Novel Fluorogenic Substrates for Phosphodiesterase I[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 guanosine 5′-monophosphate. By using this phenomenon, the bimane system was used for the fluorophor of substrates for phosphodiesterase I.

Bimanes were coupled to 5′-guanylic acid and the resulting compounds were shown to be portent fluorogenic substrates for the assay of phosphodiesterase I.

Keywords bimane; 5′-guanylic acid; quenching; fluorogenic substrate; fluorometric enzyme assay; phosphodiesterase I

During the course of our investigations in organic fluorescent reagents we have reported useful fluorogenic substrates for proteinase having 7-amino-4-methylcoumarin and 7-aminocoumarin-4-methanesulfonic acid as fluorophor.[2)] We also proposed the application of 9,10-dioxa-*syn*-3,4,6,7-tetramethylbimane (bimane) to fluorogenic substrates for hydrolytic enzymes such as chymotrypsin, aminopeptidase,[3)] carboxypeptidase A,[4)] angiotensin converting enzyme,[5)] and cathepsin C.[1)] In an effort to extend this potent fluorophor, we undertook the application of the bimane system to the substrate for nuclease.

While the example of colorimetric substrate for nuclease, *p*-nitrophenyl nucleotides is well known,[6)] there are few reports of fluorogenic substrates for nuclease. As an example of fluorogenic substrates, 1-aminonaphthalene-5-sulfonyl (ANS) nucleotides have been used for deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase[7a,b)] or snake venom phosphodiesterase,[7c)] and 4-methylumbelliferyl nucleotide has been applied to the diagnosis of cancer or zymogram.[8)]

TABLE I. Relative Fluorescence Intensity of Bimane in the Presence of Nucleotides

Nucleotide	F/F_0
5′-UMP	0.99
5′-CMP	0.99
5′-AMP	1.01
5′-GMP	0.26

Fluorescence intensity of bimane (1.04×10^{-5} M) in the presence (F) and in the absence (F_0) of nucleotides (5.0×10^{-2} M) in 0.1 M Tris–HCl buffer, pH 8.9. λex: 400 nm; λem: 484 nm.

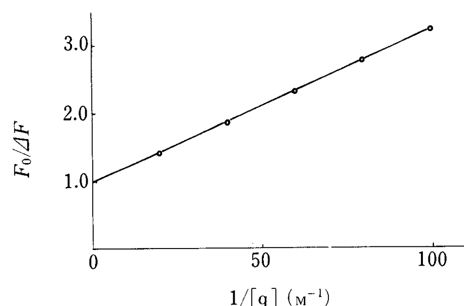

Fig. 1. Stern–Volmer Plots for Quenching of Bimane Fluorescence in the Presence of 5′-GMP

F_0: fluorescence intensity of bimane in the absence of 5′-GMP. F: fluorescence intensity of bimane in the presence of 5′-GMP. ΔF: F_0-F. [q]: concentration of quencher.

Fluorescence intensity of bimane in the presence and the absence of 5′-nucleotides was measured and the relative intensities were listed in Table I. It was interestingly revealed that guanosine 5′-monophosphate (5′-GMP) intensely quenched the fluorescence of bimane though uridine 5′-monophosphate (5′-UMP), cytidine 5′-monophosphate (5′-CMP) and adenosine 5′-monophosphate (5′-AMP) do not affect the intensity of bimane fluorescence as shown in Table I. This intermolecular quenching of fluorescence of bimane in the presence of 5′-GMP was quantitatively examined. Stern–Volmer plots on the quenching of bimane fluorescence with 5′-GMP was shown in Fig. 1. From this figure, Stern–Volmer constant ($k_q\tau$) was determined to be 45 M^{-1}.

From these intermolecular quenching experiments, a system containing bimane coupled to guanylic acid can be expected to be the fluorogenic substrate for phosphodiesterase. Bimane-nucleotides of **1a**—**b** were designed as fluorogenic substrates for phosphodiesterase I (PDE I) [enzyme code 3.1.4.1].

