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A Potentially Useful Fluorogenic Amine, 4-Amino-7-nitrobenz-2-oxa-1,3-diazole. An Application as a Substrate for a Microdetermination of Chymotrypsin[1)]

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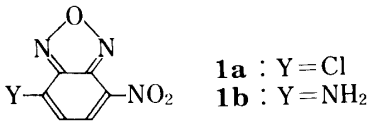
4-Amino-7-nitrobenz-2-oxa-1,3-diazole (**1b**) is a potentially useful key fluorogenic amine. As an example of the application of this amine, 7-(*N*-tosyl- and *N*-mesyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole (**2**) were prepared and shown to be good fluorogenic substrates for the assay of chymotrypsin.

Keywords——fluorometric enzyme assay; fluorogenic amine; 4-amino-7-nitrobenz-2-oxa-1,3-diazole; 7-(*N*-tosyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole; 7-(*N*-mesyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole; kinetic study

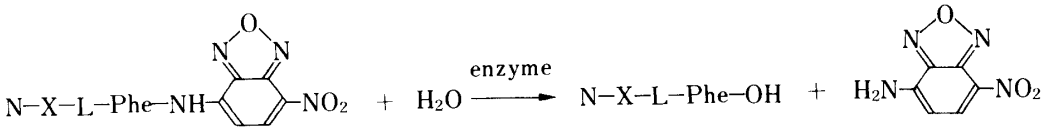
In the course of exploring sensitive fluorometric assays for amidase activity of hydrolytic enzymes[2)] such as leucine aminopeptidase,[3)] trypsin and papain,[4)] γ-glutamyltranspeptidase[5)] and cystine aminopeptidase,[1)] we have established the usefulness of 7-amino-4-methylcoumarin (AMC) and its amide derivatives as practical fluorogenic substrates. Based on the work of both our group and the Merck group,[6)] extensive applications of 7-amino-4-methylcoumarin as a key component of hydrolytic enzyme substrates have emerged.[7)]

In 1968 Ghosh and Whitehouse found that 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (**1a**) is not fluorescent but forms highly fluorescent products on reaction with amino acids.[8)] Since then, compound **1a** has been used as fluorescent probe for amino and thiol functions in biological research.[9)] In view of the wide applicability of organic fluorescent reagents,[2)] it is desirable to have available various fluorogenic amines with a spectrum of fluorescence properties for a variety of research purposes in biomedical science. Thus, we have decided to study the properties of 7-amino-4-nitrobenz-2-oxa-1,3-diazole (**1b**, NBD-amine), which is a possible candidate. In this paper, as a first example of an application of this amine, we describe the preparation of 7-(*N*-tosyl- and *N*-mesyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole (**2a** and **2b**) and their properties as sensitive fluorogenic substrates for



1a : Y=Cl
1b : Y=NH_2

1



2

2a : X=tosyl
2b : X=mesyl

1b

Chart 1

chymotrypsin.

The substrates (**2a** and **2b**) were synthesized from *N*-tosyl- or *N*-mesyl-L-phenylalanyl chloride and NBD-amine (**1b**).[10] Enzymatic hydrolysis of **2** by chymotrypsin proceeds to liberate the amine (**1b**) as shown in Chart 1. The fluorescence intensity of **2** is negligibly small (less than 0.1%) compared with that of **1b** (excitation at 472 nm, emission at 555 nm) in buffer solution (pH 7.8), as shown in Fig. 1.

Kinetic parameters are listed in Table I. Compared with the values for reported fluorogenic substrates,[6a] glutaryl-phenylalanyl-4-methylcoumarinylamide and alanyl-alanyl-phenylalanyl-4-methylcoumarinylamide (K_m: 6.7×10^{-4} M and 5.0×10^{-4} M; k_{cat}: 0.052 s^{-1} and 0.83 s^{-1}, respectively), K_m of **2a** is smaller by about one order of magnitude, although the value of K_m of **2b** is comparable, indicating that substrate **2a** has a higher affinity for chymotrypsin. The values of k_{cat} for **2a** and **2b** are higher by one to two orders of magnitude than those of the coumarin substrates. The linearities of the plots of fluorescence intensity *vs.* incubation time (for more 5 min) and also *vs.* enzyme concentration (at least 100-fold range up to 0.01 μg enzyme/ml, at a substrate concentration of 3.9 μM) were satisfactory. The present conditions allowed the measurement of as little as 0.02 μg of the enzyme, equivalent to 0.8 pmol (based on a mol wt. of 25000), as illustrated in Fig. 2.

