Structure Activity Relationship of Inhibitors Specific for Prolyl Endopeptidase†

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Structural requirements of N-blocked L-proline derivatives as specific inhibitors for prolyl endopeptidase were investigated using a series of substrate analogs. Replacement of L-proline by its D-isomer remarkably reduced the inhibition. Introduction of a sulfur atom in proline and/or in the penultimate pyrrolidine rings significantly increased the inhibition, but the introduction of oxygen rather diminished the activity. A peptide linkage (acid–amide bond) between the proline and the pyrrolidine ring was also required to keep the inhibitory activity. A benzyloxycarbonyl group was most effective as an N-blocked component of the inhibitors.

The inhibition of proteolytic activity by low molecular weight inhibitors serves to evaluate the biological role of the targetted enzyme and to control undesirable high levels of proteolytic activity within biological systems.

Prolyl endopeptidase (EC 3.4.21.26) is widely distributed in animal tissues and seems to play some role in the metabolism of biologically active peptides.[1-4] The enzyme has been purified from brains of several animals,[2-4] carrot,[5] mushrooms[6,7] and microorganisms.[8-10] On the other hand, endogeneous inhibitors of a polypeptide nature have been found in animal tissues and purified from porcine pancreas,[11] sperm of Ascidian,[12] and rat brain.[13] Potent inhibitors specific for the enzyme, Z-Gly-Pro-CH_2Cl,[14] Z-Pro-prolinal,[15,16] and the related compounds[17-20] were also synthesized and reported to show an antiamnesic effect on experimental amnesia in rat and mouse induced by scopolamine or electro-convulsive shock.[21-23]

We[24] have reported that the presence of a pyrrolidine ring in P1 subsite[25] is critical for the inhibitory activity for prolyl endopeptidase and that introduction of a sulfur atom into the pyrrolidine ring (conversion of pyrrolidine to thiazolidine) markedly enhances the inhibitory activity. We further examined the effects of the introduction of sulfur, oxygen, and sulfoxide into proline and pyrrolidine rings on the inhibition for the enzyme and stereo-specificity in the proline moiety of the inhibitor. This paper deals with the structure–function relationship of the inhibitors specific for prolyl endopeptidase.

Materials and Methods

Materials. *N*-Benzyloxycarbonyl-Gly-L-Pro-β-naphthylamide (Z-Gly-Pro-2-NNap) was prepared as described previously.[1] L-Thioproline (Thiopro) and thiazolidine were purchased from the Sigma Chemical Co. and Aldrich Chemical Co., U.S.A., respectively. Prolyl endopeptidase of bovine brain was purified as reported previously.[3]

Assays of prolyl endopeptidase and the inhibitory activity of synthetic compounds. The enzyme activity was assayed

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Abbreviations: Z-, *N*-benzyloxycarbonyl-; 2-NNap, β-naphthylamide; Thiopro-, L-thioprolyl-; DCC, *N,N'*-dicyclohexylcarbodiimide.

by the method of Yoshimoto *et al.*[1-3] with Z-Gly-Pro-2-NNap as a substrate. Inhibitory activities of synthetic compounds were examined as follows: The enzyme, 11 nM, was preincubated with varied concentrations of test compounds from 1 nM to 5 mM at 30°C and pH 7.0. After 5 min, the residual activities were assayed using 0.5 mM substrate under the standard conditions. IC_{50} values were defined as the inhibitor concentrations that resulted in 50% inhibition of the initial activity under the above conditions. The inhibitor constant, *Ki*, was estimated by the method of Dixon[26] using 25 nM enzyme, various concentrations of inhibitors, and 0.3 mM and 0.9 mM substrates at 37°C and pH 7.0.

