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Synthesis of a Key Fluorogenic Amide, L-Arginine-4-methylcoumaryl-7-amide (L-Arg-MCA) and Its Derivatives. Fluorescence Assays for Trypsin and Papain[1]

L-Arginine-4-methylcoumaryl-7-amide **2** and its derivatives, which contain 7-amino-4-methylcoumarin **1** as a key fluorescence amine, were synthesized. The L-antipodes of N^{α}-benzoyl and N^{α}-carbobenzoxy derivatives were shown to be the useful fluorogenic substrates for the sensitive determinations of trypsin and papain.

Keywords——7-amino-4-methylcoumarin; a key fluorescence amine; fluorogenic substrates; L-antipode; sensitive enzyme assay; kinetic study

In the course of our broadly based studies of organic fluorescence reagents,[2] we have been aware of special usefulness of aminocoumarin derivatives as the fluorophores,[3] and systematic studies have been undertaken on the synthesis and the application of various fluorogenic substrates containing 7-amino-4-methylcoumarin **1** as the key fluorescence amine component. For example, the leucineamide of the amine was found to be a useful fluorogenic substrate for the assay of aminopeptidase.[1a] In the present communication we wish to report the synthesis of a key fluorogenic amide, L-arginine-4-methylcoumaryl-7-amide (L-Arg–MCA) **2** and its derivatives, and sensitive determinations of trypsin and papain using these fluorogenic amide substrates; the L-antipodes of N^{α}-benzoylarginine-4-methylcoumaryl-7-amide (Bz-L-Arg–MCA) **3a** and N^{α}-carbobenzoxyarginine-4-methylcoumaryl-7-amide (Z-L-Arg–MCA) **3b**.

N^{α}-Carbobenzoxy-N^{G}-nitro-L-arginine **4a** was coupled with **1**[1a] in the presence of 1-hydroxybenzotriazole and N-ethyl-N′-3-dimethylaminopropylcarbodiimide in DMF to give the amide **5a** (41%), which was transformed, either on treatment with anhydrous HF (Sakakibara method)[4] followed by ion-exchange column chromatography (Dowex 1×2), or by catalytic hydrogenation (5% Pd-charcoal; aqueous HOAc), into **2** in good yields. In a similar manner, **5b**, which was prepared from **4b** and **1** with DCC, was treated with anhydrous HF

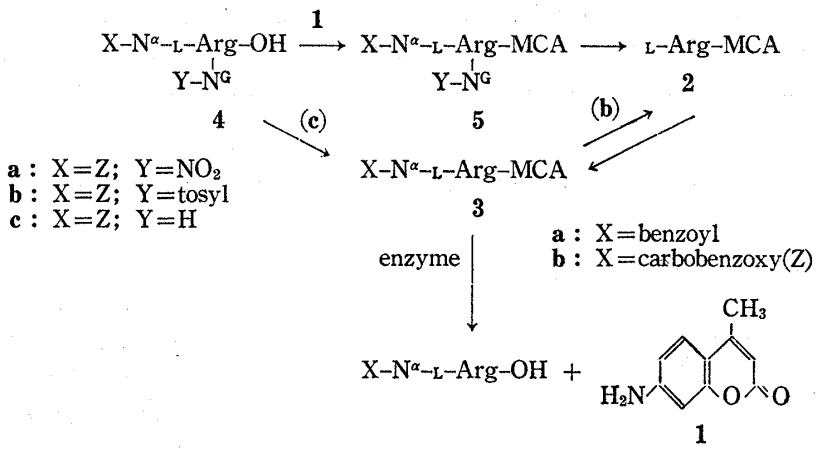

Chart 1

1) Organic Fluorescence Reagent. IV. *a*) Part III: Y. Kanaoka, T. Takahashi, and H. Nakayama, *Chem. Pharm. Bull.* (Tokyo), **25**, 362 (1977).
2) Y. Kanaoka, *Angew. Chem. Int. Ed. Eng.*, **16**, 137 (1977).
3) M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **23**, 1385 (1975)
4) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

to give **2** in 85% yield. **3a** was prepared from **2** and benzoic acid N-hydroxysuccinimide ester in aqueous DMF in 82% yield. Though **3b** can be synthesized similarly from **2** by carbobenzoxylation, it was, alternatively, prepared directly from **4c** and **1** with DCC in DMF in 29% yield. By catalytic hydrogenation (5% Pd/C, aq. HOAc) **3b** was converted to **2** in quantitative yield (Chart 1).[5]

L-Arginine-4-methylcoumaryl-7-amide (**2**): Acetate, mp 210—214.5° (dec.), $[\alpha]_D^{18}=+82.3$ ($c=0.5$, 50% HOAc); Hydrochloride, mp 275° (dec.), $[\alpha]_D^{15}=+91.9$ ($c=1.01$, 25% HOAc). N^{α}-Benzoyl-L-arginine-4-methylcoumaryl-7-amide (**3a**): Hydrochloride, mp 250° (dec., sintering above 160°), $[\alpha]_D^{15}=+29.8$ ($c=0.41$, DMF). N^{α}-Carbobenzoxy-L-arginine-4-methylcoumarly-7-amide (**3b**): Hydrochloride, mp 210—211° (dec.), $[\alpha]_D^{15}=-17.0$ ($c=2.35$, DMF).

