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Organic Fluorescent Reagents

XIV. Novel Fluorogenic Substrates for Microdetermination of Chymotrypsin and Aminopeptidase: Bimane Fluorescence Appears after Hydrolysis

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The fluorescence of 9,10-dioxa-*syn*-3,4,6,7-tetramethylbimane (bimane) was found to be quenched in the presence of tryptophan or tyrosine. Based on this observation, the bimane system was utilized as a fluorophore within proteolytic enzyme substrates. Bimane peptides containing tryptophan (**1a–1f**) were prepared and shown to be potent fluorogenic substrates for the assay of chymotrypsin and aminopeptidase.

In the course of the studies of organic fluorescence reagents by Kanaoka *et al.* (*1*), the usefulness of 7-amino-4-methylcoumarin as a fluorophore and its amide derivatives as practical fluorogenic substrates for hydrolytic enzymes has been reported (*2*). A novel application of 7-amino-4-nitro-2-oxa-1,3-diazole as a fluorogenic amine for hydrolytic enzyme substrates was also proposed (*3*).

9,10-Dioxa-*syn*-3,4,6,7-tetramethylbimane (bimane) was initially introduced as a new class of highly fluorescent compounds by one of the authors (E.M.K.) (*4*). Bimane is a bicyclic heterocyclic system and has appreciable solubility in water, in contrast to the low solubility of 7-amino-4-methylcoumarin (AMC).[2] The emission maximum of bimane is 480 nm, at a considerably longer wavelength than that of AMC (440 nm). Extensive studies have been made regarding the chemical and photophysical properties of the bimane system (*5–9*), and bimane fluorescent labels have also been reported (*10–13*). In the present work, we describe fluorogenic substrates for some proteolytic enzymes utilizing quenching properties of the bimane system.

We have observed that the fluorescence of bimane is quenched in the presence of tryptophan or tyrosine. To evaluate the influence of amino acids on the fluorescence of bimane, the fluorescence intensity of bimane in the presence and the absence of amino acids was measured; the relative intensities are listed in Table 1. Although most amino acids tested have no effect, bimane fluorescence is strongly quenched by the methyl esters of tryptophan and tyrosine. This observation sug-

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[2] Abbreviations used: AMC, 7-amino-4-methylcoumarin; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Bim, bimane.

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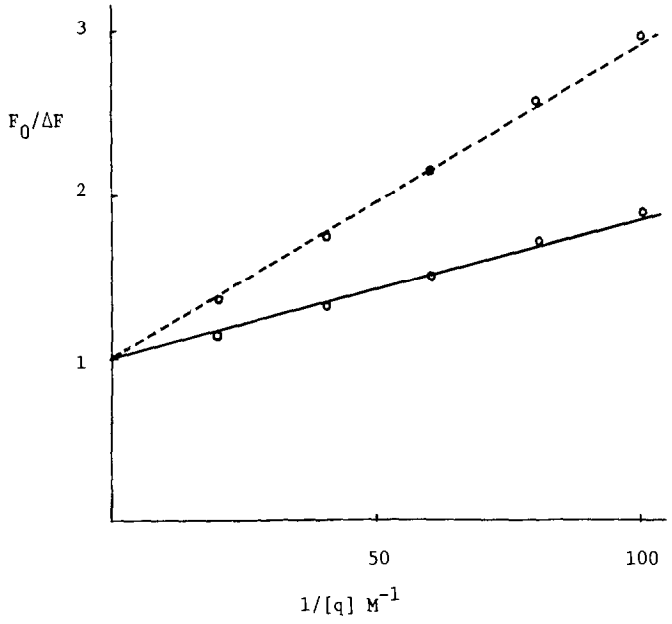


FIG. 1. Stern-Volmer plots for quenching of bimane fluorescence in the presence of tryptophan and tyrosine methyl esters. F_0, Fluorescence intensity of bimane in the absence of amino acid. F, Fluorescence intensity of bimane in the presence of amino acid. ΔF: $F_0 - F$. [q], Concentration of quenchers: tryptophan methyl ester, —; tyrosine methyl ester, ---.

gested that quenching by certain amino acids of the bimane fluorescence may be utilized in the design of new fluorogenic substrates for enzymes.

