# New Fluorogenic Substrates for Microdetermination of Cathepsin C[1)]

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**As a sensitive fluorometric assay for the activity of cathepsin C, bimane-peptides containing tryptophan were synthesized and shown to be fluorogenic substrates for the microdetermination of cathepsin C activity.**

**Keywords** bimane; tryptophan; intramolecular quenching; cathepsin; fluorogenic substrate; fluorometric enzyme assay

Cathepsins have been largely located in the lysosomal fraction of the cell. These enzymes have been considered to be the most active proteinases in the cell and important in intracellular protein catabolism.[2)] Of the cathepsins, the one most extensively studied and purified is cathepsin C (dipeptidyl aminopeptidase I, E.C. 3.4.14.1). This enzyme is a thiol proteinase and shows dipeptidyl amino-peptidase activity that cleaves dipeptidyl amide or ester having a free α-amino group in the *N*-terminal amino acid.[3)]

As a synthetic fluorogenic substrate for cathepsin C, dipeptidyl-β-naphthylamides have already been reported.[4)] To avoid the carcinogenic naphthylamine derivatives, it is preferable to develop novel fluorogenic substrates with a non-carcinogenic fluorophore.

In the course of the studies of organic fluorescent reagents, we have recently reported bimane-containing fluorogenic substrates for hydrolytic enzymes such as chymotrypsin, aminopeptidase, carboxypeptidase and angiotensin converting enzyme.[1,5)] In this paper we wish to report the preparation of three bimane-peptides containing tryptophan and the fluorometric assay for cathepsin C using these bimane-peptides as fluorogenic substrates.

The bimane-containing substrates (**1d—f**) were synthesized by usual peptide synthesis procedure (Chart 1). The amide substrates (**1d** and **1e**) were obtained by the mixed anhydride method from *N*-benzyloxycarbonyl (Z)-peptides and aminobimane (**3a**) followed by deprotection of *N*-termini. The ester substrate (**1f**) was synthesized by alkylation of the potassium salt of *N*-Z-glycyl-tryptophan (**2b**) with monobromobimane.

At an equimolar concentration ($1.09\times10^{-5}$, $0.466\times10^{-5}$, $0.246\times10^{-5}$ M for **1d** and **3d**, **1e** and **3e**, **1f** and **3f**, respectively), the relative fluorescence intensities of **1d—f** versus those of **3d—f** in 0.2 M citrate buffer solution (pH 6.0) were 0.08, 0.23, and 0.02, respectively. In these substrates the fluorescence of the bimane group is intramolecularly quenched by tryptophan incorporated in the substrate molecules. However, enzymatic release of glycyltryptophan leads to an increase in fluorescence which can be easily and continuously detected.

Kinetic parameters of **1d—f** for cathepsin C were obtained by continuous spectrofluorometric assay, and the results are listed in Table I. In order to compare the characteristics of the bimane substrates with those of previously reported fluorogenic β-naphthylamide substrates (**4** and **5**),[4)] the kinetic parameters for **4** and **5** were also measured with the same cathepsin C. Although the $k_{cat}$ value for **1d** is not high, the values for **1e** and **1f** are comparable to those for **4** and **5**. Values of $k_{cat}/K_m$ for **1e** and **1f** are comparable to that for **4**, and smaller than that for **5**.

From these kinetic results, substrate **1e** was selected and the linearity of the relationship between cathepsin C concentration and fluorescence intensity was examined. The linearity of the fluorescence enhancements *vs.* incubation time (for more than 6 min) are satisfactory. The rates of hydrolysis are proportional to enzyme concentration over an at least 600-fold range up to 17 ng/ml as shown in Fig. 1.

The kinetic parameters for these new substrates are unsatisfactory when compared with those for the naphthylamide substrate of **5**, though kinetic parameters for **1e** and **1f** are comparable with those for **4**. However, **1e** and **1f** were useful as the substrate since the bimane product

TABLE I. Kinetic Parameters of Fluorogenic Substrates for Cathepsin C

| Substrate | $K_m$ (M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| **1d** | $1.0\times10^{-3}$ | $8.4\times10^{-3}$ | 8.4 |
| **1e** | $1.3\times10^{-3}$ | $1.4\times10$ | $1.1\times10^{4}$ |
| **1f** | $1.5\times10^{-3}$ | $1.5\times10$ | $1.0\times10^{4}$ |
| H–Ala–Ala–βNA (**4**) | $5.0\times10^{-4}$ | $2.0\times10$ | $4.0\times10^{4}$ |
| H–Gly–Arg–βNA (**5**) | $3.1\times10^{-4}$ | $9.3\times10$ | $3.0\times10^{5}$ |