Bimane substrates were synthesized as shown in Chart 1. Disodium 5′-guanosine monophosphate was alkylated with monobromobimane[9)] at the phosphate site to give **1a**. Phosphor-amidate substrate **1b** was obtained from guanosine 5′-triphosphate (5′-GTP) by coupling with aminobimane (**2c**)[10)] by using water-soluble carbodiimide. The relative fluorescence intensity of **1a** and **1b** are 0.03 and 0.09, respectively, versus the fluorescence intensity of **2a** (for **1a**) or **2b** (for **1b**) (excitation at 400 nm, emission at 484 nm, **1a,b** and **2a,b** have the same excitation and emission maxima). It was illustrated that the bimane fluorescence was quenched not only intermolecularly in the presence of a high concentration of guanylic acid but also intramolecularly with an incorporated guanylic acid moiety.

Table II lists the kinetic constants for the hydrolyses of these substrates (**1a**—**b**) by PDE I. Both K_m and k_{cat} of **1b** are better than those of **1a**. As judged by the higher K_m value of **1a** than that of **1b**, the distance between bimane moiety and guanosine moiety plays a significant role in substrate binding to the enzyme. At the time of enzymatic

TABLE II. Kinetic Parameters for Phosphodiesterase I

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
1a	130	17	0.13
1b	33	34	1.0
1,5-ANS-5′-UTP (3)	42	87	2.1

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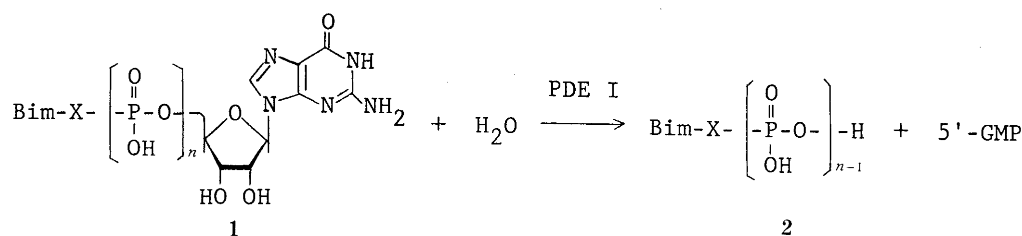

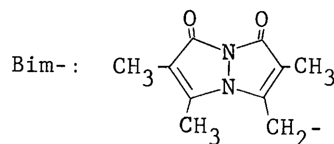

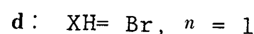

PDE I: phosphodiesterase I

Chart 1

hydrolyses of bimane-substrates, equal amounts of 5′-GMP can be released accompanied by the liberation of bimane derivatives (**2a**,**b**). Although a high concentration of 5′-GMP has a quenching effect on the bimane-system, an equivalent amount of 5′-GMP had negligible effects on the fluorescence intensity of liberated **2a** and **2b**. As direct continuous spectrofluorometric substrates for PDE I, nucleoside-5′-triphosphoro-γ-(5-sulfonic acid)naphthylamidates (1,5-ANS-5′-NTP) have been reported.[7] The fluorescence intensity of the 5′-GTP analog decreases slightly when the α,β-phosphoryl bond is cleaved. In contrast, the fluorescence intensity of the uridine 5′-triphosphate (5′-UTP) analog increases as a result of this cleavage. Therefore, the kinetic constants of 1,5-ANS-5′-UTP (**3**) were also measured with the same lot of PDE I and under the same assay conditions in order to compare the kinetic parameters of the bimane substrate of **1b**. Although bimane-5′-GTP (**1b**) has a comparable value of kinetic parameters with that of 1,5-ANS-5′-UTP (**3**) as shown in Table II, an excitation maximum of **1c** is 400 nm, a longer wavelength compared with that of ANS-5′-UTP (320 nm). For the micro-determination of fluorescent assay, substrate **1b** was used for examination of a linear response between the 100-fold range of PDE I concentration and fluorescence intensity. Substrate **1b** can be used to detect as little as around 0.017 nM (1.9 ng/ml) of PDE I using a substrate concentration of only 5.53×10^{-6} M.

The principle advantage of the bimane system is long-wave excitation, thus interference from biological materials such as reduced nicotinamide adenine dinucleotide is low.[11] As a further advantage, a bimane substrate has a slightly larger difference (11-fold for **1b**) in fluorescence intensity upon digestion with phosphodiesterase than that of ANS-analog (2- to 6-fold).[7c] It has been demonstrated that the bimane system is applicable to fluorogenic substrate for nucleotidase. Further application of bimane-nucleotide for PDE II and ribonuclease T_1 is now under investigation.

