (**11** and Z–L-Phe–L-Arg–CAMS, **12**) and papain (**9** and **10**).



1 2 3 4



ACMS **5** CAMS

peptidyl-
6: *N*-Tos–L-Phe–
7: Tri-Z–L-Arg–
8: H–L-Arg–
9: *N*-Bz–L-Arg–
10: *N*-Z–L-Arg–
11: *N*-Z–Gly–Gly–L-Arg–
12: *N*-Z–L-Phe–L-Arg–

Chart 1

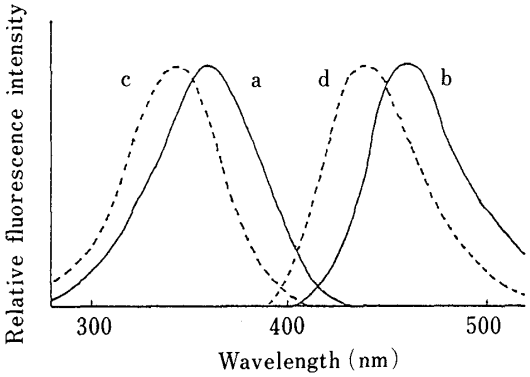


Fig. 1. Fluorescence Spectra of ACMS and AMC

Concentration: 5.40×10^{-6} M (2% EtOH in buffer for AMC) in 50 mM Tris–HCl buffer containing 100 mM $CaCl_2$, pH 7.8. Excitation spectra of ACMS (a) (em. 462 nm) and AMC (c) (em. 440 nm). Emission spectra of ACMS (b) (ex. 362 nm) and AMC (d) (ex. 345 nm).

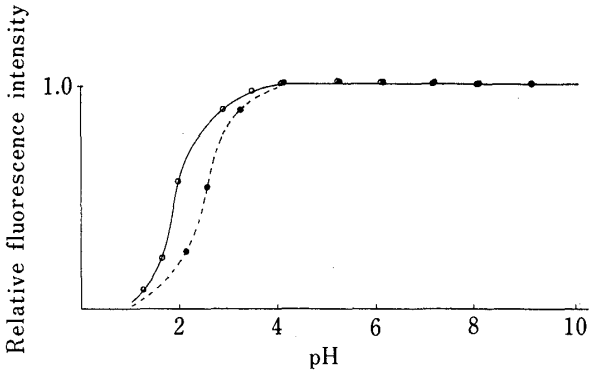


Fig. 2. pH Dependence of Fluorescence Intensity of ACMS and AMC

Concentration: 2.60×10^{-6} M. pH 1.03—3.05, 0.1 M glycine–HCl buffer; pH 2.95—6.05, 0.1 M citrate–phosphate buffer; pH 6.08—9.10, 0.1 M phosphate–borate buffer. Excitation at 380 nm and emission at 460 nm for AMC (--●--), excitation at 362 nm and emission at 462 nm for ACMS (—○—).

The route for the synthesis of ACMS (**5**) is shown in Chart 1. Ethyl 4-bromo-3-oxobutanate (**2**) and 3-carboethoxyaminophenol (**1**) were treated in concentrated sulfuric acid to give 4-bromomethyl-7-carboethoxyaminocoumarin (**3**), which was converted into the sulfonic acid derivative with sodium sulfite, producing sodium 7-carboethoxyaminocoumarin-4-methanesulfonate (**4**). ACMS (**5**) was obtained by hydrolysis of **4**. As expected, the water-solubility of ACMS is good enough for enzyme assay in aqueous media.

The fluorescence spectra of ACMS and AMC are shown in Fig. 1. Excitation and emission maximum wavelengths of ACMS (**5**) are shifted to longer wavelength (by 20 nm) compared with those of AMC,[2d] and the Stokes shift is large (100 nm). The fluorescence intensity of ACMS is as high as that of AMC.

The pH dependence of fluorescence intensity of ACMS (**5**) and AMC is shown in Fig. 2. Fluorescence intensity of ACMS is constant at higher pH than 4 and unchanged between pH 6 and pH 9, the optimum pH range of usual enzyme reactions. Since the properties of ACMS (**5**) described above indicate that ACMS (**5**) is a useful fluorescent amine, we decided to prepare fluorogenic substrates containing ACMS.

First, ACMS-containing fluorogenic substrates for chymotrypsin were prepared. As an acyl moiety of the substrate, Tos–Phe was chosen in view of the high affinity of *N*-tosylphenylalanyl chloromethyl ketone for the binding site of chymotrypsin.[3] As shown in Chart 1, *N*-tosyl-L-phenylalanyl chloride (**13**) was coupled with **5** in the presence of triethylamine in water to produce *N*-Tos–L-Phe–CAMS (**6**). In a similar manner, **13** was coupled with AMC to produce *N*-tosyl-L-phenylalanyl-4-methylcoumaryl-7-amide (Tos–L-Phe–MCA, **14**) for comparison of kinetic parameters.