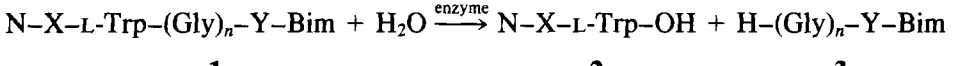
Stern-Volmer plots on quenching of bimane fluorescence with tryptophan or tyrosine are shown in Fig. 1. From these plots, the Stern-Volmer constants ($k_q\tau$)

TABLE 1

Relative Fluorescence Intensity of 9,10-Dioxa-*syn*-3,4,6,7-tetramethylbimane in the Presence of L-Amino Acids

Amino acid	F/F_0	Amino acid	F/F_0
Glycine	0.99	Cystine dimethyl ester	0.99
Leucine	1.03	Cysteine methyl ester	0.97
Proline	1.00	Methionine	0.89
Serine	1.01	Histidine	0.98
Asparagine	1.00	Phenylalanine	1.08
Lysine	1.01	Tyrosine methyl ester	0.11
Arginine	1.03	Tryptophan methyl ester	0.03

Note. The fluorescence intensity of bimane (1.19×10^{-5} M) in the presence (F) and in the absence (F_0) of amino acids (1.00×10^{-1} M) in 0.08 M Tris buffer, pH 7.80, is shown. λ_{ex}, 397 nm; λ_{em}, 480 nm.



N–Boc–Gly–NH–Bim

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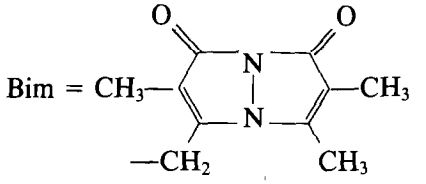
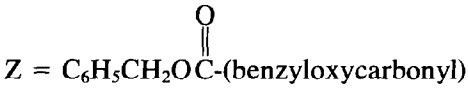
a: X = Z, Y = NH, n = 0
b: X = Z, Y = NH, n = 1
c: X = Z, Y = O, n = 0
d: X = H, Y = NH, n = 0
e: X = H, Y = NH, n = 1
f: X = H, Y = O, n = 0





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were determined to be 115 and 63 M^{-1} for tryptophan and tyrosine, respectively. Since the value for tryptophan is higher than that for tyrosine, we decided to prepare bimane peptides containing tryptophan as candidate fluorogenic substrates for proteolytic enzymes. First, bimane peptides of **1a–1c** were prepared as fluorogenic substrates for chymotrypsin.

Peptides **1a** and **1b** are synthesized by mixed anhydride method from *N*-carbobenzoxy-L-tryptophan or *N*-carbobenzoxy-L-tryptophylglycine and 9,10-dioxa-*syn*-(aminomethyl, methyl) (methyl, methyl) bimane (aminobimane) (*7*). Substrate **1c** is prepared from the potassium salt of *N*-carbobenzoxy-L-tryptophan through reaction with 9,10-dioxa-*syn*-(bromomethyl, methyl) (methyl, methyl) bimane (bromobimane) (*7*). The relative fluorescence intensities of **1a, 1b,** and **1c** are 0.04, 0.30, and 0.02, respectively, versus that of bimane (excitation at 397 nm, emission at 480 nm) in buffer solution (pH 7.8), indicating that the bimane fluorescence is quenched by intramolecularly bound tryptophan. This type of quenching interaction is known in many systems (*14, 15*). Excitation and emission curves are illustrated for peptides **1a–1c** in Fig. 2.

The kinetic parameters for the fluorogenic substrates **1a–1c** of chymotrypsin are listed in Table 2. Although the k_{cat} value of **1a** is not high, the values of **1b** and **1c** are comparable to those of reported fluorogenic substrates for chymotrypsin such as glutaryl–Phe–AMC and Ala–Ala–Phe–AMC (*16*). The linearity of the plot of fluoroescence intensity versus enzyme concentration is satisfactory with the lower limit of 0.1 nM enzyme as shown in Fig. 3.

