Bimane Fluorogenic Substrates for Microdetermination of Angiotensin Converting Enzyme Level in Serum[1)]

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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 nitrated aromatic amino acid. Bimane peptides containing nitrated amino acid (1a, b) were shown to be useful fluorogenic substrates for the assay of angiotensin I converting enzyme (ACE) from rabbit lung, similar to bimane substrate containing tryptophan (3) previously reported. Among these bimane substrates, substrate 3 was shown to be a potent fluorogenic substrate for microdetermination of the ACE level in human serum.

Keywords bimane; nitrophenylalanine; nitrotryptophan; fluorogenic substrate; angiotensin I converting enzyme; serum

Proteolytic enzymes play a key role not only in the regulation of cellular protein turnover but also in the control of many other physiological functions such as digestion, blood coagulation, inflammation, control of blood pressure and so on. In recent years, the sensitivity of their determination has been increased substantially by the use of fluorogenic substrates with 7-amino-4-methylcoumarin derivatives as fluorophores.[3)] In our search for an improved fluorescent group, we have proposed 9,10-dioxa-*syn*-3,4,6,7-tetramethylbimane (bimane) as a fluorophore for hydrolytic enzyme substrates.[4)] We have recently reported that Bim–SCH_2CO–Phe–Trp–Pro–OH was a fluorogenic substrate which allowed the sensitive determination of angiotensin I converting enzyme (ACE).[4c)]

In this paper, we wish to report a new combination of fluorophore (bimane) and quencher (nitrated aromatic amino acid) for the intramolecularly quenched substrate for the assay of ACE, and also a microdetermination of human serum ACE, which is an exopeptidase and a dipeptidyl-carboxypeptidase that cleaves the C-terminal dipeptide of substrates containing a free carboxyl group and participates in the renin-angiotensin system of blood pressure regulation.

It was found that the fluorescence of bimane can be quenched in the presence of a nitrated aromatic amino acid. To evaluate the influence of a nitrated aromatic amino acid on the fluorescence of bimane, the fluorescence intensity of bimane in the presence and in the absence of a nitrated aromatic amino acid was measured; the intermolecular relative fluorescence intensity of bimane (1.10×10^{-5} M) in the presence (*versus* absence) of large excess 4-nitrophenylalanine (1.00×10^{-1} M) was 0.01. The attempted intermolecular quenched experiment for 6-nitrotryptophan was difficult bacause of significant absorption at the wavelength of excitation for bimane (*ca.* 400 nm). This quenching efficiency of 4-nitrophenylalanine was quantitatively analyzed. Stern–Volmer plots of the quenching of bimane fluorescence with 4-nitrophenylalanine is shown in Fig. 1. From this plot, the Stern–Volmer constant ($k_q\tau$) is determined to be 100 M^{-1} for 4-nitrophenylalanine, which is almost a comparable value with that of tryptophan (115 M^{-1}).[4a)] This observation suggested that quenching by nitrated aromatic amino acids of the bimane fluorescence may be utilized in the design of fluorogenic substrates for ACE.

Bimane substrates (**1a, b**) were synthesized using an active ester method from bimane-thioglycolic acid *N*-hydroxysuccinimide ester and tripeptides. The relative fluorescence intensity (RFI) of substrates **1a** and **1b** are 0.25 and 0.058, respectively, *versus* the fluorescence intensity of Bim–

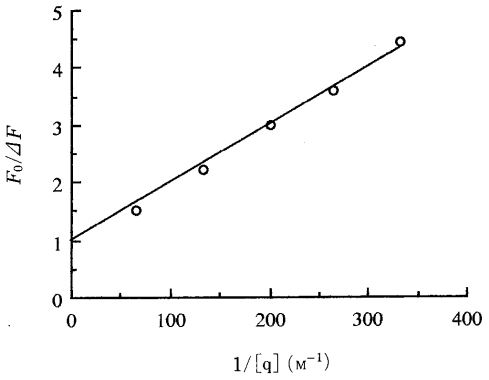

Fig. 1. Stern–Volmer Plots for Quenching of Bimane Fluorescence in the Presence of 4-Nitrophenylalanine

F_0, fluorescence intensity of bimane in the absence of 4-nitrophenylalanine. F, fluorescence intensity of bimane in the presence of 4-nitrophenylalanine. ΔF: $F_0 - F$. [q], concentration of quencher.

