Novel Fluorogenic Substrates Containing Bimane System for Microdetermination of Angiotensin I Converting Enzyme[1)]

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As a sensitive fluorometric assay for the activity of angiotensin converting enzyme, bimane-peptides containing tryptophan, *i.e.*, 1,7-dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-glycyl (or L-phenylalanyl)-L-tryptophyl-L-leucine (or L-proline), were synthesized and shown to be potent fluorogenic substrates for the micro-determination of angiotensin I converting enzyme activity.

Keywords 1,7-dioxo-2,3,5,6-tetramethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazole; tryptophan; intramolecular quenching; fluorogenic substrate; fluorometric enzyme assay; angiotensin I converting enzyme

In the course of exploring sensitive fluorometric assays for amidase activity of hydrolytic enzymes such as subtilisin,[2)] we have established the usefulness of 7-amino-4-methylcoumarin and its amide derivatives as practical fluorogenic substrates. Novel applications of 7-amino-4-nitro-2-oxa-1,3-diazole as a fluorogenic amine and 7-aminocoumarin-4-methanesulfonic acid as a water-soluble fluorogenic amine were also proposed.[3,4)] 1,7-Dioxo-2,3,5,6-tetramethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazole (bimane) has been introduced as the first member of a new class of highly fluorescent compounds.[5)] We have recently reported an application of bimane to prepare fluorogenic substrates for assay of hydrolytic enzymes such as chymotrypsin, aminopeptidase and carboxypeptidase.[1,6)]

Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase [EC 3.4.15.1] that cleaves the C-terminal dipeptide of substrates containing a free carboxy group, and participates in the renin–angiotensin system of blood pressure regulation. ACE activity has been assayed by several techniques. A synthetic substrates, hippuryl-histidyl-leucine, is commonly utilized. Free hippuric acid generated enzymatically can be assayed spectrophotometrically, or the histidyl-leucine dipeptide can be measured fluorometrically after reaction with *o*-phthalaldehyde (OPA).[7)] Several substrates which change their fluorescence intensities after cleavage by ACE have been reported. Such "intramolecularly quenched" fluorogenic substrates are: *p*-nitrobenzyloxycarbonylglycyl-L-tryptophylglycine,[8)] *o*-aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline,[9)] and dansylglycyl-*p*-nitro-L-phenylalanine.[10)]

We wish to report here a novel, rapid and sensitive spectrofluorometric assay of ACE using the bimane system as a substrate. The C-terminal amino acid of angiotensin I is leucine, and C-terminal proline is not susceptible to carboxypeptidase A. For these reasons, two types of substrates which have leucine or proline at the C-terminus of the peptide were designed. Phenylalanine was also chosen for the amino acid at the P_1 site of substrates, as the P_1 site of angiotensin I is occupied by phenylalanine. Bimane substrates **1a**—**1d** and a standard compound **1f** for fluorescence measurement were synthesized by the active ester method from bimane-thiomethylcarbonyl *N*-hydroxy-succinimide ester[1)] and the corresponding peptides (for **1a**—**1d**) or phenylalanine (for **1f**). Synthesis of the other standard compound (**1e**) was reported in the preceding paper.[1)]

The fluorescence of the bimane group is quenched by the tryptophan incorporated in the substrate, and cleavage at the amino side of tryptophan liberates the bimane moiety, thus leading to a marked increase in fluorescence which can be continuously detected. The relative fluorescence intensities of **1a**—**1d** are 0.13, 0.17, 0.20 and 0.15, respectively, with respect to **1e** (for **1a** and **1b**) or **1f** (for **1c** and **1d**) (excitation at 399 nm, emission at 483 nm).

Kinetic parameters of **1a**—**1d** for ACE 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 substrates (**4**—**6**), the kinetic parameters of **4**—**6** were also measured with the same ACE.

Although the k_{cat} values for **1a**—**1d** are comparable to the values for **3**, **5**, **6**, the values of K_m for **1a** and **1b** are smaller than the value for **3**, and comparable to the values for **4**, **5** and **6**. The K_m values for **1c** and **1d** are smaller than

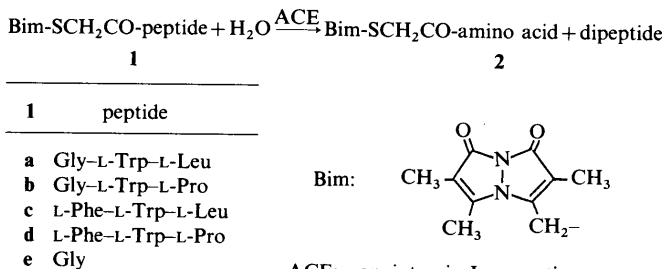

Chart 1

TABLE I. Kinetic Parameters of the Fluorogenic Substrates for Angiotensin I Converting Enzyme[a)]

