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Synthesis of a New Fluorogenic Substrate for Cystine Aminopeptidase[1)]

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S-Benzyl-L-cysteine-4-methylcoumarinyl-7-amide (CMC) was synthesized. This amide was expected to be a potentially useful substrate for fluorometric microdetermination of cystine aminopeptidase, one of the most important enzymes in the diagnosis of placental functions.

Keywords——cystine aminopeptidase; 7-amino-4-methylcoumarin; a key fluorogenic amine; fluorometric assay; placental functions

Cystine aminopeptidase (CAP) is one of the most important enzymes in the diagnosis of placental functions, because changes of its activity during pregnancy give an indication of the integrity of the developing placenta.[2)] The serum CAP activity has been determined colorimetrically[3)] or fluorometrically.[4)] However, these methods are not sufficiently sensitive or rapid. For example, a conventional assay by Watson's method[3d)] using *S*-benzyl-L-cysteine-*p*-nitroanilide requires 30 min and 50 μl of serum per reaction.

In the course of our broadly based studies of organic fluorescence reagents,[5)] we have become aware of the particular usefulness of aminocoumarin derivatives as fluorophores, and a study has been undertaken to develop a series of fluorescence reagents employing 7-amino-4-methylcoumarin (AMC; **1**) as a key fluorophore. Thus, several AMC amides of appropriate amino acid derivatives have been synthesized[6)] and successfully employed for laboratory and clinical assays of corresponding proteolytic enzymes: *e.g.*, leucine aminopeptidase,[7)] trypsin and papain,[8)] and γ-glutamyltranspeptidase.[1)] As an extension of this work, the synthesis of a key fluorogenic amide, *S*-benzyl-L-cysteine-4-methylcoumarinyl-7-amide (CMC)

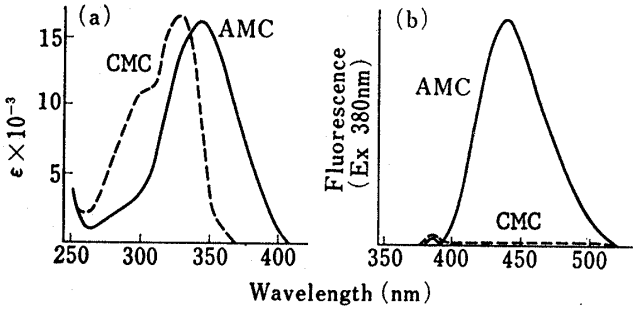


Fig. 1. (a) Ultraviolet Absorption Spectra of **1** (AMC) and the Amide (CMC) in Ethanol,[8)] (b) Fluorescence Specra of **1** (AMC) and the Amide (CMC) in Ethanol[8)]

——, AMC; ------ ,CMC.

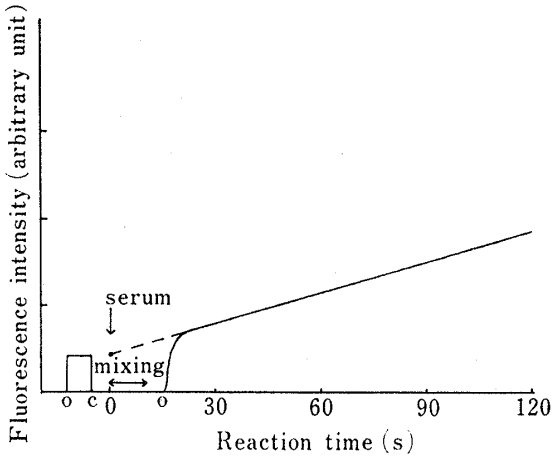


Fig. 2. Fluorescence Development due to Hydrolysis of CMC to AMC by the Serum of a Pregnant Woman at 37°C

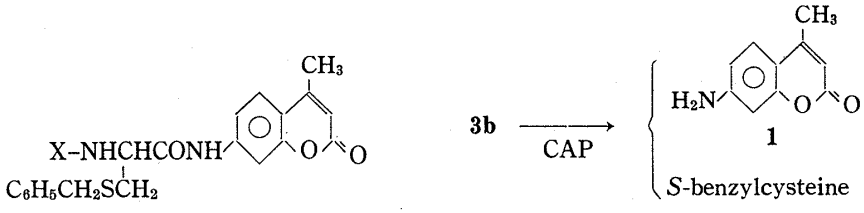
O: Shutter open. C: Shutter closed.

3b, as a useful substrate for fluorometric microdetermination of cystine aminopeptidase, is reported in the present paper.

