Pyroglutamyl Aminopeptidase I, as a Drug Metabolizing Enzyme, Recognizes Xenobiotic Substrates Containing L-2-Oxothiazolidine-4-carboxylic Acid

Koji ABE,^{*,a} Fujio SAITO,^b Makiko YAMADA,^a and Taro TOKUI^a

^aDrug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Co., Ltd.; and ^bChemtech Labo., Inc.; 1–2–58 Hiromachi, Shinagawa-ku, Tokyo 140–8710, Japan. Received September 28, 2003; accepted October 29, 2003

Pyroglutamyl aminopeptidase I (PAP-I) is known for specifically removing the L-pyroglutamate (L-pGlu) residue from the amino terminus of L-pGlu proteins and peptides. In general, substrate recognition of PAP-I as to L-pGlu moiety is tightly regulated. However, we recently identified PAP-I as a metabolic enzyme of an organic nitrate compound, RS-7897, which contains L-2-oxothiazolidine-4-carboxylic acid (L-OTCA). L-OTCA is a latent sulfhydryl group, which has moiety structurally related to L-pGlu. In this study, we investigated the substrate specificity of PAP-I toward modified L-pGlu-containing substrates using recombinant rat, mouse and human PAP-Is. PAP-I was tolerant of replacement of a carbon atom at the 4-position of the L-pGlu moiety by a sulfur atom (L-OTCA), an oxygen atom (L-2-oxooxazolidine-4-carboxylic acid, L-OOCA) and an NH group (L-2-oxoimidazolidine-4-carboxylic acid, L-OICA). The K_m values for rat PAP-I in hydrolyzing L-pGlu–L-Ala, L-OTCA–L-Ala, L-OOCA–L-Ala and L-OICA–L-Ala were 0.057, 0.43, 0.71 and 0.42 mM, respectively. Similar results were observed in mouse and human PAP-Is as well. Moreover, the hydrolysis of RS-7897 in rat and mouse liver cytosols were both completely inhibited by an antibody against rat PAP-I, strongly suggesting that PAP-I is solely involved in the hydrolysis of L-OTCA-containing compounds in rat and mouse liver cytosols.

Key words pyroglutamyl aminopeptidase I; L-pyroglutamate; substrate specificity; L-2-oxothiazolidine-4-carboxylic acid

Pyroglutamyl aminopeptidase I (PAP-I, EC 3.4.19.3) is a typical cytosolic cysteine peptidase, displaying ubiquitous tissue distribution, which hydrolytically removes the L-pyroglutamate (L-pGlu) residue from the amino terminus of L-pGlu proteins and peptides.^{1–3} Substrates for PAP-I include biologically active peptides such as thyrotropin-releasing hormone (TRH), luliberin (LH–RH) and neurotensin, and dipeptides such as L-pGlu–L-Ala and L-pGlu–L-Val.^{2–4} PAP-I shows broad substrate specificity and is also known to hydrolyze the L-pGlu amide bond in non-peptide synthetic compounds such as L-pGlu *p*-nitroanilide (L-pGlu–*p*NA) and L-pGlu β -naphthylamide.^{3–5}

On the other hand, PAP-I is very sensitive to structural changes of L-pGlu residue. Tsuru *et al.*⁴ reported that PAP-I is not capable of hydrolyzing the L-Pro–X bond. Capecchi *et al.*⁶ reported that increasing the pGlu ring size from 5 to 6 members eliminates the ability of PAP-I to cleave the adjacent peptide bond. Moss *et al.*^{7,8} reported that substitution of the NH group on the L-pGlu ring for an Nx group where x is an acyl group (N–CO–R₁) or Mannich base (N–CH₂N–R₂–R₃) confers complete resistance to PAP-I hydrolysis. One notable exception is that PAP-I recognizes the moiety of 2-oxoimidazolidine-4-carboxylic acid (OICA), in which a carbon atom at the 4-position of the pGlu moiety is replaced by an NH group.⁶