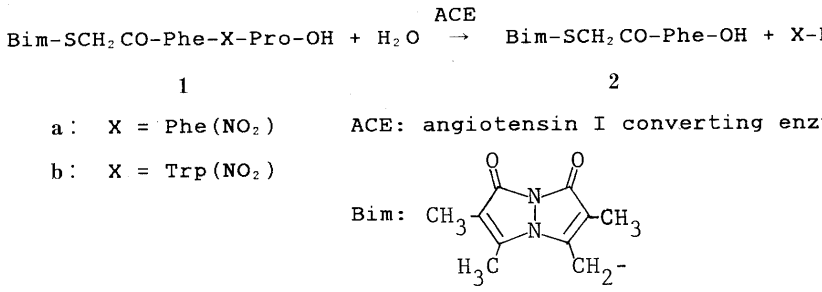

Chart 1

TABLE I. Kinetic Parameters of the Substrates for ACE

Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
1a	1.7×10^{-5}	5.1	3.0×10^{5}
1b	1.9×10^{-5}	5.4	2.9×10^{5}
Bim–SCH_2CO–Phe–Trp–Pro–OH (**3**)	2.6×10^{-5}	6.9	2.6×20^{5}
Hip–His–Leu (**4**)	1.1×10^{-3}	11	9.8×10^{3}

Hip–: benzoylglycyl-.

SCH_2CO–Phe–OH (excitation at 401 nm, emission at 481 nm). Although the substrate containing nitrophenylalanine (**1a**) has a higher RFI value than that of the previously reported bimane substrate (**3**) (0.15[4c]), the substrate containing nitrotryptophan (**1b**) has a smaller value than that of **3**, indicating a more intense quenching of nitrotryptophan than of tryptophan on the bimane system.

Kinetic parameters of **1a**, **b** for rabbit lung ACE were obtained by direct continuous spectrofluorometric assay, and the results are listed in Table I. In order to compare the characteristics of substrates containing nitrated aromatic amino acid with those of previously reported fluorogenic substrates (**3** and **4**), the kinetic parameters of **3**, **4** were also measured with the same lot of ACE under the same assay conditions. Both values of K_m and k_{cat} of **1a**, **b** are comparable to those of **3**, so bimane substrates with nitrated aromatic amino acid are also useful for the assay of ACE. Compared with the kinetic parameters of benzoylglycyl-histidyl-leucine (Hip–His–Leu) (**4**),[5] which is the most frequently used substrate for the assay of ACE, K_m values of **1a**, **b** are smaller by about two orders of magnitude compared to those of **4**, though the k_{cat} values of **1a**, **b** are smaller than **4**. So, the values of k_{cat}/K_m of **1a**, **b** are higher than those of **4**. For examination of the linearity of relationship between ACE concentration and fluorescence intensity, substrate **1b** was chosen because of its smaller RFI value compared to **1a**. The rates of hydrolysis for **1b** are proportional to enzyme concentration at least in the range 10^{-12}—10^{-10} M. This is almost the same value as that of the lowest detection limit of ACE with a bimane substrate of **3**.[4c]

As demonstrated above, bimane substrates with nitrated aromatic amino acids (**1a**, **b**) have almost comparable kinetic parameters and sensitivity as the previously reported bimane substrate with tryptophan (**3**). Therefore, substrate **3** and **1b** were used for the assay of ACE activity in human serum. The effect of substrate concentration on reaction velocity for serum ACE is given in Fig. 2. It showed human serum ACE activities with **3** and **1b** as substrate plateaus at a substrate concentration above 1.0×10^{-4} and 4.5×10^{-5} M, respectively. This value of substrate concentration is nearly one range lower in concentration of substrate compared with Hip–His–Leu as a substrate (5×10^{-3} M). The value of units (24) for **3** is almost comparable to the value of units (23) obtained with Hip–His–Leu for the same lot of human serum, though the value of units (12) for **1b** is small.[5] The ACE activities measured with **3** and **1b** are proportional to the serum volume over the range of 2—30 μl for **3** and 2—20 μl for **1b** as shown in Fig. 3, and it increased with time for at least 16 min. Thus, routine assays employ 20 μl serum and a 6 min-incubation.