The scheme used to synthesize AMC-containing fluorogenic substrates for trypsin are presented in Chart 1. Tricarbobenzoxy-L-arginine (**15**) was coupled with **5** by the use of isobutyloxycarbonyl chloride and triethylamine in tetrahydrofuran–dimethylformamide to produce 7-(tricarbobenzoxy-L-arginyl)coumarylamido-4-methanesulfonic acid (**7**), which was deprotected by treatment with methanesulfonic acid to give H–L-Arg–CAMS (**8**). Compound **8** was then coupled with benzoyl chloride in the presence of triethylamine at pH 9 in water to give Bz–L-Arg–CAMS (**9**). In a similar manner, Z–L-Arg–CAMS (**10**) was prepared from **8** with carbobenzoxy chloride. The fluorescence spectra of ACMS and the substrates **6**, **9** and **10** are shown in Fig. 3.

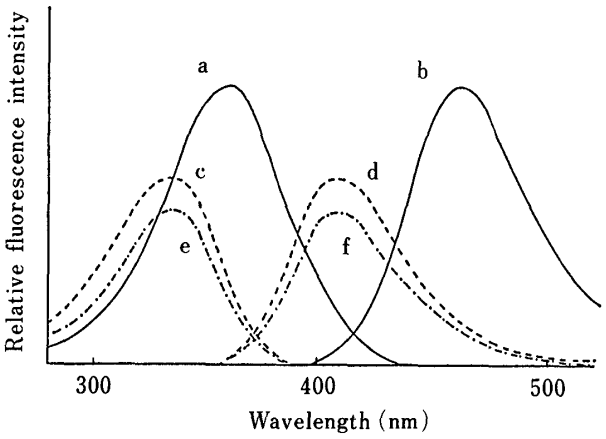


Fig. 3. Fluorescence Spectra of ACMS and Peptidyl-CAMS

——, ACMS (**5**); – – – –, Tos-L-Phe-CAMS (**6**) and Z-Gly-Gly-L-Arg-CAMS (**11**); –·–·–, Bz-L-Arg-CAMS (**9**), Z-L-Arg-CAMS (**10**) and Z-L-Phe-L-Arg-CAMS (**12**).

Concentration: 4.96×10^{-6} M in the same buffer as in Fig. 1. Excitation spectra of ACMS (a) (em. 462 nm), or Tos-L-Phe-CAMS (**6**) (c) (em. 410 nm) and Z-Gly-Gly-L-Arg-CAMS (**11**) (c) (em. 409 nm), or Bz-L-Arg-CAMS (**9**) (e) (em. 409 nm) and Z-L-Arg-CAMS (**10**) (e) (em. 409 nm) and Z-L-Phe-L-Arg-CAMS (**12**) (e) (em. 409 nm). Emission spectra of ACMS (b) (ex. 362 nm), or Tos-L-Phe-CAMS (**6**) (d) (ex. 337 nm) and Z-Gly-Gly-L-Arg-CAMS (**11**) (d) (ex. 336 nm), or Bz-L-Arg-CAMS (**9**) (f) (ex. 336 nm) and Z-L-Arg-CAMS (**10**) (f) (ex. 336 nm) and Z-L-Phe-L-Arg-CAMS (**12**) (f) (ex. 336 nm).

TABLE I. Kinetic Parameters of the Fluorogenic Substrates

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Chymotrypsin	Tos-L-Phe-CAMS (**6**)	0.76	0.0055	7.2
	Tos-L-Phe-MCA (**14**)	0.036	0.0022	6.2×10
Trypsin	Bz-L-Arg-CAMS (**9**)	0.55	0.16	3.0×10^2
	Bz-L-Arg-MCA (**16**)[2b]	0.11	0.42	3.8×10^3
	Z-L-Arg-CAMS (**10**)	0.12	0.10	9.0×10^2
	Z-L-Arg-MCA (**17**)[2b]	0.20	0.89	4.5×10^3
Thrombin	Z-Gly-Gly-L-Arg-CAMS (**11**)	0.27	0.052	1.9×10^2
	Z-Gly-Gly-L-Arg-MCA (**18**)[6]	0.11	310	2.8×10^6
Plasmin	**11**	0.51	0.058	1.1×10^2
	18[6]	0.45	1.7	3.8×10^3
	Z-L-Phe-L-Arg-CAMS (**12**)	0.098	0.10	1.0×10^3
	Z-L-Phe-L-Arg-MCA (**19**)[7]	—	0.31	—
Papain	**9**	0.36	0.22	6.2×10^2
	16[2b]	0.92	0.53	5.8×10^2
	10	0.26	0.075	3.0×10^2
	17[2b]	0.97	0.42	4.3×10^2

Excitation and emission maxima of these substrates are shifted to shorter wavelength compared with those of ACMS. The fluorescence intensity of ACMS (excitation at 362 nm, emission at 462 nm) is twice that of **9** and **10**, and one and a half times that of **6** (excitation at 336 nm, emission at 410 nm) at the respective maximum wavelengths in a buffer solution. However, when excited at 380 nm and measured at 462 nm, ACMS possesses a relative fluorescence intensity approximately 400-fold *vs.* **9**, 180-fold *vs.* **10** and 180-fold *vs.* **6** higher than those of the above substrates, respectively, so that the faint fluorescence of these substrates does not interfere with this fluorometric assay through the course of the enzymatic hydrolysis.