Recently, we found in dogs that PAP-I hydrolyzes the amide bond of 4(R)-N-(2-nitroxyethyl)-2-oxothiazolidine-4-carboxamide (RS-7897), an organic nitrate under development, and removes a L-2-oxothiazolidine-4-carboxylic acid (L-OTCA) residue from the parent compound.⁹ L-OTCA is a compound which has a sulfur atom instead of a carbon atom at the 4-position of L-pGlu. It is well known as a latent sulfhydryl group and is easily converted to L-cysteine by 5-oxoprolinase.¹⁰ These results suggest that substrate recognition of PAP-I might not be strictly rigid as to the replacement of the carbon atom at the 4-position of the L-pGlu moiety.

In the present study we investigated the substrate specificity of PAP-I toward several organic nitrates and L-pGlu–L-Ala analogues using recombinant mammalian PAP-Is⁵ and an antibody raised against rat PAP-I.⁵ We demonstrated that xenobiotic compounds, which contain modified L-pGlu residues having a sulfur atom, an oxygen atom or an NH group instead of the carbon atom at the 4-position of the L-pGlu moiety, can serve as substrates for PAP-I.

MATERIALS AND METHODS

Chemicals L-OTCA, L-pGlu, rabbit IgG and L-pGlu–L-Ala were obtained from Sigma Chemical Co. L-2-Thioxo-4-thiazolidinecarboxylic acid (L-TTCA) was obtained from Aldrich Chemical Company. All other reagents and solvents used were of commercially available, guaranteed grade.

Test Compounds RS-7897, aminoethylnitrate hydrochloride (AEN·HCl), OTCA-d₃, L-2-aminopropyl nitrate hydrochloride (L-2-APN·HCl), D-2-APN·HCl, D-OTCA–AEN, furyl-DL-OTCA–AEN, L-OTCA–L-2-APN, L-OTCA–D-2-APN, D-OTCA–L-2-APN and D-OTCA–D-2-APN were synthesized as previously described.^{9,11} L-2-Oxooxazolidine-4-carboxylic acid (L-OOCA, melting point (mp): 116–117 °C) and L-OICA (mp: 178–179 °C) were synthesized according to the methods of Falb *et al.*¹² and Shiba *et al.*,¹³ respectively. L-TTCA–AEN (mp: 128 °C), L-pGlu–AEN (mp: 85–86 °C) and L-OOCA–AEN (mp: 102–103 °C) were synthesized from L-TTCA, L-pGlu and L-OOCA, respectively, according to the method of Ishihara *et al.*¹¹ L-OTCA–ethanolamine (L-OTCA–EA, mp: 116–118 °C) was synthesized by reduction reaction of RS-7897 using zinc powder in the presence of acetic acid. L-OTCA–L-Ala (mp: 182–184 °C), L-OOCA–L-Ala (mp: 189–190 °C) and L-OICA–L-Ala (mp: 211–212 °C) were synthesized by condensation reaction of the corresponding precursors L-OTCA, L-OOCA and (S)-1-benzyloxycarbonyl-2-oxoimidazolidine-

* To whom correspondence should be addressed. e-mail: koujia@shina.sankyo.co.jp

Fig. 1. Chemical Structures of the Compounds Comprising the Substrates Tested

L-pGlu: L-pyroglutamate, L-OTCA: L-2-oxothiazolidine-4-carboxylic acid, L-OOCA: L-2-oxooxazolidine-4-carboxylic acid, L-OICA: L-2-oxoimidazolidine-4-carboxylic acid, L-TTCA: L-2-thioxo-4-thiazolidinecarboxylic acid, AEN: aminoethylnitrate, EA: ethanolamine, 2-APN: 2-aminopropylnitrate.

