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Proteolytic Emzymes. III.[1] *trans*-4-Aminomethylcyclohexane-1-carboxylic Acid Derivatives as Substrates and an Active Site Titrant of Trypsin[2]

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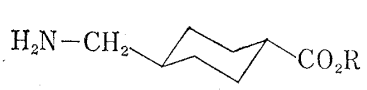
In a previous paper[4] it was reported that ethyl (EAB; **2a**) and *p*-nitrophenyl (NPAB; **2b**) *p*-amidinobenzoate hydrochloride were useful as substrates of trypsin, affording information on requirements of the trypsin active center.[1,4-6] In addition, NPAB was found to be a reagent for the active site titration of the enzyme by way of an aceyl-enzyme intermediate.[4]

Okamoto, *et al.*[7] proposed that the *trans*-4-aminomethylcyclohexane-1-carboxylic acid (*t*-AMCHA; **1**, R=H) is a very powerful inhibitor of plasmin. *t*-AMCHA is now widely used as an anti-plasmin or an anti-fibrinolytic drug in clinical field. Plasmin and trypsin are classfied both in the same "serine enzyme" which is inhibited by diisopropyl fluorophosphate (DFP).[8] They are similar not only in their catalytic function but also in their specificity, hydrolyzing peptide bonds at the carboxyl side of lysine and arginine residue. It appeared reasonable, therefore, to expect that *t*-AMCHA would be a good trypsin inhibitor. In fact, Landmann, *et al.* showed that *t*-AMCHA competitively inhibits the tryptic activation of chymotrypsinogen with K_i value of 3.5×10^{-3}M.[9] Further, Gülzow, *et al.* reported that *t*-AMCHA behaves with different efficiencies as a competitive inhibitor for trypsin and plasmin.[10] Recently Shaw, *et al.* compared the enzymatic activities of trypsin, plasmin and thrombin with guanidinobenzoate as a common substrate.[11] Such a comparison of the kinetic parameters seems important for evaluation of the relation of these proteolytic enzymes.



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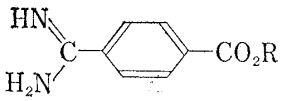

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a : R=C_2H_5 b : R=$C_6H_4NO_2$ (*p*)

In the present paper, ethyl **1a** and *p*-nitrophenyl **1b** esters of *t*-AMCHA were synthesized and their kinetic parameters were determined. The results are listed in Table I. For comparison the data of **2a** and **2b**[4] are also included. The parameters of **1a** and **1b** are not largely

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Table I. Kinetic Parameters of *t*-AMCHA Esters as the Substrates and the Titrants of Trypsin

Substrate	K_m (app) M	k_{cat} (over all) min^{-1}	k_2 (assumed and calcd.) min^{-1}	k_3 (assumed and calcd.) min^{-1}	pH	Spont. hyd. min^{-1}	N%
1a	8.3×10^{-4}	0.56	1.05	1.20	8.2	not obsd.	—
1b	$5\times10^{-7}\gg$	1.20	$\gg$600	1.20	8.2	1.8×10^{-3}	61
	$5\times10^{-7}\gg$	7.6×10^{-2}	$\gg$600	7.6×10^{-2}	5.4	not obsd.	61
2a[4]	1.4×10^{-4}	0.398	0.473	2.53	8.2	not obsd.	—
2b[4]	$5\times10^{-7}\gg$	2.53	$\gg$600	2.53	8.2	1.3×10^{-2}	61
	$5\times10^{-7}\gg$	0.166	$\gg$600	0.166	5.4	not obsd.	61

different from those of **2a** and **2b**. Definition and calculation of these parameters are those as described previously.[1]

As shown in Table I, **1b** has a so large k_2 value compared with k_3 that the optical monitoring of *p*-nitrophenol production reveals an initial "burst" followed by a much slower steady-state increase as observed with **2b**.[1,4] Therefore the compound **1b** may be suitable for normality titration of trypsin in the same manner as **2b**. The operational normality of a commercial specimen of bovine trypsin was thus determined to be 61% either at pH 8.2 or 5.4 on an absorbancy basis. The value is in substantial agreement with that obtained with the same specimen by **2b** as reported previously.[4]

Experimental[12]

Materials

Enzyme——Tripsin was obtained from Worthington Biochemical Corp., Lot TRL-6261 and TRL-7FA.

Ethyl *trans*-4-Aminomethylcyclohexane-1-carboxylate Hydrochloride 1a——A solution of *trans*-4-aminomethylcyclohexane-1-carboxylic acid (1.55 g, a gift from Daiichi Seiyaku Co., Ltd.) in 50 ml EtOH was saturated with dry HCl under cooling and allowed to stand overnight at room temp. After removal of the solvent *in vacuo* the residue was recrystallized from EtOH–ether, 2.0 g of colorless leaflet, mp 194—196°, IR ν_{max}^{Nujol} cm^{-1}: 1725 (C=O ester). *Anal.* Calcd. for $C_{10}H_{19}O_2NCl$: C, 54.11; H, 9.05; N, 6.33. Found: C, 54.24; H, 8.92; N, 6.45.