As additional examples of bimane-fluorogenic substrates, peptides **1d–1f** were prepared for aminopeptidase. Catalytic hydrogenation of **1a–1c** on palladium carbon under a hydrogen atmosphere at ordinary pressure removed a N-protecting group of tryptophan residue to afford the desired substrates **1d–1f.** These compounds were subjected to hydrolysis by leucine aminopeptidase, which has a broad substrate specificity for not only leucine but also for other amino acids (*17*)

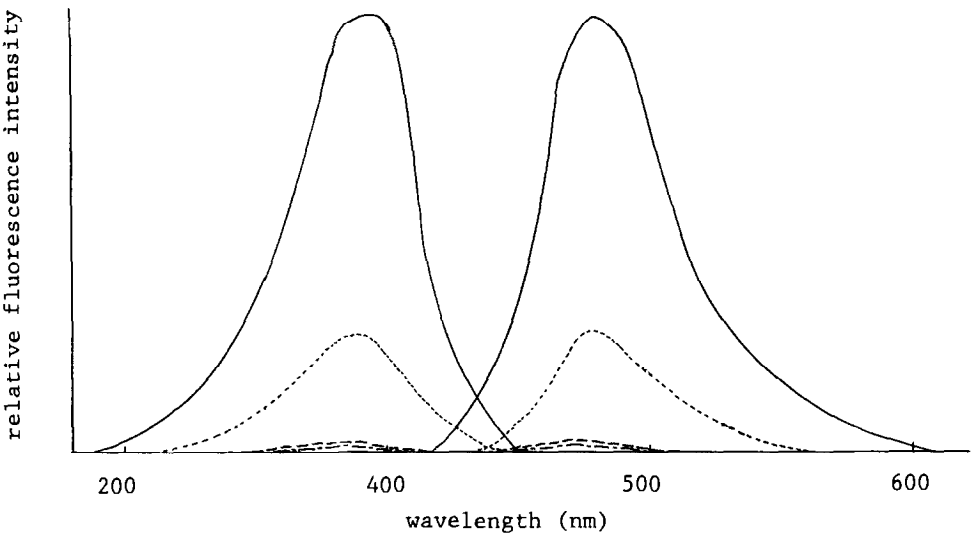

FIG. 2. Fluorescence spectra of bimane derivatives. Relative fluorescence intensity is in parentheses. Bimane (1.00), —; N-Z-L-Trp-NH-Bim (0.04), ---; N-Z-L-Trp-Gly-NH-Bim (0.30), ···; N-Z-L-Trp-O-Bim (0.02), -·-.

(Table 2). The ester substrate **1f** has a higher k_{cat} value than that of the amide substrate **1d,** but the amide substrate **1e** containing glycine between tryptophan and bimane also has a high k_{cat} value, and these substrates also have K_m values comparable to the values for fluorogenic substrates of 7-L-leucyl-4-methylcoumarinyl amide (*18*) and also with the intramolecularly quenched fluoroescence substrate of Lys(ABz)-X-ONBzl (*19*). As shown in Fig. 4, the linearity of the fluorescence intensity versus varying enzyme concentration (lowest concentration of 0.1 nM) was satisfactory.

Previous work has shown that strong donor groups can quench bimane fluorescence (*20*). Since indole rings should be donors in character, we infer that tryptophan transfers an electron to the bimane excited state. The resulting ion pair, Trp^{+} Bim^{-}, undergoes reverse electron transfer to yield the ground state, S_0. It is likely that the proximity of the indole and bimane rings favors electron transfer, so

TABLE 2

Kinetic Parameters of the Fluorogenic Substrates for Chymotrypsin and Aminopeptidase

Enzyme	Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Chymotrypsin	N-Z-L-Trp-NH-Bim	8.3×10^{-5}	5.5×10^{-4}	6.6
	N-Z-L-Trp-Gly-NH-Bim	1.0×10^{-4}	9.4×10^{-3}	9.4×10
	N-Z-L-Trp-O-Bim	4.6×10^{-5}	1.5×10	3.3×10^{5}
Aminopeptidase	H-L-Trp-NH-Bim	5.0×10^{-4}	1.4×10^{-1}	2.8×10^{2}
	H-L-Trp-Gly-NH-Bim	5.0×10^{-4}	1.9×10	3.8×10^{4}
	H-L-Trp-O-Bim	2.5×10^{-4}	3.7	1.5×10^{4}