The fluorescence intensity of NBD-amine (**1b**) is around one-hundredth of that of AMC, probably because this amine carries a nitro group, a substituent which is generally known to show a quenching effect on the fluorescence intensity of its parent compounds.[11] However,

TABLE I. Kinetic Parameters of the Fluorogenic Substrates for Chymotrypsin

Substrates	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
2a	1.92×10^{-5}	4.13	2.15×10^5
2b	3.13×10^{-4}	3.69	1.18×10^4

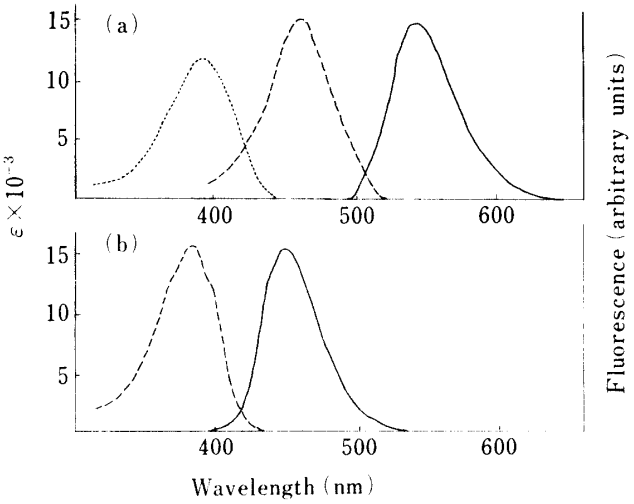


Fig. 1. (a) Absorption and Fluorescence Spectra of **1b** and **2a** in Tris-Buffer

----: absorption spectrum of **1b**. ------: absorption spectrum of **2a**. ——: fluorescence spectrum of **1b**.

(b) Absorption and Fluorescence Spectra of 7-Amino-4-methylcoumarin

----: absorption spectrum. ——: fluorescence spectrum.

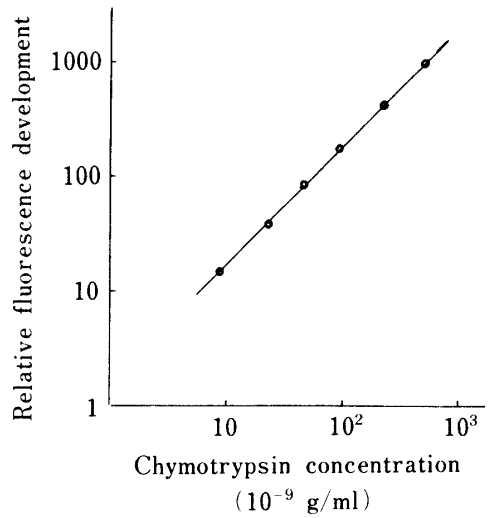


Fig. 2. Fluorescence Development *vs.* Chymotrypsin Concentration

Assays were performed as described under Experimental procedure. Substrate, 7-(*N*-tosyl-L-phenylalanyl)amino-4-nitrobenzo-2-oxa-1,3-diazole (**2a**). Chymotrypsin concentrations ranged from 9.43×10^{-9} to 4.74×10^{-7} g/ml.

there are some interesting features of these new 7-amino-4-nitrobenz-2-oxa-1,3-diazole derivatives. (1) NBD-amine showed an emission maximum at 555 nm on excitation at 472 nm; this is a region of very long wavelength. For example, 7-amino-4-methylcoumarin gives a maximum at 440 nm with 346 nm excitation (see Fig. 2). (2) There is distinct difference in fluorescence intensity between the NBD-amine (**1b**) and the corresponding amide (**2**) substrates.(3) NBD-amine gave a constant fluorescence intensity over a wide pH range of 2—9. These fluorescence characteristics of NBD-amine derivatives suggest possible new applications in biological research. For example, in histochemical studies involving fluorescence microscopy, employment of fluorogenic groups which emit in a long-wavelength region is obviously desirable. Applications of fluorogenic substrates containing NBD-amine (**1b**) for other enzymes are under study.

