Synthesis of *trans*-4-Aminomethylcyclohexanecarbonyl-L- and -D-phenylalanine-4-carboxymethylanilide and Examination of Their Inhibitory Activity against Plasma Kallikrein¹⁾

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Based on studies of structure–activity relationship, *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanine-4-carboxymethylanilide (Tra-Phe-APAA) was designed as a selective plasma kallikrein inhibitor and synthesized. Tra-Phe-APAA inhibited plasma kallikrein with a K_i value of 0.81 μM , while it inhibited glandular kallikrein, plasmin, urokinase, factor Xa and thrombin with K_i values of >500, 390, 200, >500, and >500 μM , respectively. However, its stereoisomer, Tra-D-Phe-APPA did not exhibit any detectable inhibitory activity against the above enzymes.

Keywords plasma kallikrein; selective inhibitor; L-phenylalanine derivative; *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanine-4-carboxymethylanilide; chemical synthesis

It is well known that the action of plasma kallikrein liberates bradykinin from high molecular weight kininogen.²⁾ It was also reported that plasma kallikrein can activate factor XII,³⁾ prourokinase⁴⁾ and plasminogen⁵⁾ and may enhance blood polymorphonuclear leukocyte chemotaxis.⁶⁾ These results suggest that plasma kallikrein has a broad spectrum of activities, but as yet, detailed studies of the role of plasma kallikrein remain to be performed.

Our studies were directed at the synthesis of a selective plasma kallikrein inhibitor with the objective of obtaining a valuable and powerful tool for studies on the role of plasma kallikrein and development of new types of clinical therapy. This paper deals with the synthesis of *trans*-4-aminomethylcyclohexanecarbonyl-L- and -D-phenylalanine-4-carboxymethylanilide (Tra-L- and -D-Phe-APAA) and the examination of their inhibitory activity against plasma kallikrein, glandular kallikrein, plasmin, urokinase, factor Xa and thrombin.

In our previous reports,^{7,8)} the following results were obtained: a) Tra group at the P₁ position was suitable for

interaction with negatively charged groups of enzymes such as plasmin and plasma kallikrein, b) the protection of the guanidino function of L-Arg residue at the P'₁ position by the mesitylenesulfonyl group (Mts) increased the affinity of the inhibitor with the enzymes and c) the C-terminal carboxyl group had the role of increasing the specificity of the plasma kallikrein inhibitor. From these results, we designed *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanine-4-carboxymethylanilide [Tra-L-Phe-APAA, (I)], as a selective plasma kallikrein inhibitor as shown in Fig. 1. L-Phe residue was selected because the phenyl group of L-Phe residue might interact with some part of plasma kallikrein as the phenyl group of Mts, resulting in an increase of the affinity of the inhibitor with the enzyme. The designed compound I was synthesized according to the route shown in Fig. 2.

Its inhibitory activity against plasma kallikrein, glandular kallikrein, plasmin, urokinase, factor Xa and thrombin was examined. As summarized in Table I, compound I is a highly selective plasma kallikrein inhibitor, exhibiting that our

Fig. 1. Design of Selective Inhibitor against Plasma Kallikrein

Fig. 2. Synthetic Route to Compound I

TABLE I. Enzyme Inhibition of Compound I

Enzyme	K_i (μM)
Plasma kallikrein	0.81
Glandular kallikrein	> 500
Plasmin	390
Urokinase	200
Factor Xa	> 500
Thrombin	> 500

0.05 M Tris-HCl buffer was used as a buffer and the pH was 7.8 for plasma kallikrein, 9.0 for glandular kallikrein, 7.4 for plasmin, 8.8 for urokinase and 8.3 for factor Xa and thrombin.

