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New Fluorogenic Substrates for Microdetermination of Carboxypeptidase A[1)]

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As a part of the development of a sensitive fluorometric assay for the activity of carboxypeptidase A, bimane-peptides containing tryptophan, *i.e.*, *S*-[9,10-dioxa-*syn*-(methyl, methyl)-(methylene, methyl)-bimane]-thioglycolyl-(glycyl)-L-tryptophan and *O*-{*S*-[9,10-dioxa-*syn*-(methyl, methyl) (methylene, methyl)-bimane]-thioglycolyl}-DL-3-indolelactic acid, were prepared and shown to be good fluorogenic substrates for microdetermination of carboxypeptidase A activity.

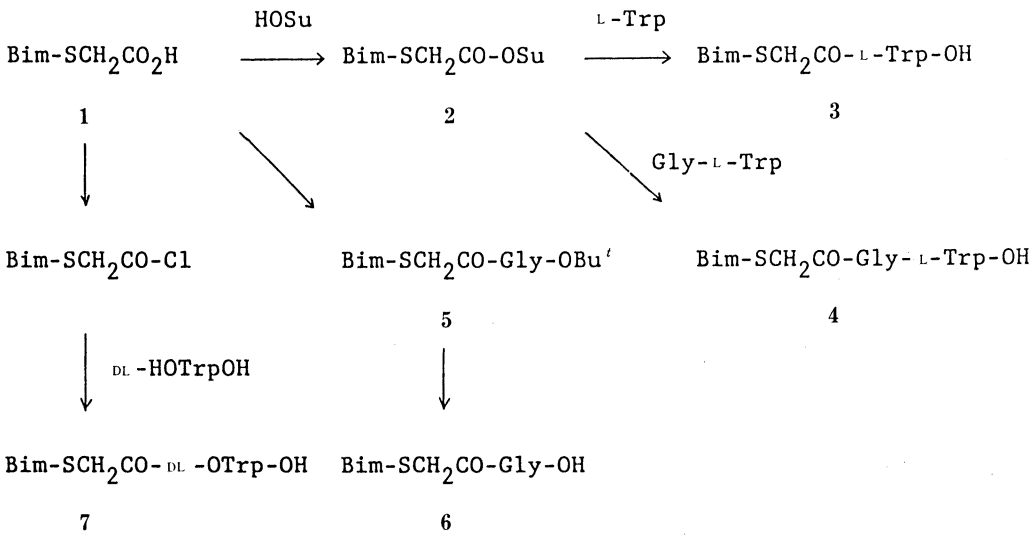
Keywords——9,10-dioxa-*syn*-3,4,6,7-tetramethylbimane; tryptophan; intramolecular quenching; fluorogenic substrate; fluorometric enzyme assay; carboxypeptidase A

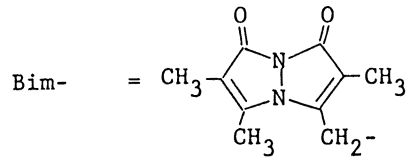
In a previous paper, we described an application of 9,10-dioxa-*syn*-3,4,6,7-tetramethylbimane (bimane) as the fluorophor in the fluorometric assay of hydrolytic enzyme substrates.[1)] In the present paper, we wish to report further applications of bimane to prepare fluorogenic substrates of carboxypeptidase A, as an extension of our work on fluorogenic substrates for microdetermination of hydrolytic enzymes.[2)]

Carboxypeptidase A is a exopeptidase hydrolyzing the peptide bond adjacent to the C-terminal end of a peptide chain. A standard method for the assay of carboxypeptidase A is a spectrophotometric procedure which utilizes hippuryl-L-phenylalanine as a substrate and involves monitoring small differences of absorbance at 254 nm.[3)] Assays of enzymic activity by the use of fluorescent substrates are faster and more sensitive than those with chromogenic substrates, and are usually designed to yield a fluorescent product. Colorimetric[4)] and fluorometric[5)] estimations of carboxypeptidase activity have also been reported, based on β-carbonaphthoxy-L-phenylalanine, by observing color development with diazonium salt or the difference in fluorescence intensities between the substrate and the product (β-naphtholate ion).