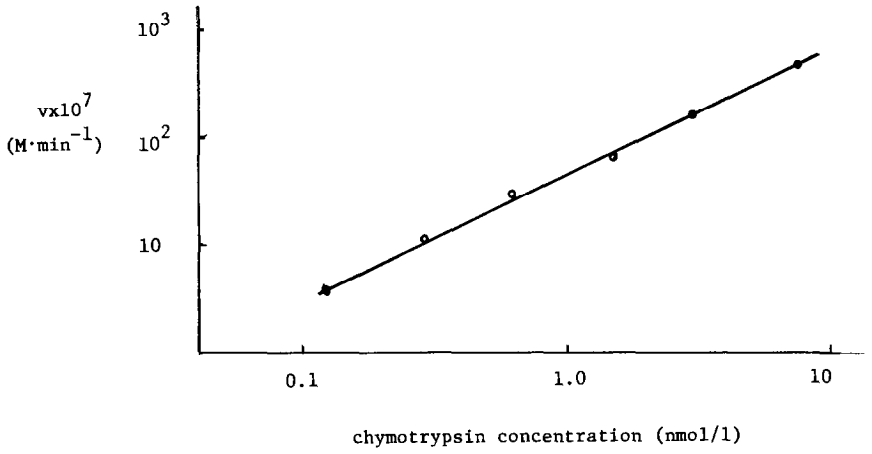

FIG. 3. Initial velocity vs chymotrypsin concentration. Assays were performed as described under Experimental. Substrate, N–Z–L-Trp–O–Bim. Time of incubation, 6 min.

that the fluorescence of the peptide **1b** is less quenched than that of **1a** or **1c**. Assuming that τ for the tryptophan and tyrosine methyl esters is about 10 ns (*21*), the quenching constant, k_q, is bout 10^{10} M^{-1} s^{-1}, i.e., diffusion controlled.

The set of molecules containing both bimane and certain aromatic amino acid residues in the peptide molecule are potent fluorogenic substrates. Further application of the bimane system for other enzymes like carboxypeptidase A and the angiotensin converting enzyme are now under investigation.

EXPERIMENTAL

All melting points are uncorrected. Infrared spectra were recorded on a JASCO IRA-1 spectrometer in Nujol nulls. Fluorescence measurements were performed

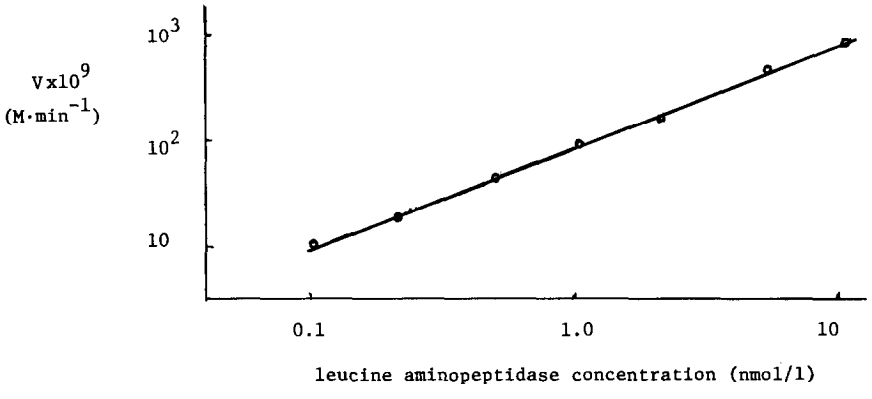

FIG. 4. Initial velocity vs leucine aminopeptidase concentration. Assays were performed as described under Experimental. Substrate, H–L-Trp–Gly–NH–Bim. Time of incubation, 8 min.

with a Hitachi Model 650-60 fluorescence spectrometer. Ultraviolet absorption spectra were obtained with a Hitachi Model 210-10 spectrophotometer.

9,10-Dioxa-syn-(N-carbobenzoxy-L-tryptophylaminomethyl, Methyl) (Methyl, Methyl) Bimane (***1a***)

To a stirred, ice–salt-cooled (−15°C) solution of 338 mg (1.0 mmol) of *N*-carbobenzoxy-L-tryptophan and 116 mg (1.0 mmol) of *N*-ethylmorpholine in 10 ml of anhydrous THF, 137 mg (1.0 mmol) of isobutyloxycarbonyl chloride was added dropwise. After the mixture was stirred for 10 min, 207 mg (1.0 mmol) of 9,10-dioxa-*syn*-(aminomethyl, methyl) (methyl, methyl) bimane (*7*) in 3 ml of anhydrous DMF was added dropwise, and the reaction mixture was stirred at −15°C for 2 h and then overnight at room temperature. Most of the THF was evaporated, and the residue was again dissolved in ethyl acetate. The organic solution was washed with saturated sodium bicarbonate solution, water, dilute hydrochloric acid, and water and then dried over anhydrous sodium sulfate. The removal of solvent leaves a solid, which was recrystallized from ethyl acetate–*n*-hexane to give the product as pale yellow fine needles, mp 145–148°C; 228 mg, 43%. $[\alpha]_D^{22} = -9.8°$ ($c = 0.63$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 1735, 1730 (sh), 1700, 1670, and 1650. *Anal.* Calcd for $C_{29}H_{29}N_5O_5$: C, 66.02; H, 5.54; N, 13.28. Found: C, 65.90; H, 5.63; N, 13.00.