Syntheses of N-blocked proline derivatives and their intermediate compounds. All the amino acids used here were of the L-configuration, unless otherwise stated. Z-Pro-pyrrolidine (**1**), Z-Pro-thiazolidine (**3**), Z-Thiopro-pyrrolidine (**4**), Z-Thiopro-thiazolidine (**6**), and 3-phenylpropionyl-Thiopro-thiazolidine (**16**) were synthesized as described previously.[24]

Z-Pro-oxazolidine (**2**) *and Z-Thiopro-oxazolidine* (**5**). 1,3-Oxazolidine was prepared by the modified procedure shown in the literature.[27,28] A solution of ethanolamine (2.40 g, 40 mmol) and paraformaldehyde (1.32 g, 44 mmol) in anhyd. benzene (50 ml) in the presence of molecular sieves 4-A (6.0 g) was stirred for 2 hr at 40°C. The benzene layer was separated into another flask. Molecular sieves were washed with CH_2Cl_2 (20 ml), and the combined organic solvents were evaporated *in vacuo* to give crude 1,3-oxazolidine (3.0 g). This material was used directly for the next reaction without further purification. Compounds **2** and **5** were prepared by using DCC from Z-proline or Z-thioproline and the crude 1,3-oxazolidine in 26% and 40% yields, respectively.

Z-Thiopro (S-oxide)-pyrrolidine (**7**). A solution of Z-Thiopro-pyrrolidine (0.64 g, 2.0 mmol) in MeOH (10 ml) was added dropwise to a stirred solution of sodium metaperiodate (0.64 g, 3 mmol) in H_2O (10 ml) at room temperature and the resulting solution was stirred for 5 hr. The mixture was filtered and the filtrate was extracted with $CHCl_3$. The combined extracts were washed with 10% citric acid, H_2O, 5% $NaHCO_3$ and brine, and dried over Na_2SO_4, then the solvent was removed *in vacuo*. The residue was crystallized from AcOEt–ether to give compound **7** (0.42 g, 63%).

Z-Pro-thiazolidine S-oxide (**8**) *and Z-Thiopro (S-oxide)-thiazolidine S-oxide* (**10**). They were prepared from Z-Pro-thiazolidine and Z-thiopro-thiazolidine in a similar manner to that described above. Their yields were 67% and 27%, respectively.

Z-Thiopro (S-oxide)-thiazolidine (**9**). This was synthesized in a 61% yield using DCC (solvent DMF–CH_2Cl_2) from Z-thioproline S-oxide, mp 185–189°C, MS *m/z*: 267 (M^+), and thiazolidine.

Z-D-Pro-pyrrolidine (**11**) *and Z-D-Pro-thiazolidine* (**12**). They were prepared from Z-D-proline by the reported procedure[24] for derivatives of the L-isomer in 31% and 67% yields, respectively.

1-[(1-Z-pyrrolidine-2-yl)methyl]pyrrolidine (**13**). To a stirred solution of Z-prolinol (0.47 g, 2.0 mmol) and carbontetrabromide (0.79 g, 2.4 mmol) in CH_3CN (10 ml) was added triphenylphosphine (0.63 g, 2.4 mmol) on ice-cooling, and the mixture was stirred for an additional 8 hr at room temperature. The solvent was evaporated and the residue was chromatographed on a silica gel (elution with 4 : 1 hexane–AcOEt) to give 0.27 g (48%) of 1-Z-2-(bromomethyl)pyrrolidine as a colorless oil, MS *m/z*: 299, 297 (M^+), IR (neat) cm^{-1}: 1700 (C=O). This bromide (1.27 g, 4.2 mmol) and pyrrolidine (0.58 g, 8.2 mmol) were dissolved in EtOH (8 ml) and the solution was heated in a sealed tube for 3 hr at 100°C. The solvent was removed *in vacuo* and the residue was chromatographed on a silica gel (elution with 99 : 1 $CHCl_3$–MeOH) to give 0.27 g (28%) of compound 13 as an oil.

3-[(1-Z-pyrrolidine-2-yl)methyl]thiazolidine (**14**) was prepared in a 25% yield from 1-Z-2-(bromomethyl)pyrrolidine and thiazolidine in a similar manner to that described above.

3-Phenylpropionyl-Pro-pyrrolidine (**15**), *4-phenylbutyryl-Pro-pyrrolidine* (**17**), *4-phenylbutyryl-Thiopro-thiazolidine* (**18**), *(1-naphthyl) acetyl-Pro-pyrrolidine* (**19**) *and (1-naphthyl) acetyl-Thiopro-thiazolidine* (**20**). These compounds were prepared from the respective N-blocked amino acids and the corresponding amines using DCC. The yields were 31%, 46%, 73%, 40%, and 88%, respectively.