Kinetic studies gave the parameters listed in Table I. Although the k_{cat} value of **6** for chymotrypsin is comparable to that of MCA-substrates (**14**), the K_m value of **6** is higher than that of **14**, suggesting lower affinity of **6** to chymotrypsin.[4] The lower k_{cat} values of **9** and **10** for trypsin account for the lower k_{cat}/K_m values of **9** and **10** than MCA substrates (**16** and **17**) reported before,[2b] implying an effect of the sulfonyl group of the substrate on the interaction with the enzyme catalytic site. The linearities of the fluorescence enhancements *vs.* incubation time (for more than 10 min) are satisfactory and the rates of hydrolysis are proportional to enzyme concentration over an at least 1000-fold range up to 100 (for **9**), 10 (for **10**) and 200

TABLE II. Solubilities of Substrates in Water[a)]

Substrate	Solubility (M)	Buffer[b)]
Tos–L-Phe–CAMS (**6**)	2×10^{-4}	A
Bz–L-Arg–CAMS (**9**)	1×10^{-4}	B
Z–L-Arg–CAMS (**10**)	4×10^{-5}	B
Z–Gly–Gly–L-Arg–CAMS (**11**)	3×10^{-5}	B
Z–L-Phe–L-Arg–CAMS (**12**)	1.2×10^{-5}	B
Tos–L-Phe–MCA (**14**)	$<6\times10^{-9}$	A
Bz–L-Arg–MCA (**15**)	4.6×10^{-6}	B
Z–L-Phe–L-Arg–MCA (**19**)	1.3×10^{-6}	B

a) Solubilities of MCA substrates were determined by ultraviolet absorption measurement after centrifugation. *b*) A: 80 mM Tris–HCl buffer (pH 7.8). B: 50 mM Tris–HCl buffer (pH 8.0).

(for **6**) ng/ml, values comparable with those of the MCA substrates for microdetermination.[5)] As the solubilities in water of these substrates **6**, **9** and **10** are higher than those of the MCA substrates, as shown in Table II, these assays can be performed without using any organic solvent, though the assay solution of MCA-substrates usually contains 1% dimethyl sulfoxide (DMSO).

As a further application of ACMS, substrates **9** and **10** were used for an assay of papain, which has a broad substrate specificity but a relatively higher affinity for basic amino acids. As shown in Table I, kinetic parameters of both **9** and **10** for papain are comparable to those of MCA substrates. It was revealed that papain activity was not affected by the sulfonyl group of the CAMS substrates.

To extend the further applicability of ACMS, several CAMS substrates were examined for the assay of physiologically important trypsin-like enzymes such as thrombin and plasmin. MCA substrates for these enzymes have been already reported to be Z–Gly–Gly–Arg–MCA[6)] for thrombin and Z–Phe–Arg–MCA[7)] for plasmin. In an attempt to improve the solubility properties of these substrates, ACMS derivatives of these peptidyl moieties were newly synthesized. Z–Gly–Gly–OH was coupled with **5** by the mixed anhydride method using isobutyloxycarbonyl chloride to give Z–Gly–Gly–L-Arg–CAMS (**11**), and the synthesis of Z–L-Phe–L-Arg–CAMS (**12**) was achieved from Z–L-Phe–OH and **5** by the same procedure as above. The fluorescence spectra of **11** and **12** are shown in Fig. 3. Although the maximum wavelength of fluorescence spectra for CAMS substrates **11** and **12** was shifted to shorter wavelength, as is observed generally, the relative fluorescence intensities of **11** and **12** are two-thirds and a half of that of ACMS, respectively, at the respective maximum wavelengths. However, when excited at 380 nm and measured at 462 nm, ACMS (**5**) has a relative fluorescence intensity approximately 250-fold and 300-fold higher than those of the amides **11** and **12**, respectively, so that the faint fluorescence of the substrate does not interfere with these fluorescence assays.

As shown in Table I, the k_{cat}/K_m value of **11** is much lower than that of the corresponding MCA substrate **18** due to the low k_{cat} value. Presumably, the sulfonyl group of the substrate retards the catalytic process. Next, kinetic parameters for plasmin were obtained by using **11** and Z–L-Phe–L-Arg–CAMS (**12**), as shown in Table I. Similarly to the results of thrombin assay, the k_{cat}/K_m value of substrate **12**, which has a Phe–Arg moiety, was about one order of magnitude higher that of **11**. When compared with the corresponding MCA substrates, although **11** has lower k_{cat} and k_{cat}/K_m values than those of **18**, the ACMS substrate **12** has a comparable k_{cat}/K_m value to that of **18** and a comparable k_{cat} value with that of the corresponding MCA substrate **19** (the K_m value is not given in literature[7)]). From these results, **12** seems to be a potent fluorogenic substrate for plasmin assay.