In general, the use of 7-peptidyl-4-methylcoumaryl amide substrates provides a very sensitive fluorometric method for hydrolytic enzyme assays.[6)] Although 4-methyl-7-aminocoumarin (AMC) may be applied to prepare fluorogenic substrates for analyses of endopeptidase, aminopeptidase, *etc.*, AMC has not yet been used for a carboxypeptidase substrate. As an example of fluorometric substrates for carboxypeptidase A, an intramolecularly quenching substrate was reported, in which the resonance energy transfer phenomenon was utilized with dansylated tryptophan-peptides as substrates.[7)] In an extension of this approach, we wish to describe here the utilization of intramolecular quenching by formation of excited complexes[1,8)] for carboxypeptidase A fluorogenic substrates. We have observed and reported in the preceding paper that the fluorescence of bimane is quenched in the presence of tryptophan, and bimane fluorescence is also quenched by intramolecularly bound tryptophan.[1)]

As fluorogenic substrates for carboxypeptidase A, bimane peptides containing tryptophan at the C-terminus were designed, and substrates **3**, **4** and **7** were synthesized as shown





HOTrpOH = 3-indolelactic acid
-OSu = *N*-hydroxysuccinimide ester
-OBu^t = *tert*-butoxy

Chart 1

in Chart 1. Monobromobimane was treated with thioglycolic acid to give bimane-thioglycolic acid (**1**). Amide substrates **3** and **4** were obtained by the active ester method from **1** with L-tryptophan or glycyl-L-tryptophan, respectively. The ester substrate **7** was synthesized by the acid chloride method from **1** and indolelactic acid.

In these substrates the fluorescence of the bimane group is intramolecularly quenched by the indole group of tryptophan incorporated in the substrate molecules. However, enzymatic cleavage of the C-terminal tryptophan leads to an increase in fluorescence which can be easily and continuously detected. The relative fluorescence intensities of **3**, **4** and **7** *versus* the fluorescence intensities of **1**, **6** and **1** are 0.05, 0.11 and 0.02, respectively (excitation at 399 nm, emission at 483 nm).

Table I lists the kinetic constants of the hydrolysis for the bimane substrates (**3**, **4** and **7**) by carboxypeptidase A. The k_{cat} values for **3** and **7** are low, but the value for **4** is comparable to the value for the reported fluorogenic substrate, dansylglycyltryptophan,[7] and values of K_m for **3**, **4**, **7** are also comparable to the reported values of carbonaphthoxy and dansyl substrates.[5,7] From these kinetic results, in spite of the poorer intramolecular quenching of **4** than **3**, substrate **4** was used for examination of the linearity of the relationship between carboxypeptidase A concentration and fluorescence intensity. Substrate **4** can be used to detect as little as around 0.1 nM (3.5 ng/ml) carboxypeptidase A using a substrate concentration of 2.03×10^{-5} M, as shown in Fig. 1.

In the present work we extended the usefulness of the bimane system to fluorogenic

TABLE I. Kinetic Parameters of the Fluorogenic Substrates for Carboxypeptidase A

Substrate	K_m(M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Bim-SCH_2CO-L-Trp-OH (**3**)	5.0×10^{-4}	2.3×10^{-3}	4.6
Bim-SCH_2CO-Gly-L-Trp-OH (**4**)	4.0×10^{-4}	2.8×10	7.0×10^{4}
Bim-SCH_2CO-DL-OTrp-OH (**7**)	2.0×10^{-4}	4.9×10^{-1}	2.5×10^{3}
β-Carbonaphthoxy-L-Phe-OH[5]	9.3×10^{-4}		
Dns-Gly-L-Trp-OH[7]	$0.75—1.30 \times 10^{-4}$	0.92—1.2	

H-OTrp-OH = 3-indolelactic acid. Dns = dansyl.

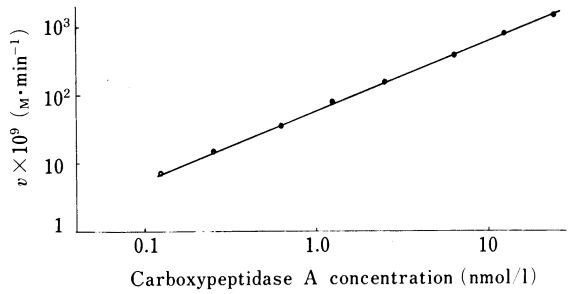


Fig. 1. Initial Velocity *vs.* Carboxypeptidase A Concentration

Assays were performed as described under Experimental. Substrate, **4**. Carboxypeptidase A concentration ranged from 0.126×10^{-9} to 24.0×10^{-9} M.

substrates for carboxypeptidase A. Compared with other fluorometric assays for carboxypeptidase, the bimane system has several advantages. 1) Bimane has a higher fluorescence intensity than naphthol.[9] 2) Bimane derivatives have a longer excitation maximum wavelength (399 nm) than others (317 nm for carbonaphthoxy substrate, 290 nm for dansyl substrate), so that assays with bimane substrates are not subject to interference, although biological materials such as reduced nicotinamide adenine dinucleotide (NADH) display strong intrinsic fluorescence under 330 to 360 nm excitation, giving rise to strong background signals.[10] 3) The bimane system has a constant fluorescence intensity over a wide pH range of 1—9. 4) An important advantage of the bimane system is its applicability to exopeptidases such as carboxypeptidase. Although AMC, which is the most popular fluorogenic amine for hydrolytic enzyme substrates, has been successfully applied to endopeptidases and aminopeptidases, it has not yet been applied to carboxypeptidase because of the difficulty of designing enzymatically hydrolyzable bonding between AMC and the amino side of amino acids.