5-carboxylic acid with L-alanine *t*-butyl ester HCl salt using diphenyl phosphoryl azide in the presence of triethylamine. The benzyloxycarbonyl group was removed by hydrogenation and the *t*-butyl group was catalyzed by acid to form carboxylic acid. The structures of the compounds comprising the substrates tested are shown in Fig. 1.

Kinetic Analysis of Hydrolysis of Synthesized Organic Nitrates and L-pGlu-L-Ala Analogues Rat, mouse and human PAP-Is functionally expressed by *Escherichia coli* as previously described⁵⁾ were used as the enzyme source. The enzymatic kinetic parameters, K_m and V_{max} values, for enzyme-catalyzed hydrolysis of synthesized organic nitrates and L-pGlu-L-Ala analogues were estimated by Eadie-Hofstee plot. To calculate K_{cat} , the subunit molecular weights of the recombinant rat, mouse and human PAP-I were used and the values are 23324, 23345 and 23549, respectively. Potassium phosphate buffer (10 mM, pH 7.4) containing 1 mM dithiothreitol (DTT) and the substrate at varying concentrations (0.02–4 mM or 0.005–1 mM) in a total volume of 40 μ l was preincubated at 37 °C for 2 min, and the reaction was started by the addition of 10 μ l of the enzyme solution. After incubation of the mixture for 5 min at 37 °C, the reaction was stopped by the addition of three volumes of acetonitrile containing 2 μ M of L-2-APN·HCl, AEN·HCl or OTCA-*d*₃ as the internal standard for the determinations of AEN, L-2-APN and D-2-APN, or L-pGlu, L-OTCA, L-OOCA and L-OICA, respectively, by LC tandem mass spectrometry (LC/MS/MS). The mixtures were centrifuged at 20000 $\times g$ for 2 min at 4 °C and the supernatants were stored at 4 °C until analysis. The concentrations of AEN, L-OTCA, L-2-APN and D-2-APN produced from the synthesized organic nitrates and the concentrations of L-pGlu, L-OTCA, L-OOCA and L-OICA produced from L-pGlu-L-Ala analogues were determined as described later. In a separate experiment it was confirmed that all of the products were stable during the testing period.

Effect of Antibody on RS-7897-Hydrolyzing Activity

Rat and mouse liver cytosols were prepared as previously described.⁵⁾ Human liver cytosols were purchased from the International Institute for the Advancement of Medicine (IIAM). Polyclonal antibody against rat PAP-I recombinant

protein was raised in rabbits as reported previously.⁵⁾ Each animal liver cytosol in 10 mM potassium phosphate buffer (pH 7.4) was preincubated on ice for 0.5–1 h with the appropriate concentration of the PAP-I antibody in the absence of DTT. RS-7897 was then added to a final concentration of 20 μ M and the reaction mixture (50 μ l) was incubated at 37 °C for 5–30 min. After incubation, the reaction was stopped by the addition of three volumes of acetonitrile containing 1 μ M of L-2-APN·HCl as the internal standard for the determination of AEN. The mixtures were centrifuged at 20000 $\times g$ for 2 min at 4 °C and the supernatants were stored at 4 °C until analysis. In a separate experiment it was confirmed that the AEN produced was stable during the testing period.