TABLE II. Comparison of IC_{50} Values (μM) of Tra-L- and -D-Phe-NH-- CH_2COOH for Various Enzymes

Enzyme	Substrate	IC_{50} (μM)	
		L	D
Plasma kallikrein	S-2302	1.3	1000 (33%)
Plasmin	S-2251	620	1000 (0%)
	Fibrin	350	1000 (24%)
Urokinase	S-2444	350	1000 (0%)
Thrombin	S-2238	1000 (0%) ^{a)}	1000 (0%)
	Fibrinogen	1000 (0%)	1000 (0%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

Fig. 3. HPLC Profiles of Compounds I and II

a) Compound I, b) compound II, c) mixture of compounds I and II. Column: YMC R-ODS-5 (4.6 $\times$ 250 mm); solvent: A = H_2O (0.05% TFA) B = CH_3CN (0.05% TFA) gradient A/B 90/10 $\rightarrow$ 5 min $\rightarrow$ 90/10 $\rightarrow$ 20 min $\rightarrow$ 60/40 $\rightarrow$ 5 min $\rightarrow$ 60/40 $\rightarrow$ 10 min $\rightarrow$ 90/10; flow rate: 1.0 ml/min; absorbance: 220 nm.

design based on the structure-activity study is correct.

Next, in order to examine the effect of racemization of the L-Phe residue of compound I on the inhibitory activity, its stereoisomer, *trans*-4-aminomethylcyclohexanecarbonyl-D-phenylalanine-4-carboxymethylanilide (II) was synthesized by the same route as shown in Fig. 2. Compound II exhibited a symmetrical single peak on high performance liquid chromatography (HPLC) at the same retention time as compound I as shown in Fig. 3. The $[\alpha]_D$ value of compound II is -20.5° , whereas that of compound I is $+20.5^\circ$. The inhibitory activities of compound II against the above enzymes were examined and the results are summarized in Table II in comparison with those of compound I. As can be seen in the Table, compound II exhibited weak inhibitory activity against plasma kallikrein even at a concentration of 1 mM and it did not show any detectable inhibitory activity against other enzymes. Regarding the differences in the inhibitory activity against plasma kallikrein between compounds I and II, we deduce

Fig. 4. Schematic Representation of Interaction of Compound I with Plasma Kallikrein

that in the case of compound II, the phenyl group of D-Phe residue or the phenyl group of 4-carboxymethylanilide moiety, which might be placed in the opposite direction against the binding part of the enzyme, can not interact with some part of the enzyme, although both phenyl groups in compound I can interact with some part of the enzyme as shown in Fig. 4.

Further, compound II did not affect the inhibitory activity of compound I as shown in Fig. 5. These results demonstrate that racemization of the L-Phe residue decreases the inhibitory activity of compound I and the racemization of the D-Phe residue increases the inhibitory activity of compound II in proportion to the degree of racemization.

Finally, it can be emphasized that compound I is a valuable and powerful experimental tool for investigating the role of plasma kallikrein. Moreover, the relatively high LD_{50} value of compound I prompts us to extend our studies to *in vivo* experimental applications. It may also play an important role in the development of new types of clinical

Fig. 5. Inhibition of Plasma Kallikrein by Compounds I and II

a) ○, inhibition by compound I; ●, inhibition by compound I and compound II (1 mM). b) ○, inhibition by compound II; ●, inhibition by compound II and compound I (2 μM). V_o , velocity of hydrolysis by plasma kallikrein in the absence of inhibitor; V_i , velocity of hydrolysis in the presence of inhibitor.

therapy.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110°C, 18 h) were determined with an amino acid analyzer (K-101 AS, Kyowa Seimitsu). For thin-layer chromatography (TLC) (Kieselgel G, Merck), R_f^1 , R_f^2 , R_f^3 and R_f^4 values refer to the systems of CHCl_3 -MeOH-AcOH (90:8:2), CHCl_3 -MeOH-H₂O (89:10:1), CHCl_3 -MeOH-H₂O (8:3:1, lower phase) and n -BuOH-AcOH-H₂O (4:1:5, upper phase), respectively.

General Procedure for Preparation of Boc-L- and -D-Phe-4-Benzoyloxycarbonylmethylanilide A mixed anhydride [prepared from Boc-Phe-OH (27 g, 10 mmol) and isobutylchloroformate (1.3 ml, 10 mmol) as usual] in THF (30 ml) was added to an ice-cold solution of 4-benzoyloxycarbonylmethylaniline-TosOH (4.3 g, 10 mmol) in DMF (50 ml), containing N -methylmorpholine (1.1 ml, 10 mmol). The reaction mixture was stirred at 0°C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na₂CO₃, 10% citric acid and water, dried over Na₂SO₄ and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and recrystallized from EtOH. Yield L: 3.4 g (69%), D: 3.4 g (69%); mp L: 147–148.5°C, D: 148–149.5°C; $[\alpha]_D^{25}$ L: +28.1° (c =0.9, MeOH), D: -28.2° (c =0.8, MeOH), R_f^1 L: 0.65, D: 0.65; R_f^2 L: 0.72, D: 0.72. *Anal.* Calcd for C₂₉H₃₂N₂O₅: C, 71.3; H, 6.60; N, 5.73. Found: L: C, 71.0; H, 6.56; N, 5.59. D: C, 71.3; H, 6.61; N, 5.72.