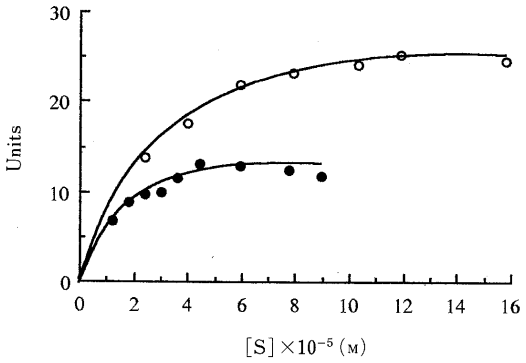

Fig. 2. The Effect of Substrate Concentration on Reaction Velocity

The unit values for Bim–SCH_2CO–Phe–Trp–Pro–OH (**3**) and Bim–SCH_2CO–Phe–Trp(NO_2)–Pro–OH (**1b**) catalyzed by human serum as a function of the concentration of the substrate. ○—○—○, **3**; ●—●—●, **1b**.

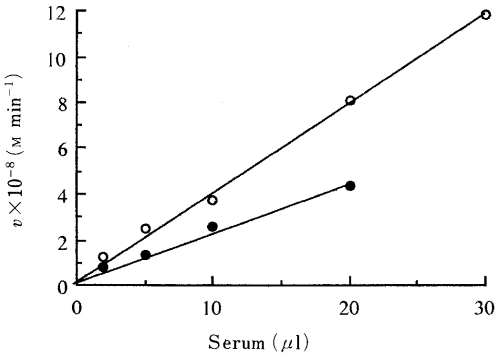

Fig. 3. Linearity with Respect to Serum Concentration of the Velocity

Linearity with respect to serum concentration of the velocity of Bim–SCH_2CO–Phe–OH formation catalyzed by human serum using Bim–SCH_2CO–Phe–Trp–Pro–OH (**3**) and Bim–SCH_2CO–Phe–Trp(NO_2)–Pro–OH (**1b**) as substrate. ○—○—○, **3**; ●—●—●, **1b**.

To make sure that the activities measured with **3** and **1b** are indeed due to ACE activity in the serum, the following experiments were carried out. These bimane substrates have a phenylalanine redsidue, so there is a possibility that they are susceptible to the action of chymotrypsin-like enzymes. However, the hydrolytic activities of serum assayed with **3** and **1b** were not affected by the treatment of human serum with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK), a selective inhibitor of chymotrypsin. Further, to exclude the participation of any cysteine proteinase activity on the fluorimetric assay, the assay was carried out in the present of *p*-chloromercuribenzoic acid (PCMB), a cysteine proteinase inhibitor. Again, the activities of human serum utilizing **3** and **1b** were not influenced. Therefore, this assay of human serum with bimane substrates **3** and **1b** is free from chymotrypsin-like enzyme activity and cysteine proteinase activity. Although enkephalinase has a similar substrate specificity to ACE, the concentration of chloride ion in buffer discriminates between their activities. Enkephalinase activity is inhibited in the presence of a chloride ion, while ACE activity is activated by it.[6] Therefore, the assay of ACE activity in human serum was carried out in the presence of 300 mM sodium chloride.