Measurements of AEN, L-2-APN, D-2-APN, L-pGlu, L-OTCA, L-OOCA and L-OICA A SCIEX API-III plus (PerkinElmer Japan Co., Ltd.) connected to an LC-10A system (Shimadzu Corp.) was used as the LC/MS/MS system. The ionization in the LC/MS/MS was performed according to the electrospray ionization (ESI) mode. The column used was CAPCELL PAK C18 UG120 Å (2.0 mm I.D. $\times$ 150 mm, Shiseido Co., Ltd.). The mobile phase used was a mixture of acetonitrile and water (50:50, v/v) containing 1 mM ammonium acetate. In the case of AEN, L-2-APN and D-2-APN, protonated molecular ions, $[M+H]^+$ (AEN: $m/z=107$, L-2-APN and D-2-APN: $m/z=121$), were selected as the precursor ion, and the product ions (AEN: $m/z=61$, L-2-APN and D-2-APN: $m/z=75$) were monitored. In the case of L-pGlu, L-OTCA, L-OOCA and L-OICA, deprotonated molecular ions, $[M-H]^-$ (L-pGlu: $m/z=128$, L-OTCA: $m/z=146$, L-OOCA: $m/z=130$, L-OICA: $m/z=128.7$ and OTCA-*d*₃: $m/z=149$), were selected as the precursor ion, and the product ions (L-pGlu: $m/z=84.2$, L-OTCA: $m/z=102.8$, L-OOCA: $m/z=99.8$, L-OICA: $m/z=42$ and OTCA-*d*₃: $m/z=105.8$) were monitored. The lower limits of quantification were estimated to be 0.1 μ M.

RESULTS

Kinetic Analysis of Hydrolysis toward Synthesized Organic Nitrates by Rat PAP-I The K_m , K_{cat} and K_{cat}/K_m (in-

trinsic clearance) values for rat PAP-I in hydrolyzing several organic nitrates are shown in Table 1. PAP-I recognized the L-OTCA (RS-7897, L-OTCA-EA, L-OTCA-L-2-APN and L-OTCA-D-2-APN) and L-OOCA (L-OOCA-AEN) moieties as well as the L-pGlu (L-pGlu-AEN) moiety, a native recognition site for PAP-I. The affinity of L-pGlu-AEN to PAP-I was the highest of all the compounds tested. The compounds containing the L-OTCA moiety such as RS-7897, L-OTCA-EA (denitrated form of RS-7897) and L-OTCA-L-2-APN showed almost the same K_m values, but had varied K_{cat} values. The intrinsic clearance of L-OTCA-D-2-APN was much lower than that of L-OTCA-L-2-APN, showing that the non-naturally occurring configuration (D form) of the side chain decreased the affinity to PAP-I. The affinity of L-OOCA-AEN to PAP-I was lower than that of RS-7897, but the K_{cat} value was higher.

PAP-I did not recognize at any of the following L-OTCA variants: a) replacement of the oxo moiety with a thioxo moiety (L-TTCA-AEN); b) modification of the configuration of the carboxylic moiety to D forms (D-OTCA-AEN, D-OTCA-L-2-APN and D-OTCA-D-2-APN); or c) introduction of a furan moiety to the carbon atom adjacent to the sulfur atom (furyl-DL-OTCA-AEN) (Table 1).

Kinetic Analysis of Hydrolysis toward L-pGlu-L-Ala Analogues by Rat, Mouse and Human PAP-Is The K_m , K_{cat} and intrinsic clearance values for rat, mouse and human PAP-Is in hydrolyzing L-pGlu-L-Ala, L-OTCA-L-Ala, L-

OOCA-L-Ala, L-OICA-L-Ala and RS-7897 are shown in Table 2. The affinity of L-pGlu-L-Ala to rat PAP-I was more than seven times higher than those of L-OTCA-L-Ala, L-OOCA-L-Ala and L-OICA-L-Ala. The kinetic parameters of hydrolysis toward L-OTCA-L-Ala and L-OICA-L-Ala by rat PAP-I were almost the same. The affinity of L-OOCA-L-Ala to rat PAP-I was slightly lower than those of L-OTCA-L-Ala and L-OICA-L-Ala, however, the K_{cat} value was higher. Both the affinity of RS-7897 to rat PAP-I and the K_{cat} value were slightly lower than those of L-OTCA-L-Ala. Similar results were observed when mouse and human PAP-Is were used as enzyme sources.