General Procedure for Preparing Boc-Tra-L- and -D-Phe-4-Benzoyloxycarbonylmethylanilide A mixed anhydride [prepared from Boc-Tra-OH (0.64 g, 2.5 mmol) and isobutylchloroformate (0.34 ml, 2.5 mmol) as usual] in THF (30 ml) was added to an ice-cold solution of H-Phe-4-benzoyloxycarbonylmethylanilide-TFA [prepared from the corresponding N^t -Boc derivative (1.25 g, 2.5 mmol), TFA (1.0 ml, 13 mmol) and anisole (0.30 ml, 2.8 mmol)] in THF (30 ml) containing N -methylmorpholine (0.27 ml, 2.5 mmol). The reaction mixture was stirred at 0°C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na₂CO₃, 10% citric acid and water, dried over Na₂SO₄ and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and recrystallized from EtOH. Yield L: 1.3 g (83%), D: 1.1 g (68%); mp L: 173–174.5°C, D: 172–173°C; $[\alpha]_D^{25}$ L: +23.1° (c =1.1, DMF), D: -23.3° (c =1.0, DMF); R_f^1 L: 0.80, D: 0.80, R_f^2 L: 0.80, D: 0.80. *Anal.* Calcd for C₃₇H₄₅N₃O₆: C, 70.8; H, 7.23; N, 6.69. Found: L: C, 70.7; H, 7.33; N, 6.72. D: C, 70.7; H, 7.30; N, 6.72.

General Procedure for Preparing H-Tra-L- and -D-Phe-4-Carboxymethyl-anilide Boc-Tra-Phe-4-benzoyloxycarbonylmethylanilide (0.50 g, 0.80 mmol) was dissolved in TFA (1.0 ml, 13 mmol) containing anisole (0.08 ml, 0.8 mmol). The reaction mixture was stored at 0°C for 5 min and at room temperature for 2 h. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. The resulting TFA salt was dissolved in MeOH (18 ml) containing 1 N NaOH (2.0 ml, 2 mmol). The reaction mixture was stirred at 0°C for 5 min and at room temperature for 90 min. The pH of the solution was adjusted to 5–6 with acetic acid to give a white precipitate, which was collected by filtration, washed with MeOH and dried. The resulting product

TABLE III. Recovery (%) of Phe Residue after the Action of L- or D-Amino Acid Oxidase

Compound	Recovery (%)	
	L-Amino acid oxidase	D-Amino acid oxidase
Tra-L-Phe-NH- -CH ₂ COOH (I)	n.d.	99
Tra-D-Phe-NH- -CH ₂ COOH (II)	91	n.d.

n.d.: not detectable.

was dissolved in water (10 ml). 1 N HCl (0.8 ml) was added to the solution, which was lyophilized to afford an amorphous powder. Yield L: 0.34 g (90%), D: 0.20 g (55.4%), $[\alpha]_D^{25}$ L: +20.5° (c =1.1, H₂O), D: -20.5° (c =1.0, H₂O). *Anal.* Calcd for C₂₅H₃₁N₃O₄·HCl·1.8H₂O: C, 59.3; H, 6.88; N, 8.29. Found: L: C, 59.4; H, 6.55; N, 8.29. D: C, 59.4; H, 6.61; N, 8.20.

Phenylalanine Racemization Evaluation⁹⁾ The compound (I or II) (5 μmol) was hydrolyzed with 6 N HCl at 110°C for 18 h. After removal of hydrochloric acid, the residue was dissolved in water (5 ml). The pH of the solution was neutralized with 1 N NaOH. An aliquot (0.5 ml) was diluted with Tris/HCl buffer (pH 7.5) to 1 ml. 4 mg of L-amino acid oxidase Type VI (Sigma Chemical Co., Lot 11F-02641, 0.3 unit/mg) or 15 mg of D-amino acid oxidase (Sigma Chemical Co., Lot 24C-0390, 0.09 U/mg) and 10 μg of catalase (Sigma Chemical Co., Lot 84C-8060, 3900 Sigma U/mg) were added to the above solution. The reaction mixture was incubated at 37°C for 24 h. The amount of the remaining amino acid was determined by amino acid analyzer. The results are summarized in Table III.