Although the kinetic parameters of some of these new substrates are unsatisfactory when compared with those of MCA substrates, substrates **9** and **10** (for papain) and substrate **12** (for plasmin) were demonstrated to be potentially useful. It is necessary to examine a variety of ACMS peptides with different combinations of amino acid residues in order to develop very sensitive fluorogenic substrates which can discriminate among the proteases. In general, the development of these new fluorogenic CAMS substrates with improved solubility in water may be useful in several areas. 1) Kinetic determination of enzymic processes in aqueous solution without organic solvent may be desirable for certain mechanistic studies of enzymes. 2) Although many routine determinations of enzyme activity are performed in the presence of a small amount of organic solvent, there are enzymes which are sensitive to organic solvent, such as enkephalinase[8a] or prolyl endopeptidase from ascidian sperm.[8b] 3) There are a number of MCA substrates which are very hydrophobic, and improvement of the solubility of such substrates may be desirable, for example, for cathepsin G[9] and elastase.[10] 4) This water-soluble fluorogenic amine ACMS could be applied not only to studies with purified enzyme samples but also to various cell- and tissue-level biological techniques including histochemical studies.

Experimental

Melting points were determined with a Yamato MP-21 melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a JASCO IRA-1 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL-FX 100 FT-NMR spectrometer with tetramethylsilane or sodium trimethylsilylpropionate-d_4 as an internal standard. Chemical shifts are given on the δ scale. Optical rotations were obtained with a JASCO DIP-4 polarimeter. Ultraviolet (UV) spectra were measured with a Hitachi 200-10 spectrophotometer. Fluorescence spectra were recorded with a Hitachi 650-60 spectrophotometer.

4-Bromomethyl-7-carbethoxyaminocoumarin (3)——A suspension of 3-hydroxyphenylurethane[11] (**1**, 14.2 g, 78.5 mmol) and 4-bromo-3-oxobutanate[12] (**2**, 17 g, 82 mmol) in concentrated sulfuric acid (60 ml) was stirred overnight at room temperature. The solution was poured into ice water (200 ml), giving a voluminous white crystalline precipitate, which was filtered off and washed with cold water. Recrystallization from ethyl acetate gave 10.1 g (38%) of yellow needles. mp 219—221 °C. IR (Nujol): 3280 (NH), 1710 (C=O), 1620 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.28 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 4.19 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 4.84 (2H, s, CH_2Br), 6.57 (1H, s, 3-H), 7.45 (1H, dd, J=9, 2 Hz, 6-H), 7.60 (1H, d, J=2 Hz, 8-H), 7.80 (1H, d, J=9 Hz, 5-H), 10.19 (1H, br s, NH). *Anal.* Calcd for $C_{13}H_{12}BrNO_4$: C, 47.88; H, 3.71; Br, 24.50; N, 4.29. Found: C, 48.12; H, 3.72; Br, 24.22; N, 4.30.

Sodium 7-Carbethoxyaminocoumarin-4-methanesulfonate (4)——A solution of sodium sulfite seven hydrate (8.2 g, 33 mmol) in water (50 ml) and a suspension of **3** (8.8 g, 27 mmol) in ethanol (350 ml) were mixed, and the mixture was refluxed for 4 h. After cooling, the solvent was removed by evaporation. The residue was suspended in hot water and filtered while warm. The filtrate was evaporated and the residue was suspended in hot methanol and filtered while warm. The filtrate was concentrated. Recrystallization from methanol gave 5.2 g (59%) of colorless needles. mp >320 °C (dec.). IR (Nujol): 3320 (NH), 1710, 1690 (C=O), 1620 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.27 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 3.99 (2H, s, CH_2SO_3Na), 4.17 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 6.24 (1H, s, 3-H), 7.33 (1H, dd, J=9, 2 Hz, 6-H), 7.55 (1H, d, J=2 Hz, 8-H), 7.85 (1H, d, J=9 Hz, 5-H), 10.10 (1H, br s, NH). *Anal.* Calcd for $C_{13}H_{12}NNaO_7S \cdot H_2O$: C, 42.51; H, 3.84; N, 3.81; S, 8.73. Found: C, 42.65; H, 3.75; N, 4.06; S, 8.46.

7-Aminocoumarin-4-methanesulfonic Acid (5)——Concentrated sulfuric acid (16 ml) and acetic acid (16 ml) were added to **4** (7.2 g, 22 mmol), and this mixture was heated with stirring a 100 °C overnight. After cooling, the solution was poured into ethanol, giving a yellow precipitate. The solid was filtered off and washed with ethanol. Recrystallization from water gave 2.5 g (46%) of yellow needles. mp >320 °C (dec.). IR (Nujol): 3180 (NH), 1720 (C=O), 1610 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 3.86 (2H, s, $C\underline{H}_2SO_3H$), 5.94 (1H, s, 3-H), 6.43 (1H, d, J=2 Hz, 8-H), 6.55 (1H, dd, J=9, 2 Hz, 6-H), 7.57 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{10}H_7NO_5S \cdot 1/4H_2O$: C, 46.24; H, 3.69; N, 5.39; S, 12.34. Found: C, 46.25; H, 3.57; N, 5.24; S, 12.35.