Experimental

Melting points are uncorrected. Fluorescence measurements were performed with a Hitachi fluorescence spectrophotometer, model 650-60. Ultraviolet absorption spectra were obtained with a Hitachi 200-10 spectrophotometer. Preparative layer chromatography (PLC) was carried out on silica gel plates (Kieselgel 60 PF_{254}, Merck, 20 × 20 cm).

7-(*N*-Tosyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole (2a)——A mixture of 192 mg (1.07 mmol) of 4-amino-7-nitrobenz-2-oxa-1,3-diazole,[10] 108 mg (1.07 mmol) of triethylamine and 5 mg of 4-dimethylaminopyridine in 10 ml of anhydrous tetrahydrofuran (THF) was added dropwise to a stirred, ice-water-cooled solution of 360 mg (1.07 mmol) of *N*-tosyl-L-phenylalanylchloride[12] in 10 ml of anhydrous THF. The mixture was stirred in an ice-bath for 1 h, then at room temperature overnight. The mixture was evaporated to dryness *in vacuo*, and the residue was taken up in AcOEt. The solution was washed successively with 10% HCl, water, 5% $NaHCO_3$ and water. The AcOEt layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was subjected to silica gel PLC developed with a mixture of CH_2Cl_2–AcOEt (20 : 1, v/v). Recrystallization of the product from MeOH gave pale yellow needles, mp 218—219 °C, 140 mg (27%). $[\alpha]_D^{18} = +29.4°$ ($c = 0.5$, dimethylformamide (DMF)), UV λ_{max}^{EtOH} nm (ε): 390 (1.17×10^4), 274 (5.25×10^3). *Anal.* Calcd for $C_{22}H_{29}N_5O_6S$: C, 54.88; H, 3.98; N, 14.55; S, 6.65. Found: C, 54.68; H, 3.86; N, 14.30; S, 6.47.

7-(*N*-Mesyl-L-phenylalanyl)amino-4-nitrobenz-2-oxa-1,3-diazole (2b)——Prepared by the same procedure as described for **2a** from 268 mg (1.0 mmol) of *N*-mesyl-L-phenylalanylchloride[13] with 180 mg (1.0 mmol) of NBD-amine. Recrystallization of the product from AcOEt–*n*-hexane afforded pale yellow fine needles, mp 203—204 °C, 90 mg (22%). $[\alpha]_D^{18} = -8.8°$ ($c = 0.5$, AcOEt), UV λ_{max}^{buffer} nm (ε): 386 (1.17×10^4), 284 (5.38×10^3). *Anal.* Calcd for $C_{16}H_{15}N_5O_6S$: C, 47.41; H, 3.73; N, 17.28; S, 7.90. Found: C, 47.36; H, 3.70; N, 17.06; S, 8.06.

Hydrolysis of 2 by Chymotrypsin——a) Linear relation of the fluorescence intensity *vs.* enzyme concentration: A solution (20—50 μl) of 4.1×10^{-8}—4.1×10^{-6} M chymotrypsin (Worthington Biochem.) was added to 3.9 μM **2a** in 0.08 M Tris-HCl buffer (2.0 ml) containing 0.1 M $CaCl_2$ (pH 7.8) and 4.7% dimethyl sulfoxide (DMSO) at 25 °C, and the increase in emission at 555 nm (appearance of **1b**) was measured (excitation at 472 nm). b) Kinetic parameter (K_m and k_{cat}) measurment: A solution (10 μl) of 4.20×10^{-6} M (for **2a**) or 6.36×10^{-6} M (for **2b**) chymotrypsin was added to 3.05—10.2 μM **2a** or 36.1—120 μM **2b** in 0.08 M Tris-HCl buffer (2.0 ml) containing 0.1 M $CaCl_2$ (pH 7.8) and 4.7% DMSO at 25 °C, and measurement was carried out in the manner described for a).

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