Assay Procedure The enzymes used were as follows: human plasmin and plasma kallikrein (KABI Co.), bovine thrombin (Mochida Seiyaku Co.), bovine factor Xa (Diagnostic Reagent Ltd.), porcine glandular kallikrein (Sigma Chemical Co.) and human urokinase (Green Cross). Enzymatic activities of plasmin, plasma kallikrein, thrombin, factor Xa, glandular kallikrein and urokinase were determined by the method described previously,¹⁰⁾ using D-Val-Leu-Lys-pNA (S-2251), D-Pro-Phe-Arg-pNA (S-2302), D-Phe-Pip-Arg-pNA (S-2238), Bz-Ile-Glu-Gly-Arg-pNA (S-2222), D-Val-Leu-Arg-pNA (S-2266) and <Glu-Gly-Arg-pNA (S-2444), respectively. Fibrin and fibrinogen were used as substrates for plasmin and thrombin, respectively. IC₅₀ values were determined as follows; 1) Antiamidolytic assay¹¹⁾: The IC₅₀ value was taken as the concentration of inhibitor which decreased the absorbancy at 405 nm by 50% compared with the absorbancy measured under the same conditions without the inhibitor. 2) Antifibrinolytic assay¹¹⁾: The IC₅₀ value was taken as the concentration of inhibitor which prolonged the complete lysis time twofold in comparison with that in the case without the inhibitor. 3) Antifibrinogenolytic assay: To a borate saline buffer (pH 7.4) a solution of various concentrations of a peptide to be tested (0.5 ml) and 0.2% bovine fibrinogen in the above buffer (0.4 ml), bovine thrombin 4 U/ml (0.1 ml) was added. The assay was carried out at 37°C and the clotting time was measured. The IC₅₀ value was taken as the concentration of inhibitor which prolonged the clotting time twofold in comparison with that in the case without the inhibitor.

References and Notes

- Standard abbreviations for amino acid and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2584 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 172 (1976). Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; Tra, *trans*-4-aminomethylcyclohexanecarbonyl; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; TosOH, *p*-toluenesulfonic acid; *n*-BuOH, *n*-butanol; AcOH, acetic acid; Hag, homoarginine.
- M. Yano, T. Nagasawa, and T. Suzuki, *J. Biochem.*, **69**, 471 (1971).
- C. G. Cochran, S. D. Revak, and K. D. Wuepper, *J. Exp. Med.*, **138**, 1564 (1973).
- A. Ichinose, K. Fujikawa, and T. Suyama, *J. Biol. Chem.*, **261**, 3486 (1986).
- R. W. Colman, *Biochem. Biophys. Res. Commun.*, **35**, 273 (1969).
- A. P. Kaplan, A. B. Kay, and K. F. Austen, *J. Exp. Med.*, **135**, 81 (1972).

- 7) N. Teno, K. Wanaka, Y. Okada, Y. Tsuda, U. Okamoto, A. Hijikata-Okunomiya, T. Naito, and S. Okamoto, *Chem. Pharm. Bull.*, **39**, 2340 (1991).
- 8) N. Teno, K. Wanaka, Y. Okada, Y. Tsuda, U. Okamoto, A. Hijikata-Okunomiya, T. Naito, and S. Okamoto, *Chem. Pharm. Bull.*, **39**, 2930 (1991).
- 9) J. H. Johnes and W. I. Ramage, *Int. J. Peptide Protein Res.*, **14**, 65 (1979).
- 10) Y. Tsuda, N. Teno, Y. Okada, K. Wanaka, M. Bohgaki, A. Hijikata-Okunomiya, U. Okamoto, T. Naito, and S. Okamoto, *Chem. Pharm. Bull.*, **37**, 3108 (1989).
- 11) Y. Okada, Y. Tsuda, N. Teno, K. Wanaka, N. Bohgaki, A. Hijikata-Okunomiya, T. Naito, and S. Okamoto, *Chem. Pharm. Bull.*, **36**, 1289 (1988).