***p*-Nitrophenyl *trans*-4-Aminomethylcyclohexane-1-carboxylate Hydrochloride 1b**——To a solution of *t*-AMCHA (1.55 g) and $NaHCO_3$ powder (1.92 g) in 50% aqueous dioxane, *tert*-butyl azidoformate (1.74 g)[13] was added dropwise with stirring at room temp. The mixture was warmed at 40—45° for hr under stirring. 75 ml of water was added and the water layer was washed with AcOEt and acidified with citric acid. Recrystallization of precipitate from benzene–hexane gave 2.0 g of colorless needles, mp 137.5—138°, IR ν_{max}^{Nujol} cm^{-1}: 1710, 1690 (C=O). *trans*-4-(*tert*-Butyloxycarbonyl) aminomethylcyclohexane-1-carboxylic acid: *Anal.* Calcd. for $C_{13}H_{23}O_4N$: C, 60.68; H, 9.01; N, 5.12. Found: C, 60.86; H, 8.93; N, 5.12. 255 mg of this N-blocked derivative was coupled with equimolar amount of *p*-nitrophenol by means of dicyclohexylcarbodiimide in ether solution as usual. Recrystallization from benzene–hexane gave 365 mg of pale-yellow needles of mp 129—130,° IR, ν_{max}^{Nujol} cm^{-1}: 1760, 1690 (C=O). *p*-Nitrophenyl *trans*-4-(*tert*-butyloxycarbonyl)aminomethylcyclohexane-1-carboxylate. *Anal.* Calcd. for $C_{19}H_{26}N_2$: C, 60.36: H, 6.93: N, 7.40. Found: C, 59.81; H, 6.71; N, 6.91. Deblocking was achieved as follows: 200 mg of the ester was dissolved in 20 ml of AcOEt saturated with dry HCl. After standing for 24 hr at room temp., the precipitate was collected and recrystallized from EtOH–AcOEt to give 143 mg of colorless crystalline powder of mp 204° (decomp.) IR ν_{max}^{Nujol} cm^{-1}: 1760 (C=O, ester). *Anal.* Calcd. for $C_{14}H_{18}O_4N_2\cdot HCl$: C, 53.42; H, 6.08. Found: C, 53.18; H, 5.93.

Method

Determination of Kinetic Parameters——The procedures of the assays are essentially the same as used in the previous paper.[1,4] The theoretical basis of estimation of k_2 and k_3 were also described previously. In the case of **1a** the hydrolytic rate was measured with Radiometer pH-stat titrator Model TTTlc. The reaction was performed in 200 ml of 0.1 M KCl containing 0.02M $CaCl_2$ at 25° and 0.02 M NaOH as a titrant under nitro-

12) Melting points are uncorrected. UV and IR spectra were determined using a Hitachi EPS-3T spectrophotometer and a JASCO IR-S infrared spectrophotometer, respectively.

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gen stream. The initial substrate concentration was 1.66×10^{-4}—2.62×10^{-3}M and enzyme concentration was 3.75×10^{-6}M. Observed initial velocities were plotted according to Lineweaver and Burk in order to determine K_m and k_{cat}.[14] In the case of *p*-nitrophenyl ester **1b**, the rate assay was carried out by observing the formation of *p*-nitrophenol at 405 or 320 mμ. The concentration of *p*-nitrophenol produced was calculated taking experimental ε value 17600 (405 mμ) at pH 8.2 or 8200 (320 mμ) at pH 5.4. Three ml of buffer solution (0.05M Tris, pH 8.2 or 0.1M acetate, pH 5.4) and 50 μl of substrate solution (in DMF) were pipetted in a 1 cm quartz cuvette placed in thermostated cuvette holder at 25°. The enzymatic hydrolysis was then initiated by introduction of 50 μl of enzyme solution; $[S]$: 1.02×10^{-5}M—1.02×10^{-3}M, $[E]$: 1.00×10^{-5}M. The normality of trypsin was determined by extrapolating the photometric trace of the steady-state back to zero time and measuring initial burst.[1,4]

Acknowledgement We are grateful to Daiichi Seiyaku Co. Ltd. for supplying *t*-AMCHA.

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Adverse Effect of Antitumor Drugs on the Prevention of Metastasis in Mice

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In 1952 Agosin, *et al.*[2] have found an increase in the incidence of metastases by the treatment of cortisone, although local tumoral growth was found to be diminished. Other investigators[3–7] also have reported that cortisone or hydrocortisone provoke disseminated metastases in the hosts when tumor cells were transplanted intravenously to the mice. The experiment reported here shows a stimulative effect of some antitumor drugs on blood-borne metastasis in mice.

The effect of hydrocortisone treatment 24 hours before, after or simultaneously with the tumor cell inoculation (10^6 and 3×10^6 cells per mouse) is shown in Fig. 1. Similarly, an adverse effect of chemotherapy on the tumor metastasis was observed in all cases so far tested for the treatment of the antitumor drugs (Fig. 2). Twenty four hours previous or simultaneous dosage of alkylating agent, antibiotic, plant-alkaloid, folic acid antagonist, antipyrimidine and antipurine were given to the mice inoculated with 3×10^6 tumor cells. The drugs were given with a dose of about 1/4 LD_{50} in all groups. When the drugs were injected prior to the inoculation of tumor cells, the survival time of most mice were shorter than those of the control. However, the life span of the host animals which had been treated simultaneously with cyclophosphamide or methotrexate was more prolonged than others. Particularly, the

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