Substrate[b)]	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
1a	1.7×10^{-4}	1.2	7.0×10^{3}
1b	3.6×10^{-4}	1.7	4.6×10^{3}
1c	5.9×10^{-5}	3.1	5.2×10^{4}
1d	2.9×10^{-5}	2.5	8.7×10^{4}
Hip-L-His-L-Leu (**3**)[7)]	1.3×10^{-3}	5.0	3.9×10^{3}
Z(NO_2)-Gly-L-Trp-Gly (**4**)[8)]	9.0×10^{-4} [c)]	—	—
Abz-Gly-L-Phe(NO_2)-L-Pro (**5**)[9)]	1.9×10^{-4}	1.5	7.7×10^{3}
Dns-Gly-L-Phe(NO_2)-L-Pro (**6**)[10)]	4.2×10^{-4}	4.1	9.6×10^{3}

a) ACE (EC 3.4.15.1): Specific activity, 2.2 units/mg protein. *b*) Hip, *N*-benzoylglycyl; Abz, *o*-aminobenzoyl; Dns, dansyl. *c*) Literature value.[8)]

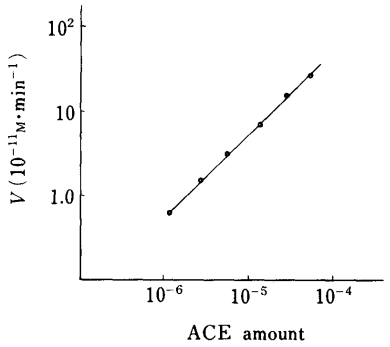

Fig. 1. Fluorescence Development *vs.* ACE Amount

Assays were performed as described under Experimental Procedure. Substrate, **1d**. ACE amount ranged from 1.1×10^{-6} to 4.4×10^{-5} units (in 2.22 ml of assay solution).

those for **1a** and **1b**. Higher affinity of **1c** and **1d** to ACE is probably due to the presence of the same amino acid (phenylalanine) at the P_1 site as in angiotensin I. Therefore, the values of k_{cat}/K_m for **1c** and **1d** are higher than those for the known fluorogenic substrates (**3**, **5**, **6**). From these kinetic results, substrate **1d** was selected and the linearity of the relationship between ACE concentration and fluorescence intensity was examined. As shown in Fig. 1, substrate **1d** can be used to detect as little as around 10^{-6} unit of ACE (in 2.22 ml of assay solution) using a substrate concentration of 4.30×10^{-6} M.

When the hydrolytic activity for **1d** was measured in the presence of *p*-chloromercuribenzoic acid in buffer solution, the activity was not decreased. Therefore, this ACE used for the assay of the bimane substrates is free from cysteine proteinase. Although hydrolyses of **1c** and **1d** containing the phenylalanine residue by a chymotrypsin-like enzyme might occur, hydrolytic activity of ACE was not affected by the treatment of ACE with *p*-tosyl-L-phenylalanyl chloromethyl ketone, which is a specific inhibitor of chymotrypsin. Enkephalinase has a similar substrate specificity to ACE, but the following assay conditions discriminate between them. Although enkephalinase is inhibited in the presence of chloride ion, ACE is rather activated[11] and maximum activity is observed with 300 mM sodium chloride.

It has thus been demonstrated that the bimane system can be used in the fluorometric assay for ACE. Compared with other fluorogenic substrates for ACE, the bimane system has several advantages. 1) Bimane substrates **1c** and **1d** have higher k_{cat}/K_m values than those for previously reported fluorescent substrates (**3**, **5** and **6**). 2) Bimane substrates can provide a continuous and rapid fluorometric assay, though a known conventional procedure [spectrofluorometric (using OPA) determination of the hydrolyzed product (his-leu) from **3**] is an end-point assay. 3) Substrates **4**—**6** can also be employed for continuous assay, but the fluorescence intensity of the bimane group is higher than that of the aminobenzoyl group (of **5**) or tryptophan (of **4**). 4) Bimane derivatives have a longer excitation maximum wavelength (399 nm) than other fluorogenic substrates (365 nm for **3** with OPA, 360 nm for **5**, 290 nm for **4** and **6**), so that interference from most biological compounds is low.[12] 5) The bimane system has a constant fluorescence intensity over a wide pH range of 1—9.

Because of these advantages, these bimane substrates are expected to be potent fluorogenic substrates for the microdetermination of ACE activity. Further application of these substrates to biochemical studies or clinical diagnoses, including monitoring of therapy with antihypertensive drugs such as captopril (which acts by inhibiting ACE) or monitoring or elevated serum ACE level in sarcoidosis, are being examined.

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. 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 fluorescence spectrophotometer.

General Procedure for the Preparation of Substrate (1) To a stirred solution of 0.6 mmol of the corresponding tripeptide (or amino acid) and 101 mg (1.2 mmol) of sodium bicarbonate in 20 ml of water, 190 mg (0.5 mmol) of 1,7-dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethanecarboxylic acid hydroxysuccinimide ester (bimane-succinimide ester)[1] in 10 ml of acetonitrile was added. After being stirred overnight at room temperature, the solution was concentrated to about two-thirds of the original volume under reduced pressure, diluted with 30 ml of water, and then washed twice with ethyl acetate. The aqueous solution was acidified with concentrated hydrochloric acid, then extracted with ethyl acetate. The extract was washed with water and dried over anhydrous sodium sulfate.