9,10-Dioxa-syn-(N-carbobenzoxy-L-tryptophyl-glycyl-aminomethyl, Methyl) (Methyl, Methyl) Bimane (***1b***)

Preparation of **1b** was carried out essentially as described for **1a** from 395 mg (1.0 mmol) of *N*-carbobenzoxy-L-tryptophyl-glycine with 207 mg (1.0 mmol) of 9,10-dioxa-*syn*-(aminomethyl, methyl) (methyl, methyl) bimane (*7*). **1b** was obtained as pale yellow needles of mp 173–176°C by recrystallization from methanol–ethyl acetate; 320 mg, 55%. $[\alpha]_D^{22} = -18°C$ ($c = 1.1$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 1735 and 1655. *Anal.* Calcd for $C_{31}H_{32}N_6O_6$ 1/2 H_2O: C, 62.72; H, 5.60; N, 14.16. Found: C, 62.86; H, 5.74; N, 13.87.

9,10-Dioxa-syn-(N-carbobenzoxy-L-tryptophyloxymethyl, Methyl) (Methyl, Methyl) Bimane (***1c***)

To a stirred, ice-water-cooled solution of 169 mg (0.5 mmol) of *N*-carbobenzoxy-L-tryptophan in 20 ml of anhydrous THF, 56 mg (0.5 mmol) of potassium *tert*-butoxide was added portionwise, the mixture was stirred for another 10 min at the same temperature, then 136 mg (0.5 mmol) of 9,10-dioxa-*syn*-(bromomethyl, methyl) (methyl, methyl) bimane (*7*) was added portionwise, and the reaction mixture was stirred at 0°C for 1 h and then overnight at room temperature. The solvent was removed under reduced pressure, and the residue was treated with ethyl acetate, which was washed with saturated sodium bicarbonate solution and water, and then dried over anhydrous sodium sulfate. Removal of the solvent leaves a solid. Recrystallization from ethanol gave pale yellow fine prisms of mp 181–184°C; 211 mg, 80%. $[\alpha]_D^{22} = -42°$ ($c = 0.92$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 1760, 1745,

1730 (sh), and 1700. *Anal.* Calcd for $C_{29}H_{28}N_4O_6$: C, 65.90; H, 5.34; N, 10.60. Found: C, 65.82; H, 5.23; N, 16.65.

*9,10-Dioxa-syn-(L-tryptophylaminomethyl, Methyl) (Methyl, Methyl) Bimane Tosylate (**1d** Tosylate)*

A mixture of 53 mg (0.1 mmol) of **1a** and 19 mg (0.1 mmol) of *p*-toluenesulfonic acid in 8 ml ethanol was hydrogenated over 5 mg of 10% Pd·–C under hydrogen atmosphere at atmospheric pressure. The Pd–C was filtered off and washed with ethanol and the combined filtrate and washings were evaporated *in vacuo*. The solid residue was recrystallized twice from ethanol to yield the **1d** tosylate as pale yellow fine prisms, mp 177–179°C; 48 mg, 85%. $[\alpha]_D^{22} = +19°$ ($c = 0.58$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 3280, 1735, and 1685. *Anal.* Calcd for $C_{28}H_{31}N_5O_6S\ 2H_2O$: C, 55.90; H, 5.86, N, 11.64; S, 5.32. Found: C, 55.93; H, 5.58; N, 11.50; S, 5.18.

*9,10-Dioxa-syn-(L-tryptophyl-glycyl-aminomethyl, Methyl) (Methyl, Methyl) Bimane Tosylate (**1e** Tosylate)*

The **1e** tosylate was obtained from 409 mg (0.7 mmol) of **1b** in 30 ml methanol in the manner described for **1d** tosylate. Recrystallization of the product from methanol–ethyl acetate gave pale yellow fine needles of mp 144–147°C; 392 mg, 90%. $[\alpha]_D^{22} = +7.8°$ ($c = 0.45$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 3290, 1735, and 1655. *Anal.* Calcd for $C_{30}H_{34}N_6O_7S\ H_2O$; C, 56.24; H, 5.66; N, 13.12; S, 5.00. Found: C, 56.45; H, 5.39; N, 12.89; S, 5.16.