These fluorescence characteristics of the bimane system are expected to be useful for the microdetermination of hydrolytic enzyme activities. Further application to other enzymes such as angiotensin converting enzyme is in progress.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrophotometer in Nujol nulls. Fluorescence measurements were performed with a Hitachi fluorescence spectrophotometer, model 650-60. Ultraviolet (UV) absorption spectra were obtained with a Hitachi 210-10 spectrometer.

9,10-Dioxa-*syn*-(carboxymethylthiomethyl, methyl)(methyl, methyl)-bimane (1)——A mixture of 386 mg (4.2 mmol) of thioglycolic acid and 424 mg (4.2 mg) of triethylamine was added dropwise to a stirred solution of 1150 mg (4.2 mmol) of 9,10-dioxa-*syn*-(bromomethyl, methyl)(methyl, methyl)-bimane[9] in 50 ml of acetonitrile. Stirring was continued overnight at room temperature, and the solvent was removed under reduced pressure. Ethyl acetate was added to the residue, then the organic solution was extracted with saturated sodium bicarbonate solution. The combined aqueous extracts were washed with ethyl acetate and then acidified with concentrated hydrochloric acid. The aqueous solution was extracted with ethyl acetate, and the extract was washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was

recrystallized from methanol to give pale yellow prisms of mp 210—212 °C; 615 mg, 52%. IR: 1725 cm^{-1}. *Anal.* Calcd for $C_{12}H_{14}N_2O_4S$: C, 51.06; H, 5.00; N, 9.95; S, 11.34. Found: C, 50.80; H, 5.01; N, 9.75; S, 11.54.

***N*-Hydroxysuccinimide Ester of *S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolic Acid (2)**——1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (383 mg, 2.0 mmol) was added portionwise to a stirred solution of 564 mg (2.0 mmol) of **1** and 230 mg (2.0 mmol) of *N*-hydroxysuccinimde in 8 ml of anhydrous dimethylformamide (DMF). After stirring overnight at room temperature, the solution was diluted with ethyl acetate, and washed with 10% hydrochloric acid and saturated sodium chloride, then dried over anhydrous sodium sulfate. Removal of solvent left a solid, which was recrystallized from ethyl acetate–acetonitrile to give pale yellow fine needles of mp 176—178 °C; 644 mg, 85%. IR: 1800, 1770, 1740, 1720 cm^{-1}. *Anal.* Calcd for $C_{16}H_{17}N_3O_6S$: C, 50.66; H, 4.52; N, 11.08; S, 8.44. Found: C, 50.42; H, 4.50; N, 11.00; S, 8.27.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-L-tryptophan (3)**——A solution of 76 mg (0.2 mmol) of **2** in 5 ml of acetonitrile was added to a stirred solution of 23 mg (0.22 mmol) of L-tryptophan and 37 mg (0.44 mmol) of sodium bicarbonate in 10 ml of water. Stirring was continued overnight at room temperature, and the solution was concentrated to about 2/3 of the original volume under reduced pressure, diluted with 30 ml of water, and then washed once with ethyl acetate. The aqueous solution was acidified with concentrated hydrochloric acid, then extracted with ethyl acetate. The extract was washed with water, and then dried over anhydrous sodium sulfate. After removal of the solvent, the residue was recrystallized from methanol to give pale yellow fine prisms of mp 215—217 °C; 67 mg, 72%. $[\alpha]_D^{22} = +16°$ ($c = 0.40$, DMF). IR: 1720, 1615 cm^{-1}. *Anal.* Calcd for $C_{23}H_{24}N_4O_5S \cdot 1/2\ H_2O$: C, 57.85; H, 5.28; N, 11.74; S, 6.70. Found: C, 58.11; H, 5.07; N, 11.69; S, 6.52.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-glycyl-L-tryptophan (4)**——Preparation of **4** was carried out as described for **3** from 57 mg (0.22 mmol) of glycyl-L-tryptophan and 76 mg (0.2 mmol) of **2**. **4** was obtained as a pale yellow powder of mp 216—220 °C (dec.) by precipitation from ethanol–ethyl acetate; 53 mg, 50%. $[\alpha]_D^{22} = +7.0°$ ($c = 0.82$, DMF). IR: 1730, 1655 cm^{-1}. *Anal.* Calcd for $C_{25}H_{27}N_5O_6S \cdot C_2H_5OH$: C, 56.73; H, 5.82; N, 12.25; S, 5.60. Found: C, 56.52; H, 5.53; N, 11.99; S, 5.44.