A synthesis of the cysteine amide (**3b**; CMC) was achieved from *N*-carbobenzoxy-*S*-benzylcysteine (**2**) and 7-amino-4-methylcoumarin (**1**; AMC). The protected amide, obtained by a mixed anhydride method, was treated with hydrobromic acid and the desired amide (**3b**) was characterized as the crystalline tosylate.

Both the amide (**3b**) and the amine **1** are fluorescent. However, as has been generally recognized,[6-8] when excited at 380 nm and measured at 460 nm, **1** possesses a relative fluorescence intensity approximately 700-fold higher than that of the amide **3b** so that the presence of the substrate amide does not interfere with the fluorometric assay. The ultraviolet absorption spectra and the fluorescence spectra of **1** and **3b** are shown in Fig. 1.

In order to see whether the amide **3b** can be a substrate for serum cystine aminopeptidase or not, a preliminary test was performed. Fig. 2 shows the fluorescence development of a solution of **3b** (CMC) initiated by the addition of a serum sample from a pregnant woman in the second trimester, known to have CAP activity. The data suggested that *S*-benzyl-L-cysteine-4-methylcoumarinyl-7-amide **3b** might well be a suitable substrate for a new fluorometric determination of serum cystine aminopeptidase (Chart 1). Since fluorescence measurements are generally several orders of magnitude more sensitive than colorimetric ones,[1,5] this amide (**3b**) was expected to provide a basis for a new microdetermination method for the enzymes. The detailed results on a clinical level will be reported elsewhere.[9]



3a: X=$C_6H_5CH_2OCO$–
3b: X=H(CMC)

Chart 1

Experimental

Melting points are uncorrected. Fluorescence measurements were performed with a Hitachi MPF-2 spectrofluorometer.

Synthesis of CMC——*N*-Carbobenzoxy-*S*-benzyl-L-cysteine-4-methylcoumarinyl-7-amide (**3a**): Isobutyl chloroformate (1.37 g, 10 mmol) was added to a solution of *Z*-Cys(Bzl)–OH (**2**)[1] (2.11 g, 10 mmol) and Et_3N (1.01 g, 10 mmol) in THF (10 ml) at −5°C with stirring. After 10 min, a cooled solution of 7-amino-4-methylcoumarin (**1**; AMC)[1,6-8] (1.75 g, 10 mmol) in DMF (20 ml) was added to this mixture. The solution was stirred in an ice-bath for 1 h, then at room temperature overnight. The solution was evaporated to dryness *in vacuo*, and the residue was taken up in CH_2Cl_2 (20 ml). The solution was washed successively with cold 10% HCl, water, 5% $NaHCO_3$, and water. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was recrystallized from AcOEt; yield 3.57 g (71%), mp 172—174°C. IR ν_{max}^{Nujol} cm^{-1}: 3280 (–NH), 1685, 1620 (C=O; coumarin, Z, amide). *Anal.* Calcd for $C_{28}H_{26}O_5N_2S$; C, 66.91; H, 5.21; N, 5.57; S, 6.38. Found: C, 67.05; H, 5.25; N, 5.62; S, 6.17.

S-Benzyl-L-cysteine-4-methylcoumarinyl-7-amide *p*-Toluenesulfonate (**3b**·TsOH; CMC·TsOH): **3a** (1.06 g, 2 mmol) was treated with 25% HBr/AcOH (6 ml) at room temperature for 40 min. Dry ether was added and the resulting syrupy precipitate was dissolved in EtOH (3 ml) and treated with *p*-toluenesulfonic acid monohydrate (600 mg). Ether was added and the resulting powder was recrystallized from EtOH–ether; yield 822 mg (76%), mp 174—177°C, $[\alpha]_D^{20}$ +32.2° (c=1.3, AcOH). IR ν_{max}^{Nujol} cm^{-1}: 1690, 1600 (C=O; coumarin, amide). UV $\lambda_{max}^{H_2O}$ nm (log ε): 325 (4.2). *Anal.* Calcd for $C_{27}H_{28}O_{26}N_2S_2$: C, 59.98; H, 5.22; N, 5.18; S, 11.86. Found: C, 59.77; H, 5.35; N, 5.17; S, 11.82.

Hydrolysis of CMC by Serum—Preliminary Assay (Fig. 2)——Reaction Mixture: 2.0 ml containing 50 mM KH_2PO_4 (pH 7.0) and 27 μM CMC. Fluorescence development initiated by the addition of 10 μl of the serum of a pregnant woman in the second trimester was measured; ex. 365 nm and em. 440 nm.

References and Notes

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