Triethylammonium 7-(*N*-Tosyl-L-phenylalanyl)coumarinylamido-4-methanesulfonate (6)——A solution of *N*-tosyl-L-phenylalanyl chloride[13] (**13**, 676 mg, 2.0 mmol) in tetrahydrofuran (10 ml) was added, to a solution of **5** (255 mg, 1.0 mmol) and triethylamine (0.14 ml, 2.0 mmol) in water (6 ml), and the mixture was stirred at room temperature at pH 9 (adjusted with triethylamine). After 1 h, additional **13** (676 mg, 2.0 mmol) in tetrahydrofuran (10 ml) was added, and the reaction was continued. After 5 h, the solvent was removed by evaporation to give a yellow precipitate. Ethyl acetate and 1 N hydrochloric acid was added to the residue and the mixture was stirred. The solid was filtered off and washed with ethyl acetate, 1 N hydrochloric acid and water. Recrystallization from 60% ethanol gave 328 mg (54%) of colorless needles. mp 256.5—258 °C. IR (Nujol): 3140 (NH), 1690 (C=O), 1610 (C=C) cm^{-1}.

NMR (DMSO-d_6) δ: 1.17 (9H, t, J=7 Hz, $NCH_2C\underline{H}_3$), 2.18 (3H, s, $C\underline{H}_3Ph$), 2.87 (2H, t, J=7 Hz, $PhC\underline{H}_2$), 3.10 (6H, q, J=7 Hz, $NC\underline{H}_2CH_3$), 3.92—4.30 (1H, m, $PhCH_2C\underline{H}$), 3.99 (2H, s, $C\underline{H}_2SO_3H$), 6.27 (1H, s, 3-H), 7.12 (2H, d, J=8 Hz, 3, 5-H of tosyl group), 7.21 (6H, s, $\underline{Ph}CH_2$ and 6-H), 7.38 (1H, d, J=2 Hz, 8-H), 7.51 (2H, d, J=8 Hz, 2, 6-H of tosyl group), 7.83 (1H, d, J=9 Hz, 5-H), 8.30 (1H, d, J=9 Hz, NH), 10.27 (1H, s, NH). $[\alpha]_D$: +48.43° (c=0.351 in DMSO). *Anal.* Calcd for $C_{32}H_{39}N_3O_8S_2 \cdot 1/4H_2O$: C, 58.03; H, 6.01; N, 6.35; S, 9.68. Found: C, 58.06; H, 5.93; N, 6.34; S, 9.88.

***N*-Tosyl-L-phenylalanyl-4-methylcoumarinyl-7-amide (14)**——A solution of 7-amino-4-methylcoumarin[2c,11] (175 mg, 1.0 mmol) in distilled tetrahydrofuran (16 ml) was cooled to 0 °C, and triethylamine (0.14 ml, 1.0 mmol) and *N*-tosyl-L-phenylalanyl chloride (**13**, 338 mg, 1.0 mmol) were added. After 2 h, additional triethylamine (0.14 ml, 1.0 mmol) and **13** (339 mg, 1.0 mmol) were added. After a further 2 h, the solution was brought to room temperature and stirred overnight. The solvent was removed by evaporation, and the residue was dissolved in ethyl acetate. The organic solution was washed with 3 N hydrochloric acid, water, saturated sodium bicarbonate and water. The organic solution was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. Recrystallization from ethyl acetate gave 125 mg (28%) of colorless needles. mp 220—222 °C. IR (Nujol): 3340 (NH), 1690 (C=O), 1610 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 2.16 (3H, s, $C\underline{H}_3PhSO_2$), 2.40 (3H, s, 4-CH_3), 2.70—2.96 (2H, m, $PhC\underline{H}_2$), 4.07 (1H, br, $PhCH_2C\underline{H}$), 6.28 (1H, s, 3-H), 7.11 (2H, d, J=9 Hz, 3, 5-H of tosyl group), 7.21 (6H, s, $\underline{Ph}CH_2$ and 6-H), 7.42 (1H, d, J=2 Hz, 8-H), 7.49 (2H, d, J=9 Hz, 2, 6-H of tosyl group), 7.68 (1H, d, J=9 Hz, 5-H), 8.32 (1H, d, J=9 Hz, NH), 10.32 (1H, br s, NH). $[\alpha]_D$: +43.13° (c=0.517, DMSO). *Anal.* Calcd for $C_{26}H_{24}N_2O_5S$: C, 65.53; H, 5.07; N, 5.87; S, 6.73. Found: C, 65.39; H, 5.05; N, 5.87; S, 6.70.