N-Blocked amino acids were prepared by acylation of proline or thioproline with the corresponding acyl chlorides in the usual manner.

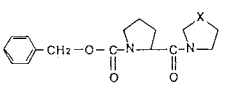
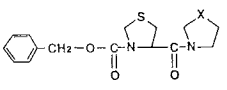
3-Phenylpropionyl-proline: mp 83–85°C, IR (KBr) C=O cm^{-1}: 1758, 1610, $[\alpha]_D$ −192° (*c*=0.5, EtOH), MS *m/z*: 261 (M^+); 4-phenylbutyryl-proline: mp 83–84°C, IR (KBr) C=O cm^{-1}: 1715, 1590, $[\alpha]_D$ −79° (*c*=0.6, AcOEt), MS *m/z*: 261 (M^+); 4-phenylbutyryl-thioproline: mp 87–90°C, IR (KBr) C=O cm^{-1}: 1715, 1610, $[\alpha]_D$ −90° (*c*=0.5, AcOEt), MS *m/z*: 279 (M^+); 1-naphthylacetyl-proline: mp 180–182°C, IR (KBr) C=O cm^{-1}: 1730, 1600, $[\alpha]_D$ −44° (*c*=0.8, dimethylsulfoxide), MS *m/z*: 283 (M^+); 1-naphthyl-thioproline: mp 146–148°C, IR (KBr) C=O cm^{-1}: 1740, 1600, $[\alpha]_D$ −86° (*c*=0.8, dimethylsulfoxide), MS *m/z*: 301 (M^+).

All melting points were measured with a Yanaco micro melting point apparatus and are uncorrected. The purities of these intermediates and the final products were confirmed by thin layer chromatography, elemental analyses, and spectral measurements: infrared (JASCO