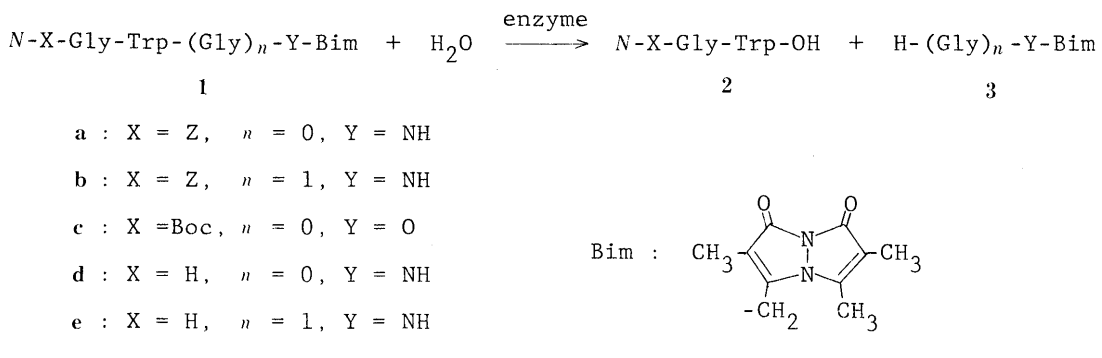


Chart 1

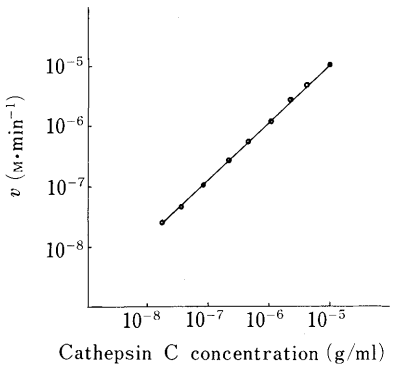


Fig. 1. Fluorescence Development *vs.* Cathepsin C Amount

Assays were performed as described under Experimental procedure. Substrate, **1e**. Cathepsin C amount ranged $1.70 \times 10^{-8}$ to $1.03 \times 10^{-5}$ g/ml.

(**3**) has higher fluorescence quantum yield (0.76 for **3d**, 0.79 for **3f**,[6] 0.50 for β-naphthylamine[7]) and longer fluorescence excitation wavelength (398 nm) than β-naphthylamine (335 nm).

It is also necessary to examine a variety of bimane-peptides with different combinations of amino acid residues in order to develop specific and sensitive substrates for each type of cathepsins, because there are many types of cathepsin (type A, B, C, H, L *etc.*).[2a,8] Further applications of the bimane system for other types of cathepsin are now under investigation.

## Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrophotometer in Nujol nulls. Optical rotations were obtained with a JASCO DIP-4 polarimeter. Fluorescence measurements were performed with a Hitachi 650-60 fluorescence spectrophotometer. Ultraviolet (UV) absorption spectra were obtained with a Hitachi 210-10 spectrometer. Analytical samples were dried *in vacuo* at 60 °C for 16 h. Thin layer chromatography (TLC) was carried out on silica gel (Kieselgel 60 $F_{254}$, Merck) using *n*-butanol–acetic acid–water (solvent ratio, 4:1:2) as eluent.

**9,10-Dioxa-*syn*-(*N*-Z-glycyl-tryptophyl-aminomethyl, methyl)(methyl, methyl)bimane (1a)** To the ice-salt cooled, stirred solution of *N*-Z–Gly–Trp–OH (395 mg, 1.0 mmol) and *N*-ethylmorphorine (115 mg, 1.0 mmol) in anhydrous tetrahydrofuran (THF) (20 ml), isobutyl chloroformate (131 mg, 1.0 mmol) was added dropwise. After 10 min, **3d**[6] (207 mg, 1.0 mmol) in dimethylformamide (DMF) (4 ml) was added dropwise, and then allowed to come to room temperature. After stirring over night, the solution was evaporated and ethyl acetate was added to the residue. Organic layer was washed with saturated sodium bicarbonate, water, 10% aq. citric acid and water, and then dried over anhydrous sodium sulfate. After removal of solvent, the residue was precipitated from MeOH–ether. Yellow powder, 320 mg (55%), mp 185—190 °C. IR (Nujol): 3280, 1740, 1715, 1655 cm$^{-1}$. $[\alpha]_D^{20}$ −4.8° ($c$=1.25, DMF). *Anal.* Calcd for $C_{31}H_{32}N_6O_6 \cdot 0.5H_2O$: C, 62.72; H, 5.60; N, 14.16. Found: C, 62.91; H, 5.45; N, 14.32.

**9,10-Dioxa-*syn*-(glycyl-tryptophyl-aminomethyl, methyl)(methyl, methyl)bimane *p*-Toluenesulfonic Acid (1d)** **1a** (117 mg, 0.2 mmol) was hydrogenated in the presence of 10% Pd–C (20 mg) and *p*-toluenesulfonic acid (40 mg) in MeOH (4 ml) under a hydrogen atmosphere. After filtration of Pd–C, the filtrate was evaporated *in vacuo*. The residue was precipitated from MeOH–ether. Pale yellow powder, 117 mg (94%). mp 222—226 °C (dec.). IR (Nujol): 1725, 1650 cm$^{-1}$. $[\alpha]_D^{21}$ −5.3° ($c$=1.18, DMF). *Anal.* Calcd for $C_{23}H_{26}N_6O_4 \cdot C_7H_8O_3S \cdot 2H_2O$: C, 54.70; H, 5.82; N, 12.76; S, 4.89. Found: C, 54.80; H, 5.95; N, 12.61; S, 4.67. *Rf* value of TLC is 0.49.