Experimental

Ultraviolet (UV) absorption spectra were obtained with a Hitachi 210-10 spectrophotometer. Fluorescence spectra were recorded on a Hitachi 650-10 fluorescence spectrophotometer. pH was measured with a Hitachi-Horiba M-7 pH meter.

Guanosine 5′-(12-Bimane)phosphate Triethylammonium Salt (α-Bimane-5′-GMP, 1a) Dimethylformamide solution (8 ml) of bimane monobromide (**2d**)[9] (54 mg, 0.2 mmol) was added to the aqueous solution (8 ml) of 5′-GMP·2Na (82 mg, 0.2 mmol), and warmed at 80 °C with stirring for 3 h. The reaction mixture was charged on a diethylaminoethyl (DEAE) cellulose column (1.4 × 32 cm) and then eluted with triethylamine–bicarbonate buffer (pH 7.5, linear gradient 0.02 to 0.4 M). A combined solution of the products was evaporated *in vacuo* and the residue was triturated with acetonitrile and collected by suction to give a pale yellow powder 17 mg (12%). *Anal.* Calcd for $C_{20}H_{24}N_7O_{10}P \cdot (C_2H_5)_3N \cdot 3H_2O$: C, 44.07; H, 6.40; N, 15.81. Found: C, 44.12; H, 6.42; N, 15.50.

Guanosine 5′-Triphosphoro-γ-(12-bimane)amidate Tri-triethylammonium Salt (γ-Bimane-5′-GTP, 1b) To the mixture of 5′-GTP·3Na (50 mg, 0.085 mmol) and aminobimane (**2c**)[10] (21 mg, 0.1 mmol) in an aqueous solution (10 ml), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (192 mg, 1.0 mmol) was added. The pH of the solution was adjusted to 6.5 and maintained with 0.1 M HCl aq. for 6 h. The product was isolated by the same procedure as above. After evaporation of the product fraction, the residue in 2 ml of water was lyophilized to give 64 mg (58%) of a pale yellow powder. *Anal.* Calcd for $C_{20}H_{27}N_8O_{15}P_3 \cdot 3[(C_2H_5)_3N] \cdot 5H_2O$: C, 41.27; H, 7.47; N, 13.93. Found: C, 41.18; H, 7.20; N, 14.10.

Pyrophosphoro-(12-bimane)amidate Tri-triethylammonium Salt (2b) Sodium diphosphate decahydrate (89 mg, 0.20 mmol) was coupled to aminobimane (**2c**)[10] (42 mg, 0.2 mmol) with EDC·HCl (383 mg, 2.0 mmol) by the same procedure as with **1b**. 55 mg (33%) of a pale yellow powder was obtained. *Anal.* Calcd for $C_{10}H_{15}N_3O_8P_2 \cdot 3[(C_2H_5)_3N] \cdot 9H_2O$: C, 40.37; H, 9.44; N, 10.09. Found: C, 40.31; H, 9.72; N, 9.84.

Hydrolyses of 1a—b by PDE I a) Kinetic parameters (K_m, k_{cat}) measurement: A solution (10 μl) of PDE I (from Crotalus durissus. Boehringer Mannheim Biochem.) (8.76×10^{-8} M for **1a** and **1b**) was added to the substrate solution (0.6—2.0 ml) of **1a** (1.64×10^{-5} M) or **1b** (9.95×10^{-6} M) in 0.1 M Tris–HCl buffer (pH 8.9 containing 0.11 M NaCl and 15 mM $MgCl_2$) (0—1.4 ml) at 25 °C and the increase in emission at 484 nm (appearance of **2a** or **2b**) 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 hydroxybimane (**2a**) for **1a**, pyrophosphorylaminobimane (**2b**) for **1b**. Kinetic parameters for the hydrolyses were obtained from Lineweaver–Burk plots. b) Linear relation of the fluorescence intensity *vs.* enzyme concentration: A solution (10—50 μl) of PDE I (1.20×10^{-9}—1.20×10^{-8} M) was added to the solution of **1b** (100 μl, 1.19×10^{-4} M) in 0.1 M Tris–HCl buffer (2.00—2.04 ml) at 25 °C, and measurement was carried out in the manner described in a).

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