Communications to the Editor

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A New Fluorogenic Substrate for Aminopeptidase[1)]

7-Amino-4-methylcoumarin (**2**) was proposed as a candidate key fluorogenic amine. Thus 7-L-leucyl-4-methyl-coumarinylamide (hydrochloride) (**1**) was prepared and shown to be a useful fluorogenic substrate for the assay of aminopeptidase.

Keywords——7-amino-4-methylcoumarin; a key fluorescence amine; 7-leucyl-4-methylcoumarinylamide; fluorometric enzyme assay; kinetic study

Fluorescence measurements are generally several orders of magnitude more sensitive than colorimetric ones since in fluorescence one measures an increase in signal over a zero background, while in spectrophotometry a decrease in a large standing current is measured. Thus fluorescence methods have found increasing usage in a wide spectrum of biological studies.[1b,2)] In enzymology, for example, fluorometry has been successfully applied to many enzyme assays.[1b,2)]

Determination of aminopeptidase activity in various body samples has been a subject of intensive study because of its general clinical importance.[3)] In the course of our broadly based studies of organic fluorescence reagents,[1b)] we have been aware of special usefulness of aminocoumarin derivatives as the fluorophores[4)] and a study has been undertaken to develop a series of fluorescence reagents employing aminocoumarins as a key fluorophore. A recent paper on 7-glutarylphenylalaninamido-4-methylcoumarin as a fluorogenic substrate for chymotrypsin by Zimmerman, *et al.*[5)] prompted us to report some of our results on the application of 7-L-leucyl-4-methylcoumarinylamide hydrochloride **1** which is a good substrate for aminopeptidase (AP).

7-Amino-4-methylcoumarin (**2**)[6)] [mp 221—224° (dec.); UV_{max} (Tris-buffer pH 8.0) nm (ε) 343 (25500)] was coupled with carbobenzyloxy-L-leucine by the mixed anhydride method (isobutyl chloroformate) followed by catalytic hydrogenation to give **1** [colorless needles from AcOH, mp 248—251° (dec.), $[\alpha]_D^{20}=+42.5$ (c=1.6, EtOH) UV_{max} (Tris-buffer, pH 8.0) nm (ε): 324 (26800)]. *Anal.* Calcd. for $C_{16}H_{21}O_3N_2Cl\cdot 1/3H_2O$: C, 58.09; H, 6.60; N, 8.47; Cl, 10.72. Found: C, 58.37; H, 6.54; N, 8.42; Cl, 10.83. Enzymatic hydrolysis of **1** by leucine aminopeptidase proceeds to liberate the amine (**2**) as shown in Chart 1. Fluorescence intensity[7)] of **2** (excitation at 345 nm, emission at 440 nm) is only six times as high as that of the substrate (**1**) (excitation at 327 nm, emission at 390 nm) at each maximum wave-length in a buffer solution (pH 8.0). However, when excited at 380 nm and measured at 440 nm, **2** possesses a relative fluorescence intensity approximately 300-fold higher than that of equimolar **1** so that the faint

1) Organic Fluorescence Reagents. III. *a*) Part I: H. Nakayama and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **21**, 2804 (1973); *b*) Part II: Y. Kanaoka, *Angew. Chem.*, German and International Edn., in press; *c*) Presented at the Annual Meeting of the Biochemical Society of Japan, Sapporo, September, 1976; T. Takahashi, H. Nakayama, and Y. Kanaoka, *Seikagaku*, **48**, 539 (1976).

2) *a*) S. Udenfriend, "Fluorescence Assay in Biology and Medicine," vol. 2, Academic Press, New York, 1969; *b*) G.G. Guilbault, "Enzymatic Methods of Analysis," Pergamon Press, London, 1970; *c*) G.G. Guilbault (ed.), "Practical Fluorescence," Marcel Dekker, New York, 1973; *d*) R.F. Chen and H. Edelhoch ed., "Biochemical Fluorescence: Concept," vol. 1, Marcel Dekker, New York, 1975, vol. 2, 1976.

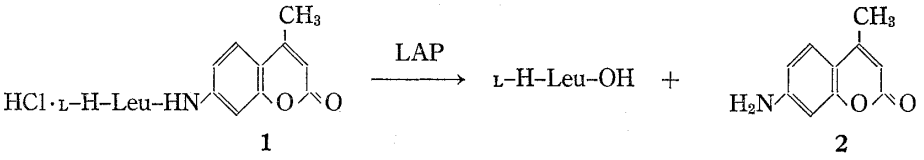
3) For example see: *a*) L.J. Greenberg, *Biochem. Biophys. Res. Comm.*, **9**, 430 (1962); *b*) H. Oya, T. Yamamoto and T. Nagatsu, *Archs. Oral Biol.*, **13**, 941 (1968).

4) *a*) M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **23**, 1385 (1975); *b*) M. Machida, N. Ushijima, T. Takahashi, and Y. Kanaoka, *ibid.*, **25**, in press.

5) M. Zimmerman, E. Yureuicz, and G. Patel, *Anal. Biochem.*, **70**, 258 (1976).

6) H. von Pechmann and O. Schwarz, *Chem. Ber.*, **32**, 3696 (1899).

7) Fluorescence spectra were recorded on a Hitachi-Fluorescence Spectrophotometer, MPF-2A.



1 2

Chart 1

fluorescence of **1** does not interfere with this fluorometric assay through the course of the enzymatic hydrolysis.

Kinetic studies gave the following parameters; $K_m = 0.16$ mM, specific activity = 4.0 μmoles/mg of enzyme/min, which were comparable with the values for a conventional fluorogenic substrate, L-leucyl-β-naphthylamide[3] (0.15 mM, 5.5 μmoles/mg of enzyme/min, respectively). The linearities of the fluorescence intensities *v.s.* incubation time (for more than 10 min), and also *v.s.* varying enzyme concentrations (at least 10-fold range up to 1.1 μg enzyme/ml, at substrate concentration of 0.2 mM) were satisfactory. Assays were typically performed as follows: To 0.03—0.5 mM of **1** in 50 mM Tris–HCl buffer (3 ml) containing 0.5% (v/v) of dimethyl sulfoxide (DMSO), was added the solution (50 μl) of 0.03—0.3 mg/ml of leucine aminopeptidase (Sigma, Type-IV, crystalline suspension) at 25°, and the increase in emission at 440 nm (appearance of **2**) was measured (excitation at 380 nm). The present conditions allow the measurement of activity of at least 0.1 μg of the enzyme, equivalent to 0.1 pmole (based on mol. wt. of 300000[8]).

It is of advantage to adopt the 7-amino-4-methylcoumarin derivatives for the fluorometric enzyme assay over other methods for the following reasons: (1) The aminocoumarin (**2**) is highly fluorescent with its maximum fluorescence intensity, for example, 14-fold greater than that of a conventional fluorescence amine, β-naphthylamine. (2) There is distinct difference in the fluorescence intensity between the aminocoumarin and the corresponding amide substrates such as **1**. Systematic studies of syntheses and the application of various fluorogenic substrates containing the aminocoumarin (**2**) as the key fluorescence amine component are now in progress.

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