7-(Tricarbobenzoxy-L-arginyl)coumarinylamido-4-methanesulfonic Acid (7)——A solution of tricarbobenzoxy-L-arginine[14] (**15**, 576 mg, 1.0 mmol) and *N*-methylmorpholine (0.12 ml, 1.0 mmol) in distilled tetrahydrofuran (4 ml) was cooled to −15 °C, and isobutyloxycarbonyl chloride (0.13 ml, 1.0 mmol) was added. After 4 min, a solution of **5** (255 mg, 1.0 mmol) and *N*-methylmorpholine (0.14 ml, 1.2 mmol) in distilled dimethylformamide (18 ml) was added, and the reaction mixture was stirred for 1 h at −15 °C, then overnight at room temperature. The solvent was removed by evaporation, and the residue was suspended in ethyl acetate. This ethyl acetate layer was washed with 1 N hydrochloric acid and concentrated by evaporation. The solid was filtered off and washed with ethyl acetate. The yield of **7** was 330 mg (41%). IR (Nujol): 3280 (NH), 1720, 1710, 1680 and 1660 (C=O), 1610 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.68 (4H, br s, $CH_2C\underline{H}_2C\underline{H}_2CH$), 3.80—4.30 (3H, br, $C\underline{H}_2CH_2CH_2C\underline{H}$), 4.02 (2H, s, $C\underline{H}_2SO_3H$), 5.03 (2H, s, $PhCH_2$), 5.18 (4H, s, $PhC\underline{H}_2$), 6.28 (1H, s, 3-H), 7.10—7.50 (18H, m, Ph, 6-H and NH), 7.64 (1H, br, NH), 7.78 (1H, d, J=2 Hz, 8-H), 7.90 (1H, d, J=9 Hz, 5-H), 10.47 (1H, br s, NH).

7-L-Arginylcoumarinylamido-4-methanesulfonic Acid Methanesulfonic Acid Salt (8)——**7** (100 mg, 0.12 mmol) and anisol (0.2 ml, 1.8 mmol) were dissolved in methanesulfonic acid (2.4 ml, 36 mmol), and the solution was stirred at room temperature.[15] After 30 min, diethyl ether was added, then the precipitate was filtered off, washed with diethyl ether, ethanol and diethyl ether, and dried *in vacuo* at 60 °C. The yield of **8** was 55 mg (88%). IR (Nujol): 3160 (NH), 1700 (C=O), 1620 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.56 (2H, br, $CH_2CH_2C\underline{H}_2CH$), 1.81 (2H, br, $CH_2C\underline{H}_2CH_2CH$), 2.38 (3H, s, $C\underline{H}_3SO_3H$), 3.08 (2H, m, $C\underline{H}_2CH_2CH_2CH$), 4.05 (3H, br, $CH_2CH_2CH_2C\underline{H}$ and $C\underline{H}_2SO_3H$), 6.31 (1H, s, 3-H), 7.05 (3H, br, NH), 7.39 (1H, dd, J=9, 2 Hz, 6-H), 7.49 (1H, br, NH), 7.79 (1H, d, J=2 Hz, 8-H), 7.94 (1H, d, J=9 Hz, 5-H), 8.30 (2H, br, NH), 10.86 (1H, br, NH). $[\alpha]_D$: +283.4° (c=0.561, H_2O). *Anal.* Calcd for $C_{17}H_{25}N_5O_9S_2 \cdot H_2O$: C, 38.85; H, 5.18; N, 13.33; S, 12.20. Found: C, 38.66; H, 5.30; N, 13.05; S, 11.95.

7-(N^{α}-Benzoyl-L-arginyl)coumarinylamido-4-methanesulfonic Acid (9)——A solution of **8** (100 mg, 0.2 mmol) in water (10 ml) was cooled to 0 °C, and the pH was adjusted to 9 with triethylamine. To this solution, benzoyl chloride (0.02 ml, 0.2 mmol) was added three times at intervals of 2 h at pH 9 (adjusted with triethylamine). After 6 h, the solution was stored at room temperature and stirred overnight. The precipitate was filtered and washed with water. Recrystallization from 80% ethanol gave 50 mg (49%) of colorless needles. mp 225.5—228.5 °C. IR (Nujol): 3160 (NH), 1710, 1680 and 1660 (C=O), 1610 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.57 (2H, br, $CH_2CH_2C\underline{H}_2CH$). 1.78 (2H, br, $CH_2C\underline{H}_2CH_2CH$), 3.17 (2H, br, $C\underline{H}_2CH_2CH_2CH$), 4.02 (2H, s, $C\underline{H}_2SO_3H$), 4.66 (1H, br, $CH_2CH_2CH_2C\underline{H}$), 6.27 (1H, s, 3-H), 7.00 (3H, br, NH), 7.32—8.06 (9H, m, PhCO, 5, 6, 8-H and NH), 8.72 (1H, d, J=7 Hz, NH), 10.56 (1H, s, NH). $[\alpha]_D$: +42.78° (c=0.554, DMSO). *Anal.* Calcd for $C_{23}H_{25}N_5O_7S \cdot 3/2H_2O$: C, 50.92; H, 5.20; N, 12.91; S, 5.91. Found: C, 50.68; H, 4.94; N, 12.77; S, 6.03.