***tert*-Butyl Ester of *S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-glycine (5)**——EDC (43 mg, 0.25 mmol) was added to a stirred solution of **1** (74 mg, 0.25 mmol), glycine *tert*-butylester hydrochloride (42 mg, 0.25 mmol) and triethylamine (25 mg, 0.25 mmol) in 2 ml of anhydrous DMF. Stirring was continued overnight at room temperature, and the solution was diluted with ethyl acetate. The extract was washed with saturated sodium bicarbonate, saturated sodium chloride, 10% citric acid and saturated sodium chloride, and then dried over anhydrous sodium sulfate. Recrystallization from ethyl acetate gave pale yellow leaflets of mp 147—148 °C; 48 mg, 49%. IR: 3380, 1755, 1680 cm^{-1}. *Anal.* Calcd for $C_{18}H_{25}N_3O_5S$: C, 54.67; H, 6.37; N, 10. 63; S, 8.09. Found: C, 54.75; H, 6.37; N, 10.57; S, 7.94.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl) (methylene, methyl)-bimane]-thioglycolyl-glycine (6)**——Compound **5** (40 mg, 0.1 mmol) was treated with 0.5 ml of 5 N hydrogen chloride in dioxane for 2 h at room temperature. Excess reagent and solvent were removed under reduced pressure. The residue was treated twice with methylene chloride. Recrystallization from water afforded pale yellow fine needles of mp 265—268 °C; 27 mg, 79%. IR: 3310, 1720, 1655 cm^{-1}. *Anal.* Calcd for $C_{14}H_{17}N_3O_5S$: C, 49.55; H, 5.05; N, 12.39; S, 9.43. Found: C, 49.82; H, 5.24; N, 12.09; S, 9.15.

***O*-{*S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl}-DL-3-indolelactic Acid (7)**——A suspension of 70 mg (0.25 mmol) of **1** in 4 ml of thionyl chloride was stirred for 2 h at room temperature. Excess reagent was removed under reduced pressure at room temperature, and the residue was treated twice with absolute benzene to remove traces of reagent. To a stirred, ice-water-cooled solution of the residual oil in 4 ml of anhydrous tetrahydrofuran, a mixture of 51 mg (0.25 mmol) of DL-3-indolelactic acid and 51 mg (0.5 mmol) of triethylamine in 1 ml of anhydrous tetrahydrofuran was added dropwise. The whole was stirred for a further 1 h at 0 °C then overnight at room temperature. The solution was diluted with ethyl acetate, washed with 10% hydrochloric acid, water and then dried over anhydrous sodium sulfate. Recrystallization from ethyl acetate gave pale yellow fine needles of mp 195—196 °C (dec.); 48 mg, 40%. IR: 3340, 1730 cm^{-1}. *Anal.* Calcd for $C_{23}H_{23}N_3O_6S \cdot 1/2\ H_2O$: C, 57.73; H, 5.06; N, 8.78; S, 6.69. Found: C, 57.72; H, 4.93; N, 8.62; S, 6.89.

Hydrolysis of 3, 4 and 7 by Carboxypeptidase A——a) Linear Relation of the Fluorescence Intensity *vs.* Enzyme Concentration: A solution (10—200 μl) of 2.65×10^{-8}—2.65×10^{-7} M carboxypeptidase A (Sigma Chem. Comp.; activity, 50 units per mg protein) in 10% aqueous lithium chloride was added to a solution of 10 μl of **4** (4.28×10^{-3} M, 41% dimethyl sulfoxide (DMSO)) and 2.0 ml of 0.025 M Tris–HCl buffer containing 0.5 M sodium chloride (pH 7.5) at 25 °C (final concentration of DMSO: 1.9%), and the increase in emission at 483 nm (appearance of **6**) was recorded (excitation at 399 nm). The rate of hydrolysis was established by comparing the rate of increase of fluorescence intensity with the fluorescence intensity of a standard solution of **6**.

b) Kinetic Parameter (K_m and k_{cat}) Measurement: A solution (20 μl) of 2.65×10^{-4} M carboxypeptidase A in 10% aqueous lithium chloride was added to a solution of 10 μl of **3** (0.646×10^{-4}—2.15×10^{-4} M), **4** (0.606×10^{-4}—2.02×10^{-4} M) or **7** (0.676×10^{-4}—2.25×10^{-4} M) in 0.025 M Tris–HCl buffer containing 0.5 M sodium chloride (pH 7.5, final concentration of DMSO, 1.9%) at 25 °C, and measurement was carried out in the same manner as described for a).

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