Effects of Antibody against Rat PAP-I on L-pGlu-pNA and RS-7897-Hydrolyzing Activity The effects of the antibody against rat PAP-I on the hydrolysis of L-pGlu-pNA (20 μ M) and RS-7897 (20 μ M) as representative substrates containing L-pGlu and L-OTCA, respectively, were investigated in rat, mouse and human liver cytosols (Table 3). The hydrolyzing activities toward L-pGlu-pNA and RS-7897 in rat and mouse liver cytosols were completely inhibited by the addition of the antibody at a concentration of 1 mg-protein/ml. The control rabbit IgG did not affect the hydrolyzing activities at all. The hydrolyzing activities toward

Table 2. Kinetic Parameters of Hydrolysis by Rat, Mouse and Human PAP-Is toward L-pGlu-L-Ala Analogues and RS-7897

Substrate	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (mM ⁻¹ s ⁻¹)
Rat PAP-I			
L-pGlu-L-Ala	0.057	3.7	64.2
L-OTCA-L-Ala	0.43	3.4	7.8
L-OOCA-L-Ala	0.71	8.1	11.3
L-OICA-L-Ala	0.42	3.6	8.4
RS-7897	0.55	2.3	4.2
Mouse PAP-I			
L-pGlu-L-Ala	0.046	3.2	70.3
L-OTCA-L-Ala	0.33	2.5	7.5
L-OOCA-L-Ala	0.57	6.6	11.7
L-OICA-L-Ala	0.37	2.7	7.2
RS-7897	0.58	2.3	3.9
Human PAP-I			
L-pGlu-L-Ala	0.045	2.4	53.3
L-OTCA-L-Ala	0.30	2.3	7.6
L-OOCA-L-Ala	0.55	6.4	11.5
L-OICA-L-Ala	0.33	2.4	7.2
RS-7897	0.50	1.8	3.7

K_m and K_{cat} values were determined as described in Materials and Methods.

Table 1. Kinetic Parameters of Hydrolysis by Rat PAP-I toward Synthesized Organic Nitrates

Substrate	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (mM ⁻¹ s ⁻¹)
RS-7897 (L-OTCA-AEN)	0.55	2.3	4.2
L-pGlu-AEN	0.14	3.5	24.1
L-OOCA-AEN	1.53	7.6	5.0
D-OTCA-AEN	n.h. ^{a)}		
L-TTCA-AEN	n.h.		
Furyl-DL-OTCA-AEN	n.h.		
L-OTCA-EA	0.62	0.9	1.5
L-OTCA-L-2-APN	0.57	3.2	5.5
L-OTCA-D-2-APN	1.43	1.5	1.0
D-OTCA-L-2-APN	n.h.		
D-OTCA-D-2-APN	n.h.		

K_m and K_{cat} values were determined as described in Materials and Methods.

a) n.h.: not hydrolyzed.

Table 3. Effect of Anti-rat PAP-I on L-pGlu-pNA- and RS-7897-Hydrolyzing Activities of Rat, Mouse and Human Liver Cytosols

Treatment	Activity (pmol/min/mg-protein)		
	Rat	Mouse	Human
L-pGlu-pNA-Hydrolyzing activity ^{a)}			
No antibody	458.3±69.4 (100.0)	318.2±24.7 (100.0)	203.5±15.1 (100.0)
Control IgG	434.2±36.6 (95.4)	326.3±12.6 (102.8)	202.2±2.6 (99.7)
PAP-I antibody	n.d. ^{b)} (0.0)	n.d. (0.0)	33.7±9.8 (16.8)
RS-7897-Hydrolyzing activity			
No antibody	28.0±0.7 (100.0)	12.7±0.7 (100.0)	13.2±2.0 (100.0)
Control IgG	28.6±0.4 (102.1)	12.3±0.3 (97.7)	12.6±1.9 (95.8)
PAP-I antibody	n.d. (0.0)	n.d. (0.0)	0.5±0.4 (3.7)

Following preincubation with the antibody for 0.5–1 h on ice, residual L-pGlu-pNA and RS-7897 hydrolyzing activities were assayed, as described in Materials and Methods. Both control IgG and the antibody concentrations were 1 mg-protein/ml. Parentheses show the mean of the residual activity as a percentage of the control activity (100%). Data are expressed as mean±S.D. of 3 experiments. a) From ref. 5. b) n.d.: not detected.

these substrates in human liver cytosol were decreased to 16.8% for L-pGlu-pNA and 3.7% for RS-7897 by the addition of the antibody at the same concentration.