*9,10-Dioxa-syn-(L-tryptophyloxymethyl, Methyl) (Methyl, Methyl) Bimane (**1f** Tosylate)*

The **1f** tosylate was prepared by the procedure described for **1d** tosylate from 53 mg (0.1 mmol) of **1c** in 6 ml of ethanol. Recrystallization of the product from ethanol afforded pale yellow fine prisms of mp 160–163°C; 54 mg, 93%. $[\alpha]_D^{22} = -15°$ ($c = 0.39$, DMF). ir ν_{max}^{Nujol} cm^{-1}: 3300 and 1725. *Anal.* Calcd for $C_{28}H_{30}N_4O_7S\ H_2O$: C, 58.42; H, 5.43; N, 9.73; S, 5.57. Found: C, 58.43; H, 5.35; N, 9.67; S, 5.54.

*9,10-Dioxa-syn-(N-tert-butoxycarbonyl-glycyl-aminomethyl, Methyl) (Methyl, Methyl) bimane (**4**)*

To a stirred solution of 175 mg (1.0 mmol) of *N-tert*-butoxycarbonyl-glycine and 207 mg (1.0 mmol) of 9,10-dioxa-*syn*-(aminomethyl, methyl) (methyl, methyl) bimane (*7*) in 4 ml of DMF, 172 mg (1.0 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added. After the reaction mixture was stirred overnight at room temperature, it was diluted with 50 ml of ethyl acetate, washed with 10% citric acid, saturated sodium chloride solution, saturated sodium bicarbonate, and saturated sodium chloride, and then dried over anhydrous sodium sulfate. Removal of the solvent leaves a solid, which was recrystallized from ethanol, of pale yellow fine prisms of mp 193–194°C, 153 mg, 43%. Ir ν_{max}^{Nujol} cm^{-1}: 1745, 1675, and 1630. *Anal.* Calcd for $C_{17}H_{24}N_4O_5$: C, 56.03; H, 6.64; N, 15.38. Found: C, 55.74; H, 6.89; N, 15.66.

*9,10-Dioxa-syn-(glycylaminomethyl, Methyl) (Methyl, Methyl) Bimane (**3e**)*

An aliquot of 182 mg (0.5 mmol) of **4** was treated in 10 ml of 1 N HCl-AcOH for 20 min, the solution was evaporated, and the residue was treated twice with anhydrous methanol. Recrystallization from methanol afforded pale yellow fine prisms of mp 286–288°C, 126 mg, 84%. ir ν_{max}^{Nujol} cm^{-1}: 1720, 1680, and 1640. *Anal.* Calcd for $C_{12}H_{16}N_4O_3$ HCl: C, 47.92; H, 5.70; N, 18.63; Cl, 11.79. Found: C, 47.62; H, 5.64; N, 18.51; Cl, 11.56.

*Hydrolyses of **1a–1e** by Chymotrypsin*

(a) Linear relation of the fluorescence intensity vs enzyme concentration. Solutions (10–50 μl) of 1.26×10^{-8}–1.52×10^{-6} M chymotrypsin (Worthington Biochemical) were added to 100 μl of 6.39×10^{-4} M **1c** in 0.08 M Tris HCl buffer (2.0 ml) containing 0.1 M $CaCl_2$ (pH 7.8) and 4.7% EtOH at 25°C, and the increase in emission at 483 nm (appearance of HO-bimane) was measured (excitation at 399 nm).

(b) Kinetic parameters (K_m and k_{cat}) measurement. A solution (100 μl, **1a;** 50 μl, **1b;** 10 μl, **1c**) of 1.34×10^{-4} M (for **1a** and **1b**) or 3.20×10^{-7} M (for **1c**) chymotrypsin was added to 12.3–41.1 μM **1a,** 15.6–51.9 μM **1b,** or 8.21–27.4 μM **1c** in 0.08 M Tris–HCl buffer (2.0 ml) containing 0.1 M $CaCl_2$ (pH 7.8) and 4.7% EtOH at 25°C, and measurement was carried out in the manner described for (a).