7-(N^{α}-Carbobenzoxy-L-arginyl)coumarinylamido-4-methanesulfonic Acid (10)——**10** was obtained from **8** (100 mg, 0.2 mmol) and carbobenzoxy chloride (0.02 ml, 0.2 mmol) by the same procedure as described for **9**. Recrystallization from 60% ethanol gave 70 mg (65%) of colorless needles. mp 214.5—218.5 °C. IR (Nujol): 3260 (NH), 1720, 1690 (C=O), 1610 (C=C) cm^{-1}. NMR (DMSO-d_6) δ: 1.05 (3H, t, J=7 Hz, $C\underline{H}_3CH_2OH$), 1.61 (4H, br, $CH_2C\underline{H}_2C\underline{H}_2CH$), 3.08 (2H, br, $C\underline{H}_2CH_2CH_2CH$), 3.44 (2H, q, J=7 Hz, $CH_3C\underline{H}_2OH$), 4.02 (2H, s, $C\underline{H}_2SO_3H$), 4.17 (1H, br, $CH_2CH_2CH_2C\underline{H}$), 5.05 (2H, s, $PhC\underline{H}_2$), 6.27 (1H, s, 3-H), 6.98 (3H, br, NH), 7.36 (7H, br, Ph, 6-H and NH), 7.70 (1H, br, NH), 7.85 (1H, d, J=2 Hz, 8-H), 7.90 (1H, d, J=9 Hz, 5-H), 10.49 (1H, br, NH). $[\alpha]_D$: +17.75° (c=0.507, DMSO). *Anal.* Calcd for $C_{24}H_{27}N_5O_8S \cdot C_2H_5OH$: C, 52.78; H, 5.62; N, 11.83; S, 5.41. Found: C, 52.67; H, 5.65; N, 11.83; S, 5.31.

7-(*N*-Benzyloxycarbonylglycylglycyl-L-arginyl)aminocoumarin-4-methanesulfonic Acid (11)——A solution of Z-Gly-Gly-OH (53 mg, 0.2 mmol) and *N*-methylmorphorin (0.022 ml, 0.2 mmol) in distilled tetrahydrofuran (4 ml) was

cooled to −15 °C, and then isobutylchlorocarbonate (0.027 ml, 0.2 mmol) was added with stirring. After 3 min, a solution of **8** (105 mg, 0.2 mmol) and *N*-methylmorphorin (0.022 ml, 0.2 mmol) in distilled dimethylformamide (12 ml) was added dropwise at the same temperature. After 1 h, the solution was allowed to come to room temperature and stirred overnight. After removal of solvent, ethanol was added to the residue. The precipitates were collected by suction, and washed with ethanol and then with water. Recrystallization from 60% ethanol gave 68 mg (50%) of colorless needles. mp 180.5—184.5 °C. IR (Nujol): 3180 (NH), 1710, 1700 and 1670 cm^{-1}. NMR (DMSO-d_6) δ: 1.56 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH$), 3.08 (2H, s, $C\underline{H}_2CH_2CH_2CH$), 3.67 (2H, d, J=5 Hz, CH_2 of Gly), 3.81 (2H, d, J=5 Hz, CH_2 of Gly), 4.01 (2H, s, $C\underline{H}_2SO_3H$), 4.45 (1H, s, $CH_2CH_2CH_2C\underline{H}$), 5.03 (2H, s, $PhC\underline{H}_2$), 6.27 (1H, s, 3-H), 6.99 (3H, br, NH), 7.34 (7H, br, Ph, 6-H and NH), 7.54 (1H, br, NH), 7.81(1H, d, J=2 Hz, 8-H), 7.90 (1H, d, J=9 Hz, 5-H), 8.21 (2H, br, NH), 10.41 (1H, br, NH). $[\alpha]_D$: −3.77° (c=0.530, DMSO). *Anal.* Calcd for $C_{28}H_{33}N_7O_{10}S \cdot H_2O$: C, 49.43; H, 5.21; N, 14.46; S, 4.73. Found: C, 49.89; H, 5.14; N, 14.25; S, 4.69.

7-(*N*-Benzyloxycarbonyl-L-phenylalanyl-L-arginyl)aminocoumarin-4-methanesulfonic Acid (12)——**12** was obtained by the same procedure as described for **11** from Z-L-Phe-OH (60 mg, 0.2 mmol) and **8** (105 mg, 0.2 mmol). Recrystallization from 60% ethanol gave 38 mg (27%) of colorless needles. mp 185.5—190.5 °C. IR (Nujol): 3180 (NH), 1710 and 1660 (C=O) cm^{-1}. NMR (DMSO-d_6) δ: 1.56 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH$), 3.07 (4H, m, $C\underline{H}_2CH_2CH_2CH$ and $PhC\underline{H}_2CH$), 3.99 (2H, s, $C\underline{H}_2SO_3H$), 4.44 (2H, m, $CH_2CH_2CH_2C\underline{H}$ and $PhCH_2C\underline{H}$), 4.92 (2H, s, $PhC\underline{H}_2OCO$), 6.25 (1H, s, 3-H), 6.97 (3H, br, NH), 7.25 (13H, br, Ph, 6-H and NH), 7.76 (1H, d, J=2 Hz, 8-H), 7.88 (1H, d, J=9 Hz, 5-H), 8.38 (1H, d, J=7 Hz, NH), 10.50 (1H, br, NH). $[\alpha]_D$: +13.8° (c=0.500, DMSO). *Anal.* Calcd for $C_{33}H_{36}N_6O_9S \cdot H_2O$: C, 55.77; H, 5.39; N, 11.82; S, 4.51. Found: C, 55.50; H, 5.21; N, 11.76; S, 4.49.