Further evidence that assay with bimane substrates of **3** and **1b** monitors specific ACE activity is shown by experiments utilizing captopril, which is a potent and specific inhibitor of ACE.[7] The K_i values for this inhibitor are similar for both substrates employed, with captopril

displaying a K_i of about 1.8 and 1.6 nM by assay with **3** and **1b**, respectively, and they are closely similar to the value of 1.7 nM obtained for ACE utilizing an assay with Hip–His–Leu.[7] Based on these experimental results, it may be concluded that fluorimetric assay in human serum using these bimane substrates selectively evaluates ACE activity in serum.

In summary, it could be illustrated that bimane substrates containing nitrated aromatic amino acid can also be used together with the already reported substrate **3** in continuous fluorimetric assay for ACE, and based on the results of assay for human serum as shown in Figs. 2 and 3, bimane substrate **3** is a potent fluorogenic substrate for the assay of ACE level in human serum. Compared with previously reported fluorescent substrates for the microanalysis of ACE level in human serum, the bimane system has the following advantages. 1) Although Hip–His–Leu is most widely utilized for assay of ACE activity,[8] it is a time-consuming end-point assay. With Hip–His–Leu, hydrolysis of the substrate has been monitored by quantitation of His–Leu liberated by the reaction with *o*-phthalaldehyde. The bimane system can provide a continuous and rapid fluorimetric assay. Recently, it was reported that the values were underestimated in the Hip–His–Leu assay in earlier studies for ACE activity, which depended on the fluorimetric determination of His–Leu. This dipeptide can be rapidly hydrolyzed by membrane dipeptidase.[9] 2) As a continuous fluorescent method, Abz–Gly–Phe(NO_2)–Pro was reported as an intramoleculary quenched fluorogenic substrate for the assay of human serum ACE activity,[10] but the fluorescence intensity of the bimane group is higher than that of the *o*-aminobenzoyl (Abz) group, and the emission wavelength of the bimane system (480 nm) is longer than that of the Abz group (360 nm). These fluorescence characteristics of the bimane system are useful to sensitive assay for serum ACE activity.

In the present work, we extended the usefulness of the bimane system to a fluorogenic substrate for the microdetermination of ACE activity. The proposed method is widely applicable, and further application of these bimane substrates to the clinical field is expected.

Experimental

Melting points were determined on a Yamato MP-21 apparatus and are uncorrected. Infrared (IR) spectra were obtained with a JASCO IRA-1 infrared spectrometer. Ultraviolet (UV) and visible absorption spectra were obtained with a Hitachi 210-10 spectrophotometer. Fluorescence spectra were recorded on a Hitachi 650-10 fluorescence spectrophotometer.

Tripeptides were prepared from *N*-boc-amino acid *N*-hydroxysuccinimide ester and either amino acid or dipeptide by the standard active ester method, followed by deblocking of the protective group with 5 M hydrogen chloride in dioxane. Angiotensin converting enzyme (rabbit lung) was purchased from Sigma Chemical Company. Human serum was obtained from the Hokkaido Red Cross Blood Center.

Synthesis of Substrates (1a, b) **1a**: To a solution of Phe–Phe-(4-NO_2)–Pro·HCl (196 mg, 0.4 mmol) and sodium bicarbonate (101 mg, 1.2 mmol) in water (10 ml), a solution of bimane-thioglycolic acid *N*-hydroxysuccinimide ester (Bim–OSu) (152 mg, 0.4 mmol) in acetonitrile (10 ml) was added. The solution was stirred at room temperature overnight, and concentrated to about 1/2 of the original volume under reduced pressure, diluted with water (50 ml), and then washed with ethyl acetate. The aqueous solution was acidified with concentrated hydrochloric acid and salted out, then extracted with ethyl acetate. The extract was washed with saturated sodium chloride solution, then dried over anhydrous sodium sulfate. **1a** was obtained from methanol–ethyl acetate by condensation as a pale yellow powder of mp 120—139 °C. 192 mg, 67%. IR (Nujol): 1740, 1630, 1520 cm^{-1}. $[\alpha]_D^{25}$ −9.3° (c=0.332, MeOH). *Anal.* Calcd for $C_{35}H_{38}N_6O_9S \cdot CH_3OH$: C, 57.59; H, 5.64; N, 11.19; S, 4.27. Found: C, 57.50; H, 5.52; N, 10.98; S, 4.45.