Both of the fluorogenic substrates described here and their common hydrolytic product, 7-amino-4-methylcoumarin **1**,[1a] are highly fluorescent. However, when excited at 380 nm and measured at 440 nm, **1** possesses a relative fluorescence intensity approximately 700-fold higher than that of the each amide substrate so that the faint fluorescence of substrate does not interfere with these fluorometric assays.

Table I. Kinetic Parameters of the Fluorogenic Substrates for Trypsin and Papain

Substrate	Trypsin			Papain		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Bz-L-Arg–MCA	0.11	0.42	3800	0.92	0.53	580
Z-L-Arg–MCA	0.20	0.89	4500	0.97	0.42	430
Bz-L-Arg-*p*-nitroanilide	0.49	0.83	1700	3.8	0.15	40

Kinetic studies gave the parameters listed in Table I. With respect to k_{cat}/K_m, both fluorogenic compounds were shown to be better amide substrates for trypsin and papain than the conventional chromogenic substrate, Bz–Arg-*p*-nitroanilide.[6-8] The linearities of the fluorescence enhancements *v.s.* incubation time (for more than 10 min) were satisfactory and the rates of hydrolysis were proportional to enzyme concentration between at least 30—600 ng/ml in the case of trypsin. Assays were typically performed as follows: To 0.01—0.1 mM of the substrate in 50 mM Tris-HCl buffer, pH 8.0 (3 ml) containing 20 mM $CaCl_2$ and 1% (v/v) of dimethyl sulfoxide (DMSO), was added the solution (50 μl) of 0.04—0.4 mg/ml of trypsin (Worthington, TRL, 2X crystallized) at 25°, and the increase in emission at 440 nm was measured (excitation at 380 nm). Papain (Worthington, Mercuripapain HGPAP, crystalline suspension in 70% EtOH) was assayed similarly except that assay medium was changed to 0.02—0.2 mM of the substrate in 50 mM Tris-HCl buffer, pH 7.5 containing 5 mM L-cysteine, 2 mM EDTA and 1% (v/v) of DMSO.

It is of advantage to adopt the fluorogenic substrates, Bz-L-Arg–MCA **3a** and Z-L-Arg–MCA **3b** for the assays of trypsin and papain over the conventional chromogenic substrate, Bz-L-Arg-*p*-nitroanilide, in several points: (1) the values of k_{cat}/K_m, which refers to specificity index of enzymatic catalysis, are higher and therefore they are more specific substrates. (2) Sensitivity of the measurement increases at least 100 times. (3) The solubility in water is

5) All new compounds gave satisfactory elemental analyses.
6) The L-antipode was first synthesized by Noguchi *et al.*,[7] and shown to be a better substrate than the racemate.[8]
7) N. Nishi, S. Tokura, and J. Noguchi, *Bull. Chem. Soc. Japan.*, **43**, 2900 (1970).
8) *a*) H. Nakata and S. Ishii, *Biochem. Biophys. Res. Comm.*, **41**, 393 (1970); *b*) *Idem.*, *J. Biochem.* (Tokyo), **72**, 281 (1972).

similar or slightly higher. (4) In addition, by using the L-antipode, it is possible to analyse the kinetic parameters of enzymatic hydrolysis more directly and precisely than with the DL-isomer because the D-isomer contained in the racemate behaves as a competitive inhibitor.[8–10] Thus L-Arg–MCA **2** is expected to be a very important intermediate for the syntheses of a series of fluorogenic peptide derivatives. Studies on fluorescence assays of trypsin-like enzymes employing the amino acid and peptide substrates which contain the L-Arg–MCA moiety as the key fluorophore will be reported in forthcoming papers.

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9) B.F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).
10) When the manuscript was in preparation, Zimmerman *et al.*, independently reported synthesis and application of several Arg–MCA derivatives including Z-L-Arg–MCA and Bz-DL-Arg–MCA, which were prepared *via* different routes from ours.[11] Evidently Bz-L-Arg–MCA **3a**, synthesized from our key-intermediate **2** is superior as the assay reagent to the DL counterpart. For example, **3a** is a much better substrate than the latter by a factor of 5 in terms of k_{cat}/K_m.
11) M. Zimmerman, B. Ashe, E.C. Yurewicz, and G. Patel, *Anal. Biochem.*, **78**, 47 (1977).