DISCUSSION

We investigated the substrate specificity of PAP-I using recombinant rat, mouse and human PAP-Is toward several organic nitrates and L-pGlu-L-Ala analogues. We demonstrated that PAP-I allowed the replacements of the carbon atom at the 4-position of the L-pGlu moiety with a sulfur atom (L-OTCA), an oxygen atom (L-OOCA) or an NH group (L-OICA), as shown in Tables 1 and 2, even though the affinities of the compounds containing these moieties to PAP-I were decreased compared to that of the L-pGlu-containing compound.

The substrate recognition mechanism has previously been studied using bacterial PAPs.^{14,15} Because the amino acid residues comprising the catalytic triad (Cys-His-Glu) are highly homologous between bacterial PAPs and mammalian PAP-Is, the mechanism in bacterial PAPs was thought to be functionally identical to that in mammalian PAP-Is.¹⁶ Ito *et al.*¹⁵ reported that the NH group and the oxygen atom of the L-pGlu moiety are necessary to form the hydrogen bond with the oxygen atom and the NH group of Gln71, respectively, of PAP in *Bacillus amyloliquefaciens*, and concurrently the L-pGlu moiety is necessary for insertion into the hydrophobic pocket of PAP. These findings well explain the reason why PAP-I is strictly sensitive to the conversions of the NH group at the 1-position and the oxo moiety at the 5-position of the L-pGlu moiety for substrate recognition by PAP-I. It was thought that any unrecognized substrate by PAP-I could not form hydrogen bond with the catalytic region of PAP-I nor insert into the hydrophobic pocket of PAP-I.

The carbon atom at the 4-position of the L-pGlu moiety was located in the hydrophobic pocket of PAP-I,¹⁵ suggesting that compounds having L-OTCA, L-OOCA or L-OICA moiety could insert into the hydrophobic pocket of PAP-I. However, the affinities of the substrates having such L-pGlu variants to PAP-I were much lower than those having the L-pGlu moiety. The relatively large molecular size of the sulfur atom or the hydrophilic nature of the oxygen and nitrogen atoms compared to the carbon atom at the 4-position of the L-pGlu moiety might interfere with the insertion of the moieties into the hydrophobic pocket of PAP-I.

There were no species differences with regard to the substrate hydrolyzing property of recombinant rat, mouse or human PAP-I toward L-pGlu-L-Ala, L-OTCA-L-Ala, L-OOCA-L-Ala, L-OICA-L-Ala or RS-7897, as shown in Table 2. We had already clarified that the molecular and biochemical properties of recombinant rat, mouse and human PAP-Is were highly conserved by using L-pGlu-pNA as a substrate.⁵ Moreover, we strongly suggested that PAP-I is solely involved in the hydrolysis of the amide bond of the L-pGlu- or L-OTCA-containing compounds in rat and mouse liver cytosols, since the hydrolyzing activities toward these substrates in rat and mouse liver cytosolic fractions were both completely inhibited by the addition of the antibody against rat PAP-I.

A major obstacle to the application of peptides as clinical

drugs is their poor biomembrane penetration and rapid enzymatic degradation.⁸ The derivatization of bioactive peptides by L-OTCA may enhance the properties of absorption and biomembrane penetration compared to that by L-pGlu, because the L-OTCA moiety is more lipophilic than the L-pGlu moiety. Moreover, the intrinsic clearance of hydrolysis of the L-OTCA derivatives was much lower than that of the L-pGlu derivatives (see Tables 1, 2), suggesting that first pass metabolism of the L-OTCA derivatives would be much lower than L-pGlu derivatives.