**9,10-Dioxa-*syn*-(*N*-Z-glycyl-tryptophyl-glycyl-aminomethyl, methyl)(methyl, methyl)bimane (1b)** **1b** was obtained by the same procedure with **1a** from *N*-Z–Gly–Trp–Gly–OH (452 mg, 1.0 mmol) and **3d**[6] (207 mg, 1.0 mmol). The product was obtained as glass (370 mg, 58%), which was used for the next step without further purification.

**9,10-Dioxa-*syn*-(glycyl-tryptophyl-glycyl-aminomethyl, methyl)(methyl, methyl)bimane *p*-Toluenesulfonic Acid (1e)** **1e** was prepared by the same procedure with **1d** from **1b** (370 mg, 0.58 mmol). Precipitation from EtOH–ether gave a pale yellow powder, 294 mg (75%), mp 220—225 °C (dec.). IR (Nujol): 1725, 1660 cm$^{-1}$. $[\alpha]_D^{21}$ −4.2° ($c$=1.68, DMF). *Anal.* Calcd for $C_{25}H_{29}N_7O_5 \cdot C_7H_8O_3S \cdot 2H_2O$: C, 53.69; H, 5.77; N, 13.70; S, 4.48. Found: C, 53.49; H, 5.65; N, 13.74; S, 4.30. *Rf* value of TLC is 0.46.

**9,10-Dioxa-*syn*-(*N*-Boc-glycyl-tryptophyloxymethyl, methyl)(methyl, methyl)bimane (1c)** Potassium *tert*-butoxide (71 mg, 0.63 mmol) was added to the solution (20 ml) of **2c** (227 mg, 0.63 mmol) in THF. After gas ceased evolving (30 min), monobromobimane[9] (**3**: $n$=0, HY=Br) (171 mg, 0.63 mg) was added and the reaction mixture was stirred at room temperature over night. After evaporation of solvent, AcOEt was added to the residue. Organic layer was washed with saturated sodium bicarbonate and water, and then dried over anhyd. sodium sulfate. After removal of solvent, the residue was recrystallized from EtOH. Pale yellow fine needles, 280 mg (81%). mp 202—204 °C (dec.). IR (Nujol): 3320, 1750, 1695, 1655 cm$^{-1}$. $[\alpha]_D^{20}$ −22.8° ($c$=0.939, DMF). *Anal.* Calcd for $C_{28}H_{33}N_5O_7$: C, 60.97; H, 6.03; N, 12.70. Found: C, 60.92; H, 6.12; N, 12.71.

**9,10-Dioxa-*syn*-(glycyl-tryptophyloxymethyl, methyl)(methyl, methyl)bimane Hydrochloride (1f)** **1c** (110 mg, 0.2 mmol) was treated with 1 N HCl–AcOH (2.5 ml) at room temperature for 30 min. After addition of ether, precipitates were collected by suction and washed with ether. Recrystallization from MeOH–ether gave pale yellow needles, 96 mg (98%), mp 185—188 °C. IR (Nujol): 3320, 1735, 1670 cm$^{-1}$. $[\alpha]_D^{20}$ −15.2° ($c$=0.525, DMF). *Anal.* Calcd for $C_{23}H_{25}N_5O_5 \cdot HCl \cdot 1.5H_2O$: C, 53.64; H, 5.68; N, 13.60; Cl, 6.88. Found: C, 53.89; H, 5.53; N, 13.63; Cl, 6.82. *Rf* value of TLC is 0.71.

**Hydrolysis of 1d—f by Cathepsin C** a) Kinetic parameter ($K_m$ and $k_{cat}$) measurement: Cathepsin C from bovine spleen is a product of Sigma Chem. Comp. (activity: 15 units per mg protein) and the concentration of enzyme was estimated from optical density (1.54 when 1 mg/ml[10]) at 280 nm and molecular weight of 210000.[4] A solution of enzyme (100 μl for **1d**, 20 μl for **1e** and **1f**) was added to the substrate solution (2.1 ml containing 0.47% DMSO, final concentration of substrates: 49—165 μM for **1d**, 70—233 μM for **1e**, 146—487 μM for **1f**) in 0.2 M citrate buffer containing 40 mM mercaptoethylamine hydrochloride (pH 6.0) at 37 °C, and the increase in emission at 481 nm (appearance of **3**) was recorded (excitation at 398 nm). The rate of hydrolysis was established by comparing the rate of increase of fluorescence intensity with the fluorescence intensity of a standard bimane derivatives (**3d** for **1d**, **3e** for **1e**, **3f** for **1f**). Kinetic parameters for hydrolysis were obtained from Lineweaver–Burk plots. b) Linear relation of fluorescence intensity *vs.* enzyme concentration: Fluorescence measurement was carried out in the manner described in a). Final enzyme concentrations were $1.70 \times 10^{-8}$—$1.03 \times 10^{-5}$ g/ml and the substrate (**1e**) concentration was $1.05 \times 10^{-4}$ M.

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