Table I. PHYSICOCHEMICAL PROPERTIES OF SOME N-BLOCKED AMINO ACID DERIVATIVES

Compound name (No.)	mp (°C) Recryst. solvent	$[\alpha]_D$ (Temp., °C) (*c*, solvent)	IR cm^{-1} KBr (C=O)	Formula (MS *m/z*: M^+)	Analysis (%) Calcd (Found)			
					C	H	N	S
Z-Pro-oxazolidine (**2**)	105–106 ether	+24.3° (13) (*c*=0.46, AcOEt)	1700 1650	$C_{16}H_{20}N_2O_4$ (304)	63.14 (63.27	6.62 6.44	9.21 9.22)	
Z-Thiopro-oxazolidine (**5**)	Oil	−83.8° (13) (*c*=0.5, AcOEt)	1700 1660 (neat)	$C_{15}H_{18}N_2O_4S$ (322)	55.89 (55.57	5.63 5.77	8.69 8.39	9.93 9.67)
Z-Thiopro(S-oxide)-pyrrolidine (**7**)	108–110 AcOEt–ether	−17.4° (23) (*c*=1.1, MeOH)	1708 1642 1052 (S–O)	$C_{16}H_{20}N_2O_4S$ (336)	57.12 (57.14	6.00 5.99	8.33 8.23	9.53 9.50)
Z-Pro-thiazolidine-(S-oxide) (**8**)	116–118 AcOEt–ether	−34.3° (23) (*c*=1.4, MeOH)	1700 1650 1068 (S–O)	$C_{16}H_{20}N_2O_4S$ (336)	57.12 (57.19	6.00 6.01	8.33 8.16	9.53 9.23)
Z-Thiopro(S-oxide)-thiazolidine (**9**)	153–155 AcOEt	−61.1° (19) (*c*=1.0, MeOH)	1718 1650 1058 (S–O)	$C_{15}H_{18}N_2O_4$ (354)	50.82 (50.84	5.13 5.13	7.90 7.80	18.09 17.59)
Z-Thiopro(S-oxide)-thiazolidine(S-oxide) (**10**)	183–185 MeOH–AcOEt	−84.2° (19) (*c*=1.1, MeOH)	1718 1652 1055 (S–O)	$C_{15}H_{18}N_2O_5S_2$ (370)	48.63 (48.46	4.91 4.86	7.56 7.51	17.31 16.63)
Z-D-Pro-pyrrolidine (**11**)	131–133 AcOEt–ether	+12.3° (18) (*c*=0.4, MeOH)	1700 1640	$C_{17}H_{22}N_2O_3$ (302)	67.52 (67.39	7.33 7.39	9.27 9.33)	
Z-D-Pro-thiazolidine (**12**)	117–119 AcOEt	+13.0° (18) (*c*=0.4, MeOH)	1700 1645	$C_{16}H_{20}N_2O_3S$ (320)	59.99 (60.05	6.29 6.42	8.75 8.76)	
1-[(1-Z-Pyrrolidine-2-yl)methyl]pyrrolidine (**13**)	Oil	−52.7° (22) (*c*=0.52, AcOEt)	1700 (neat)	$C_{17}H_{24}N_2O_2$ (288)	70.80 (70.28	8.39 8.39	9.71 9.51)	
3-[(1-Z-Pyrrolidine-2-yl)methyl]thiazolidine (**14**)	Oil	−82.0° (22) (*c*=0.5, AcOEt)	1697 (neat)	$C_{16}H_{22}N_2O_2S$ (306)	62.72 (62.49	7.24 7.18	9.14 9.10	10.44 10.54)
3-Phenylpropionyl-Pro-pyrrolidine (**15**)	70–71 ether	+6.8° (20) (*c*=0.5, AcOEt)	1650 1640	$C_{18}H_{24}N_2O_2$ (300)	71.97 (71.23	8.05 7.94	9.33 9.20)	
4-Phenylbutyryl-Pro-pyrrolidine (**17**)	Oil	+20.5° (20) (*c*=0.8, AcOEt)	1640 (br.) (neat)	$C_{19}H_{26}O_2N_2 \cdot 1/2H_2O$ (314)	70.56 (70.27	8.35 8.35	8.66 8.45)	
4-Phenylbutyryl-Thiopro-thiazolidine (**18**)	84–86 AcOEt–ether	−71.3° (20) (*c*=0.5, AcOEt)	1655 1640	$C_{17}H_{22}N_2O_2S_2$ (350)	58.27 (58.36	6.32 6.30	8.00 8.04	18.27 18.14)
(1-Naphthyl)acetyl-Pro-pyrrolidine (**19**)	Oil	−9.2° (20) (*c*=0.52, AcOEt)	1650 (br.) (neat)	$C_{21}H_{24}N_2O_2 \cdot 1/2H_2O$ (336)	73.01 (73.00	7.24 7.22	8.10 7.92)	
(1-Naphthyl)acetyl-Thiopro-thiazolidine (**20**)	Oil	−82.8° (20) (*c*=0.5, AcOEt)	1658 (br.) (neat)	$C_{19}H_{20}N_2O_2S_2$ (372)	61.28 (60.74	5.41 5.71	7.52 7.15	17.19 16.82)

Table II. CHANGE IN THE INHIBITORY ACTIVITY TOWARD PROLYL ENDOPEPTIDASE BY INTRODUCTION OF SULFUR AND OXYGEN INTO Z-PRO-PYRROLIDINE DERIVATIVES

Subsites		P3 P2 P1			P3 P2 P1	
						
X	Compd. No.	Inhibitory activity (μM)		Compd. No.	Inhibitory activity (μM)	
		IC_{50}	*Ki*		IC_{50}	*Ki*
CH_2	**1**	2.1	2.4	**4**	0.16	0.21
O	**2**	3.0	2.9	**5**	0.59	0.59
S	**3**	0.26	0.11	**6**	0.02	0.02

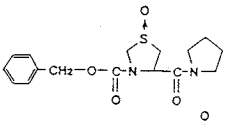
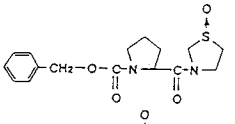
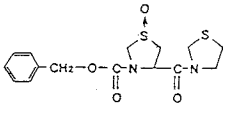
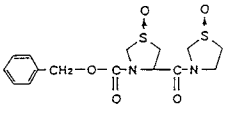
IR-810 or IRA-2 spectrophotometer), mass (JEOL JMS-DX-303 spectrometer using the electron impact ionization method), and NMR (JEOL JMN FX-90Q and Hitachi R-600 spectrometers). Their optical rotations were measured with a JASCO DIP-181 polarimeter. The physicochemical data of the final products are summarized in Table I.