Assay Procedure——α-Chymotrypsin: A solution (0.05 ml) of 1—2 mg/ml of α-chymotrypsin (Worthington, 3×crystallized) was added to 0.02—0.1 mM substrate in 80 mM Tris–HCl buffer (pH 7.8, 2 ml) containing 100 mM calcium chloride at 25 °C, and the increase in emission at 462 nm was measured (excited at 380 nm). In the assay for **14**, the assay solution contained 10% (v/v) DMSO.

Trypsin: A solution (0.05 ml) of 10—30 mg/ml of trypsin (Worthington, 2×crystallized) was added to 0.01—0.1 mM substrate in 50 mM Tris–HCl buffer (pH 8.0, 2 ml) containing 20 mM calcium chloride at 25 °C, and the increase in emission at 462 nm was measured (excited at 380 nm).

Papain: A solution (0.05 ml) of 1.1—5.5 mg/ml of papain (Sigma, 2×crystallized) was added to 0.016—0.072 mM substrate in 50 mM Tris–HCl buffer (pH 7.5, 2 ml) containing 5 mM L-Cys and 2 mM ethylenediaminetetraacetic acid (EDTA) at 25 °C.

Thrombin: A solution (0.05 ml) of 13 mg/ml of thrombin (Mochida Pharmaceutical Co.) was added to 0.016—0.093 mM substrate in 50 mM Tris–HCl buffer (pH 8.0, 2 ml) containing 100 mM calcium chloride at 37 °C.

Plasmin: A solution (0.05 ml) of 4.6—68 mg/ml of plasmin (Sigma) in 50 mM Tris–HCl buffer (pH 9.0) containing 20 mM L-Lys, 100 mM NaCl, 3 mM EDTA and 25% glycerol was added to 0.015—0.095 mM substrate in 50 mM Tris–HCl buffer (pH 9.0, 2 ml) containing 20 mM L-Lys and 100 mM NaCl at 37 °C.

References and Notes

1) Part XIII of "Organic Fluorescent Reagents." For Part XII: See Y. Kanaoka, T. Takahashi, H. Nakayama, and K. Tanizawa, *Chem. Pharm. Bull.*, **33**, 1721 (1985).
2) *a*) Y. Kanaoka, T. Takahashi, and H. Nakayama, *Chem. Pharm. Bull.*, **25**, 362 (1977); *b*) Y. Kanaoka, T. Takahashi, H. Nakayama, T. Kimura, and S. Sakakibara, *ibid.*, **25**, 3136 (1977); *c*) T. Sekine, H. Itakura, T. Namihisa, T. Takahashi, H. Nakayama, and Y. Kanaoka, *ibid.*, **29**, 3286 (1981); *d*) Y. Kanaoka, T. Takahashi, H. Nakayama, and T. Sekine, *ibid.*, **30**, 1485 (1982).
3) G. Schoellmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).
4) It is possible to measure the hydrolytic activity of chymotrypsin (concentration: 8.44×10^{-7} M) with **6** in 10 min, even though the k_{cat}/K_m value is small.
5) M. Zimmerman, B. Ashe, E. C. Yurewicz, and G. Patel, *Anal. Biochem.*, **78**, 47 (1977).
6) P. A. Pierzchala, C. P. Dorn, and M. Zimmerman, *Biochem. J.*, **183**, 555 (1979).
7) T. Morita, H. Kato, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara, *J. Biochem.* (Tokyo), **82**, 1495 (1977).
8) *a*) R. S. Rush, M. Mitas, J. C. Powers, T. Tanaka, and L. B. Hersh, *Arch. Biochem. Biophys.*, **231**, 390 (1984); *b*) M. Nishikata, H. Yokosawa, and S. Ishii, *Chem. Pharm. Bull.*, **34**, 2931 (1986).
9) N. Yoshida, M. T. Everitt, H. Neurath, R. Woodbury, and J. C. Powers, *Biochemistry*, **19**, 5799 (1980).
10) M. J. Castillo, K. Nakajima, M. Zimmerman, and J. C. Powers, *Anal. Biochem.*, **99**, 53 (1979).
11) R. L. Atkins and D. E. Bliss, *J. Org. Chem.*, **43**, 1975 (1978).
12) A. Burger and G. Ullyot, *J. Org. Chem.*, **12**, 346 (1947).
13) E. A. Popenoe and V. de Vigneaud, *J. Am. Chem. Soc.*, **76**, 6202 (1954).
14) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
15) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).