1b: From Bim–SCH_2CO–OSu (60 mg, 0.16 mmol) and Phe–Trp-(6-NO_2)–Pro (74 mg, 0.15 mmol) with triethylamine (42 μl, 0.3 mmol) using the same procedure as **1a**, **1b** was obtained from methanol–ethyl acetate by condensation as a yellow powder of mp 147—157 °C. 70 mg, 62%. IR (Nujol): 3280, 1735, 1630, 1520 cm^{-1}. $[\alpha]_D^{29}$ −17° (c=0.3165, dimethylformamide (DMF)). *Anal.* Calcd for $C_{37}H_{39}N_7O_9S \cdot 5/3H_2O$: C, 56.41; H, 5.42; N, 12.44; S, 4.07. Found: C, 56.67; H, 5.15; N, 12.17; S, 4.21.

Hydrolyses of 1a, b by Angiotensin I Converting Enzyme a) Kinetic Parameters (K_m, k_{cat}) Measurement: A solution (50 μl) of angiotensin I converting enzyme (from rabbit lung, 3.1 units/mg protein) (2.745×10^{-7} M) was added to the substrate solution (30—100 μl) of **1a** or **1b** (1.46×10^{-3} or 1.68×10^{-3} M, respectively, 25% dimethylsulfoxide) in 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM sodium chloride and 2 ml buffer solution, with additional buffer solution (70—0 μl) to compensate for the substrate solution to the same tatal volume at 37 °C. The increase in emission at 480 nm (appearance of 2) was measured (excitation at 400 nm). Rates of hydrolyses were established from the rates of increase in fluorescence intensity based on the fluorescence intensity of 2. Kinetic parameters for the hydrolyses were obtained from Lineweaver–Burk plots.

b) Linear Relation of the Fluorescence Intensity *vs.* Enzyme Concentration: A solution (5—200 μl) of ACE (6.38×10^{-10} M) was added to the solution of **1b** (20 μl, 1.68×10^{-4} M) in 50 mM Tris–HCl buffer containing 300 mM sodium chloride and the same buffer (2.195—2.000 ml) at 37 °C, and measurement was carried out in the manner described in a).

Assay for Human Serum a) ACE Activity in Human Serum (Fig. 2): Instead of ACE, human serum (20 μl) was used, and the concentrations of the substrate were 2.38×10^{-5}—15.8×10^{-5} M for **3** and 1.19×10^{-5}—8.92×10^{-5} M for **1b**. The unit values were defined as Bim–SCH_2CO–Phe–OH liberated n moles/min/ml serum. Measurement was carried out in the manner described in a) of the hydrolyses of **1a**, **b** by ACE.

b) Linear Relation of the Initial Velocity *vs.* Serum Volume (Fig. 3): Serum (2—30 μl) was added to the solution of 2.0 ml of buffer and 130 μl for **3**, 75 μl for **1b** (1.40×10^{-3} M for **3**, 1.56×10^{-3} M for **1b**: 25% DMSO, finally 1.5% DMSO) of substrate stock solution (final concentration of substrate: 8.44×10^{-5} M for **3**, 5.39×10^{-5} M for **1b**) at 37 °C. Measurement was carried out in the same way described in b) of the hydrolyses of **1a**, **b** by ACE.

Inhibition of ACE Activity by Captopril The K_i values for captopril were measured using **3** and **1b** as substrates in the manner described for the assay for human serum. Final substrate concentrations used were 6.87×10^{-6} and 1.37×10^{-5} M for **3**, 7.01×10^{-6} and 1.40×10^{-5} M for **1b**, and the final captopril concentration was 7.94×10^{-10}—3.97×10^{-9} M for both **3** and **1b**. The human serum amount was 20 μl. The values of K_i were obtained using Dixon plots.

References and Notes

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