Results and Discussion

Change in inhibitory activity on prolyl endopeptidase by introduction of sulfur, oxygen, and sulfoxide into Z-Pro-pyrrolidine derivatives

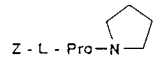
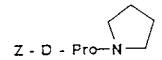
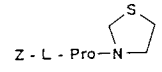
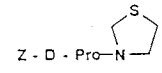
We[24] have reported that Z-Pro-thiazolidine and Z-Thiopro-thiazolidine are potent inhibitors specific for prolyl endopeptidase and that they interact with the enzyme at P3, P2 and P1 subsites according to the subsite-mapping theory proposed by Schechter and Berger.[25] Table II shows the inhibitory activity of Z-Pro- and Z-Thiopro- derivatives that contain thiazolidine, oxazolidine or pyrrolidine at P1 subsite. Introduction of a sulfur atom in proline and particularly in pyrrolidine (conversion to thiazolidine) markedly increased the inhibitory activity, but that of oxygen rather diminished the activity. The conversion of sulfur to sulfoxide in P1 and P2 subsites also reduced the inhibitory activity towards prolyl endopeptidase (Table III). Oxygen is the most electro-negative of the three atoms, S, C, and O. It seems likely that a high electron density in this region is undesirable to the enzyme-inhibitor interaction. Furthermore, introduction of sulfoxide to P1 and/or P2 subsites seems to cause steric hindrance for the binding of inhibitors to the enzyme. On the other hand, the interatomic distances of S–C, O–C and C–C are 1.84, 1.44 and 1.10 Å, respectively, indicating that the size of the five-membered ring of thiazolidine is somewhat larger than those of oxazolidine and pyrrolidine. In brief, sulfur in thiazolidine at P1 subsite seems to locate closer to and interact with a certain functional group

Table III. CHANGE IN INHIBITORY ACTIVITY CAUSED BY THE CONVERSION OF SULFUR TO SULFOXIDE IN P1 AND P2 SUBSITES

Compound No.	Structure	IC_{50} (μM)	*Ki* (μM)
7		0.78 (0.16)*	0.42
8		1.55 (0.26)	1.61
9		1.00 (0.02)	1.24
10		15.80	15.00

* Derivatives without coordinated oxygen (see Table II).

Table IV. Activity Changes by Conversion of L-Pro to D-Isomer

Compound No.	Structure	IC_{50}(μM)	*Ki* (μM)
1		2.1	2.4
11		1,000	1,000
3		0.26	0.11
12		1,000	1,000

of the enzyme: the most plausible candidate is a protonated histidine residue, which has been reported to be a functional group of prolyl endopeptidase.[3,14]

Stereo-specificity in proline moiety of the enzyme inhibitor

Subsite mapping studies on substrate specificity of prolyl endopeptidase have indicated the high stereo-specificity at position of P2 subsite.[2,3,5-7,9] As shown in Table IV, replacement of L-proline by the D-isomer in the inhibitors almost completely abolished the inhibitory activity. This result suggests that stereo-specificity is consistent with that found

Table V. Activity Change Caused by Conversion of Carbonyl Group Linking the P1 and P2 Subsites to Methylene Group

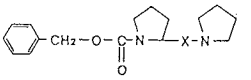
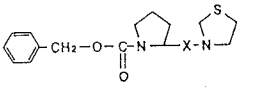
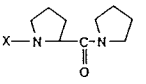
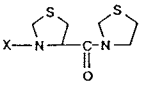
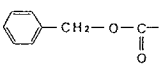
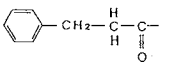
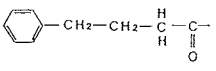
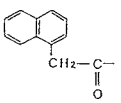
	Structure				
X		Compd. No.	IC_{50} (μM)	Compd. No.	IC_{50} (μM)
		**1**	2.1	**3**	0.26
		**13**	>1,000	**14**	>1,000

Table VI. Comparison of Inhibitory Activity of Pro-pyrrolidine and Thiopro-thiazolidine with Various N-Blocking Groups

					
X (P3)		Compd. No.	IC_{50} (μM)	Compd. No.	IC_{50} (μM)
		**1**	2.1	**6**	0.02
		**15**	8.9	**16**	0.40
		**17**	2.1	**18**	0.18
		**19**	8.2	**20**	0.74

in these enzyme-substrate interaction, confirming that the inhibitors bind to the substrate-binding region of the enzyme.