L-OTCA has an interesting feature of intracellular L-cysteine delivery.¹⁰ Recently, we have demonstrated in dogs that PAP-I hydrolyzes RS-7897 to produce active metabolite AEN and L-OTCA.⁹ Supplying L-cysteine molecules *via* L-OTCA to vascular smooth muscle cells has been suggested to decrease the development of nitrate tolerance, which is one of the undesirable effects of organic nitrates.^{11,17} Furthermore, since L-OTCA is known as a very safe compound in humans,¹⁰ it is conceivable that L-OTCA has a desirable property for derivatization of peptides and xenobiotics.

In conclusion, we found that PAP-I hydrolyzes L-pGlu variants containing heteroatoms instead of the carbon atom at the 4-position. We also postulated on the usefulness of L-OTCA, a latent sulfhydryl group, as a leaving group of prodrugs activated by PAP-I.

Acknowledgements We wish to thank Ms. M. Saito, Drs. S. Ishihara, S. Ito and A. Kasuya for their support and many valuable discussions.

REFERENCES

- 1) McDonald J. K., Barrett A. J., "Mammalian Proteases: A Glossary and Bibliography," Vol. 2, Academic Press, London, 1986.
- 2) Browne P., O'Cuinn G., *Eur. J. Biochem.*, **137**, 75–87 (1983).
- 3) Cummins P. M., O'Connor B., *Int. J. Biochem. Cell Biol.*, **28**, 883–893 (1996).
- 4) Tsuru D., Sakabe K., Yoshimoto T., Fujiwara K., *J. Pharmacobio-Dyn.*, **5**, 859–868 (1982).
- 5) Abe K., Watanabe N., Kosaka T., Yamada M., Tokui T., Ikeda T., *Biol. Pharm. Bull.*, **26**, 1528–1533 (2003).
- 6) Capecchi J. T., Loudon G. M., *J. Med. Chem.*, **28**, 140–143 (1985).
- 7) Bundgaard H., Moss J., *J. Pharm. Sci.*, **78**, 122–126 (1989).
- 8) Moss J., Bundgaard H., *Acta Pharm. Nord.*, **4**, 301–308 (1992).
- 9) Abe K., Yamada M., Terao T., Mizuno H., Matsuoka Y., Yorikane R., Tokui T., Ikeda T., *Drug Metab. Pharmacokin.*, **18**, 373–380 (2003).
- 10) Leaf C. D., Pace G. W., *Exp. Opin. Invest. Drugs*, **3**, 1293–1302 (1994).
- 11) Ishihara S., Saito F., Ohhata Y., Kanai M., Mizuno H., Fujisawa M., Yorikane R., Koike H., *Bioorg. Med. Chem. Lett.*, **13**, 1527–1530 (2003).
- 12) Falb E., Nudelman A., Hassner A., *Synthetic Commun.*, **23**, 2839–2844 (1993).
- 13) Shiba T., Koda A., Kusumoto S., Kaneko T., *Bull. Chem. Soc. Jpn.*, **41**, 2748–2753 (1968).
- 14) Odagaki Y., Hayashi A., Okada K., Hirotsu K., Kabashima T., Ito K., Yoshimoto T., Tsuru D., Sato M., Clardy J., *Structure*, **7**, 399–411 (1999).
- 15) Ito K., Inoue T., Takahashi T., Huang H. S., Esumi T., Hatakeyama S., Tanaka N., Nakamura K. T., Yoshimoto T., *J. Biol. Chem.*, **276**, 18557–18562 (2001).
- 16) Dando P. M., Fortunato M., Strand G. B., Smith T. S., Barrett A. J., *Protein Expr. Purif.*, **28**, 111–119 (2003).
- 17) Needleman P., Johnson E. M., Jr., *J. Pharmacol. Exp. Ther.*, **184**, 709–715 (1973).