1,7-Dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-glycyl-L-tryptophyl-L-leucine (1a) Obtained from ethyl acetate by condensation as a pale yellow fine powder of mp 190—195 °C. 227 mg, 71%. IR (Nujol): 1730, 1650, 1635 cm^{-1}. $[\alpha]_D^{22}$ −11.1° (c=0.738, DMF). *Anal.* Calcd for $C_{31}H_{38}N_6O_7S\cdot1/2H_2O$: C, 57.48; H, 6.07; N, 12.97; S, 4.95. Found: C, 57.35; H, 6.13; N, 12.76; S, 4.76.

1,7-Dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-glycyl-L-tryptophyl-L-proline (1b) Obtained from ethanol–ethyl acetate by condensation; 162 mg, 52%. IR (Nujol): 1730, 1625 cm^{-1}. $[\alpha]_D^{24}$ −17.6° (c=1.04, DMF). *Anal.* Calcd for $C_{30}H_{34}N_6O_7S\cdot H_2O$: C, 56.24; H, 5.66; N, 13.12; S, 5.00. Found: C, 56.53, H, 5.63; N, 13.03; S, 5.17.

1,7-Dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-L-phenylalanyl-L-tryptophyl-L-leucine (1c) Obtained from methanol–ethyl acetate by condensation as a pale yellow powder of mp 156—166 °C (dec.). 220 mg, 60%. IR (Nujol): 1730, 1650 cm^{-1}. $[\alpha]_D^{20}$ −0.042° (c=0.497, DMF). *Anal.* Calcd for $C_{38}H_{44}N_6O_7S\cdot H_2O$: C, 61.10; H, 6.20; N, 11.25; S, 4.29. Found: C, 61.04; H, 5.96; N, 11.16; S, 4.40.

1,7-Dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-L-phenylalanyl-L-tryptophyl-L-proline (1d) The peptide dissolved in water (20 ml) and dimethyl sulfoxide (30 ml) as a cosolvent was treated with the ester. **1d** was obtained as a pale yellow powder from ethyl acetate by condensation, mp 153.5—160.5 °C. 259 mg, 73%. IR (Nujol): 1740, 1640, 1605 cm^{-1}. $[\alpha]_D^{20}$ −0.27° (c=0.275, DMF). *Anal.* Calcd for $C_{37}H_{40}N_6O_7S\cdot H_2O$: C, 60.80; H, 5.79; N, 11.50; S, 4.39. Found: C, 60.64; H, 5.62; N, 11.21; S, 4.50.

1,7-Dioxo-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazol-3-yl-methylthiomethylcarbonyl-L-phenylalanine (1f) Prepared from 40 mg (0.24 mmol) of phenylalanine and 76 mg (0.20 mmol) of bimane-succinimide ester.[1] Recrystallization from acetonitrile gave pale yellow needles of mp 169—170 °C. 63 mg, 61%. IR (Nujol): 3300, 1725, 1630, 1615, 1600 cm^{-1}. $[\alpha]_D^{20}$ +0.15° (c=0.213, DMF). *Anal.* Calcd for $C_{21}H_{23}N_3O_5S$: C, 58.73; H, 5.40; N, 9.79; S, 7.45. Found: C, 58.61; H, 5.31; N, 9.67; S, 7.40.

Assay Procedure for ACE with Substrates 1a—1d a) Kinetic Parameter (K_m and k_{cat}) Measurement: A solution of ACE (50 μl) was prepared at 9.5×10^{-2} unit/ml (concentration of ACE was estimated from the optical density at 280 nm based on a molar absorptivity of 204000 M^{-1}cm^{-1} and a molecular weight of 130000[13]) (ACE, EC 3.4.15.1, from rabbit lung (Sigma Chem. Co.); Specific activity, 2.2 units/mg protein with hippuryl-L-histidyl-L-leucine; one unit of enzyme activity is the amount required to catalyze the formation of 1.0 μmol of hippuric acid per min at 37 °C). To this solution, 30—100 μl of the substrate solution [1.91×10^{-3} M for **1d**, 25% dimethyl sulfoxide (DMSO) (final concentration of DMSO was 1.2%)] in 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM NaCl and

2.0 ml of buffer solution were added, and the increase in emission at 483 nm (appearance of Bim–SCH_2CO–L-Phe–OH, emission at 399 nm) was measured. Rates of hydrolysis were established from the rate of increase in fluorescence intensity based on the fluorescence intensities of various concentrations of standard bimane derivative (**1f**). Kinetic parameters for the hydrolysis were obtained from Lineweaver-Burk plots. Kinetic parameters for other substrates (**1a**—**1c**) were also obtained by similar procedures.

b) Linear Relation of Fluorescence Intensity *vs.* Enzyme Concentration: A solution (5—200 μl) of ACE (2.2×10^{-4} unit/ml) in buffer solution was added to a solution of 2.0 ml of buffer solution and 20 μl (1.91×10^{-4} M; 2.5% DMSO, finally 0.023% DMSO) of substrate (**1d**) (final concentration: 4.30×10^{-6} M) at 37 °C, and measurement was carried out in the manner described in a).

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