Conversions of carbonyl group linked between P1 and P2 subsites to methylene group and of N-blocked Z-group in P3 subsite to the other groups

Table V shows the effects of replacement of the carbonyl group of the peptide bond linking the P1 and P2 subsites by methylene group on the inhibitory activity towards the enzyme. Although this position does not correspond to the scissile bond of the substrate, this slight change caused a remarkable loss of the inhibitory activity. It is of interest that the presence of a peptide bond, which does not correspond to the cleavage site of the substrate, is essential for the inhibitor to interact with prolyl endopeptidase. The carbonyl group linked between P1 and P2 subsites seems to form a hydrogen bond to the enzyme and act in fixation of the inhibitor.

Previous studies[3,5,6,9,24] have shown that the presence of a hydrophobic or bulky group in P3 subsite favors the enzyme-substrate interaction and the elongation of the main chain by introducing an L-amino acid in P3 position of the inhibitor rather reduces the inhibitory activity. In the present experiments, we further examined the inhibitory activity of the compounds which have varied lengths of N-protecting groups in the P3 subsite. As shown in Table VI, replacement of a Z-group by a phenylpropionyl one reduced the activity, although the chain length of both groups are almost the same. Phenylbutyryl group was effective as a P3 component to the same extent as a Z-group in Pro-pyrrolidine but less effective in Thiopro-thiazolidine. Furthermore, the replacement of a Z-group by a 1-naphthylacetyl- one, which is more bulky, decreased the inhibitory activity. Cinnamoyl-, tosyl-, phenylacetyl-, and isonicotinyl- groups are also less effective than a Z-group as a P3 subsite component.[24] It remains ambiguous why the Z-group is most effective as a P3 subsite component of inhibitors. To explain these results, however, the following is possible: The Z-group has an ester structure and resonance will occur in this structure, which will make it possible to interact with a positively charged group of prolyl endopeptidase or to form a hydrogen bonding to the enzyme. To confirm this assumption, it is necessary to visualize the active site structure of the enzyme.

Prolyl endopeptidase belongs to a class of serine proteases and is supposed to catalyze peptide bond cleavage by a mechanism similar

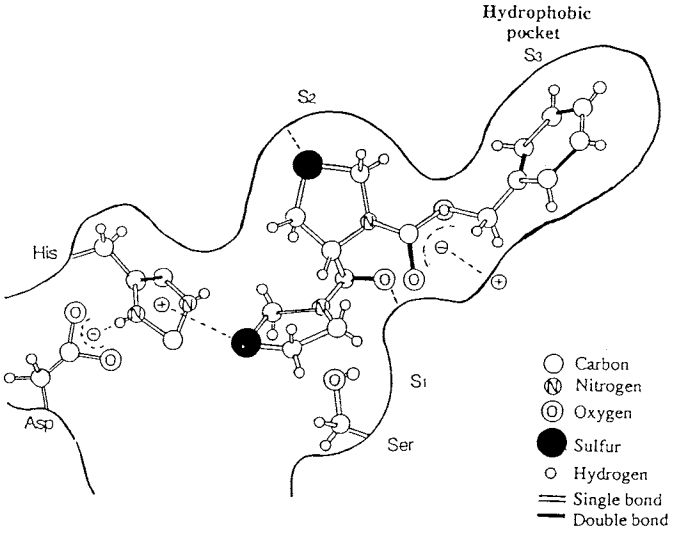


Fig. 1. A Postulated Interaction of Z-Thiopro-thiazolidine with the Active Site of Prolyl Endopeptidase.

to those by other serine proteases such as α-chymotrypsin, trypsin, and subtilisins. Taking into account the above observations, we tentatively propose a model of the interaction of Z-Thiopro-thiazolidine with the active site region of prolyl endopeptidase (Fig. 1). Studies on the active site structure of the enzyme are now in progress and will be reported elsewhere, which will give a clue to elucidate the enzyme-inhibitor interaction in more detail.