*Hydrolyses of **1d–1f** by Leucine Aminopeptidase*

(a) Linear relation of the fluorescence intensity vs enzyme concentration. A solution (5–100 μl) of 2.2×10^{-8}–2.24×10^{-7} M leucine aminopeptidase (Sigma Chemical Co., porcine kidney) was added to 2.22×10^{-5} M **1e** in 0.05 M Tris–HCl buffer (2.0 ml) containing 0.01 M $MgCl_2$ (pH 8.0) and 1.2% DMSO at 25°C, and measurement was carried out in the manner described for (a) for chymotrypsin.

(b) Kinetic parameter (K_m and k_{cat}) measurement. A solution (50 μl, **1d;** 20 μl, **1e;** 10 μl, **1f**) of 1.57×10^{-6} M (for **1d–1f**) leucine aminopeptidase was added to 53.4–178 M **1d,** 66.4–221 μM **1e,** or 44.9–150 μM **1f** in 0.05 M Tris–HCl buffer (2.0 ml) containing 0.01 M $MgCl_2$ (pH 8.0) and 1.2% DMSO at 25°C, and measurement was carried out in the manner described for (a).

REFERENCES

1. Sato, E., Matsuhisa, A., and Kanaoka, Y. (1988) *Chem. Pharm. Bull.* **36,** in press.
2. Kanaoka, Y., Takahashi, T., Nakayama, H., Ueno, T., and Sekine, T. (1982) *Chem. Pharm. Bull.* **30,** 1485, and references cited therein. See also: Kanaoka, Y. (1977) *Angew. Chem. Int. Ed. Engl.* **16,** 137.
3. Sato, E., Miyakawa, M., and Kanaoka, Y. (1984) *Chem. Pharm. Bull.* **32,** 336.
4. Kosower, E. M., Pazhenchevsky, B., and Hershkowitz, E. (1978) *J. Amer. Chem. Soc.* **100,** 6516.
5. Huppert, D. H., Dodiuk, H., Kanety, H., and Kosower, E. M. (1979) *Chem. Phys. Lett.* **65,** 164.
6. Kosower, E. M., and Pazhenchevsky, B. (1980) *J. Amer. Chem. Soc.* **102,** 4983.

7. KOSOWER, E. M., PAZHENCHEVSKY, B., DODIUK, H., KANETY, H., AND FAUST, D. (1981) *J. Org. Chem.* **46,** 1666.
8. KANETY, H., DODUIK, H., AND KOSOWER, E. (1982) *J. Org. Chem.* **47,** 207.
9. KOSOWER, E. M., HEBEL, E., ROZEN, S., AND RADKOWSKI, A. E. (1985) *J. Org. Chem.* **50,** 4152 and references cited therein.
10. KOSOWER, N. S., KOSOWER, E. M., NEWTON, G. L., AND RANNEY, H. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3382.
11. HULBERT, P. B., AND YAKUBU, S. I. (1983) *J. Pharm. Pharmacol.* **35,** 384.
12. DANIELSOHN, A., AND NOTTE, A. (1987) *Histochemistry* **86,** 281.
13. KOSOWER, N. S., AND KOSOWER, E. M. (1987) *in* Methods in Enzymology (Jakoby, W. B., and Griffiths, O. W., Eds.), Vol. 143, p. 76, Academic Press, Orlando, FL.
14. GUILBAULT, G. G. (ed.) (1961) *in* Fluorescence, pp. 349, Dekker, New York.
15. YARON, A., CARMEL, A., AND KATCHALSKI-KATZIR, E. (1979) *Anal. Biochem.* **95,** 228.
16. ZIMMERMAN, M., ASHE, B., YUREWICZ, E. C., AND PARTEL, G. (1977) *Anal. Biochem.* **78,** 47.
17. SMITH, E. L., AND SPACKMAN, D. H. (1955) *J. Biol. Chem.* **212,** 271.
18. KANAOKA, Y., TAKAHASHI, T., AND NAKAYAMA, H. (1977) *Chem. Pharm. Bull.* **25,** 362.
19. CARMEL, A., KESSLER, E., AND YARON, A. (1977) *Eur. J. Biochem.* **73,** 617.
20. KOSOWER, E. M., KANETY, H., DODIUK, H., AND HERMOLIN, J. (1982) *J. Phys. Chem.* **86,** 1270.
21. CANTOR, C. R., AND SCHIMMEL, P. R. (1980) Biophysical Chemistry, Part II, pp. 443–444, Freeman, San Francisco.