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References

1) T. Yoshimoto, K. Ogita, R. Walter, M. Koida and D. Tsuru, *Biochim. Biophys. Acta*, **569**, 184 (1979).
2) T. Yoshimoto, W. H. Simmons, T. Kita and D. Tsuru, *J. Biochem.*, **90**, 325 (1981).
3) T. Yoshimoto, T. Nishimura, T. Kita and D. Tsuru, *J. Biochem.*, **94**, 1179 (1983).
4) P. C. Andrews, C. M. Hines and J. E. Dixon, *Biochemistry*, **19**, 5494 (1980).
5) T. Yoshimoto, A. K. M. Abdus Sattar, W. Hirose and D. Tsuru, *Biochim. Biophys. Acta*, **916**, 29 (1987).
6) T. Yoshimoto, A. K. M. Abdus Sattar, W. Hirose and D. Tsuru, *J. Biochem.*, **104**, 622 (1988).
7) A. K. M. Abdus Sattar, N. Yamamoto, T. Yoshimoto and D. Tsuru, *J. Biochem.*, **107**, 256 (1990).
8) T. Yoshimoto and D. Tsuru, *Agric. Biol. Chem.*, **42**, 2417 (1978).
9) T. Yoshimoto, R. Walter and D. Tsuru, *J. Biol. Chem.*, **255**, 4786 (1980).
10) T. Yoshimoto, F. Matsuo, H. Oyama, T. Honda, A. K. M. Abdus Sattar and D. Tsuru, *Biochem (Life Sci. Adv.)*, **7**, 313 (1988).
11) T. Yoshimoto, K. Tsukumo, N. Takatsuka and D. Tsuru, *J. Pharmaco-Dyn.*, **5**, 734 (1982).
12) H. Yokosawa, M. Miyata, H. Sawada and S. Ishii, *J. Biochem.*, **94**, 1067 (1983).
13) S. Soeda, N. Yamakawa, M. Ohyama, H. Shimeno and A. Nagamatsu, *Chem. Pharm. Bull.*, **33**, 2445 (1985).
14) T. Yoshimoto, R. C. Orlowski and R. Walter, *Biochemistry*, **16**, 2942 (1977).
15) S. Wilk and M. Orlowski, *J. Neurochem.*, **41**, 69 (1983).
16) T. C. Friedman, M. Orlowski and S. Wilk, *J. Neurochem.*, **42**, 237 (1984).
17) H. Yokosawa, M. Nishikawa and S. Ishii, *J. Biochem.*, **95**, 1819 (1984).
18) M. Nishikawa, H. Yokosawa and S. Ishii, *Chem. Pharm. Bull.*, **34**, 2931 (1986).
19) T. Yoshimoto, K. Kawahara, F. Matsubara, K. Kado and D. Tsuru, *J. Biochem.*, **98**, 975 (1985).
20) T. Yoshimoto, K. Kado, F. Matsubara, N. Koriyama, H. Kaneto and D. Tsuru, *J. Pharmacobio-Dyn.*, **10**, 730 (1987).
21) T. Yoshimoto, *Nippon Nōgeikagaku Kaishi*, **58**, 1147 (1984).
22) T. Yoshimoto and D. Tsuru, *Kagaku to Seibutu*, **25**, 554 (1987).
23) D. Tsuru and T. Yoshimoto, *Bio-industry*, **4**, 788 (1987).
24) D. Tsuru, T. Yoshimoto, N. Koriyama and S. Furukawa, *J. Biochem.*, **104**, 580 (1988).
25) I. Schechter and A. Berger, *Biochem. Biophys. Res. Commun.*, **27**, 157 (1967).
26) M. Dixon, *Biochem. J.*, **55**, 170 (1953).
27) P. A. Laurent, *Bull. Soc. Chim. Fr.*, **1967**, 571.
28) G. G. Habermehl and W. Ecsy, *Heterocycles*, **7**, 1027 (1977).