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SYNTHESIS AND INHIBITORY ACTIVITY OF ACYL-PEPTIDYL-PROLINAL DERIVATIVES TOWARD POST-PROLINE CLEAVING ENZYME AS NOOTROPIC AGENTS

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Several prolinal derivatives were synthesized and examined for their inhibitory activity on post-proline cleaving enzymes from *Flavobacterium meningosepticum* and bovine brain and their possible properties as nootropic agents. Almost all the compounds tested inhibited the activity of both enzymes at low IC_{50} values of the order of nM, but a specificity difference was observed with alkylacyl-prolinal derivatives which strongly inhibited only the bacterial enzyme. Prolyl-prolinal derivatives were the most effective inhibitors for both enzymes. In the passive avoidance test using amnesic rats experimentally induced with scopolamine, the prolinal derivatives that have potent inhibitory activity toward post-proline cleaving enzymes showed also strong anti-amnesic activities at doses of $10 \sim 1000\,\mu g/kg$, i.p. Some of the compounds showed a bell-shape dose dependency. These results suggest that the post-proline cleaving enzymes play an important role in the regulation of learning and memory consolidation in the brain and inhibitors of these enzymes are suggested as possible candidates for nootropic agents, particularly for an anti-amnesic drug.

KEY WORDS: Acyl-Peptidyl-Prolinal derivatives, Post-proline cleaving enzyme, Nootropic agents, anti-amnesic acitivity.

INTRODUCTION

Vasopressin is a cyclic nonapeptide hormone, [H-Cys[1]-Tyr[2]-Phe[3]-Glu[4]-Asn[5]-Cys[6]-Pro[7]-Arg[8]-Gly[9]-NH_2], which constricts peripheral blood vessels and acts as an antidiuretic principle. The antidiuretic action of vasopressin is exerted on the absorption of water in the kidney to conserve free water in the body. Recently, de Wied *et al.* reported that [Lys[8]]-vasopressin (in which the [Arg[8]] residue was replaced by lysine residue) ameliorates impaired avoidance learning of a shuttle box avoidance response in hypophysectomized rats,[1] and in intact rats both facilitates passive avoidance learning[2] and increases resistance to extinction of a shuttle box avoidance on a pole jumping avoidance response.[3] They also showed that in rats with a hereditary blockade of vasopressin ([Arg[8]]-vasopressin) synthesis (Brattleboro strain), which display

Abbreviations used are as follows: Cys, cysteine residue; Tyr, tyrosine residue; Glu, glutamic acid residue; Asn, aspargine residue; Pro, proline residue; Arg, arginine residue; Gly, glycine residue; Ala, alanine residue, Val, valine residue; Leu, leucine residue; nLeu, norleucine residue; Met, methionine residue; Phe, phenylalanine residue; EDTA, ethylenediaminetetraacetic acid; WSCD, N-ethyl-N′, N′-dimetylaminoprop carbodiimide; Et_3N, triethylamine; THF, tetrahydrofuran; $NaBH_4$, sodium borohydride; SO_3-pyridine, sulfur trioxide-pyridine complex; DMSO, dimethylsulfoxide.

a severe memory deficit in a one trial passive avoidance situation,[4] removal of vasopressin from the cerebrospinal fluid by arginine- vasopressin antiserum inhibits memory expression in the rat.[5] Furthermore, de Wied and co-workers also isolated a peptide that accumulated as the major product during the proteolysis of arginine-vasopressin by rat brain synaptic membranes, and the chemical structure was found to be a hexapeptide [H-Cys[1]-OH pGlu[4]-Asn[5]-Cys[6]-Pro[7]-Arg[8]-Gly[9]-NH_2]. When administrated intracerebroventricularly in extremely low doses, this vasopressin fragment and its desglycineamide derivative facilitated memory consolidation in a passive avoidance situation in $10^{-3} \sim 10^{-4}$ lower concentration than that of vasopressin. These peptide metabolites of vasopressin constitute highly potent neuropeptides with selective effects on memory and related processes. Because the behaviourally active metabolites were devoid of direct pressor activity on the peripheral vasculature, it was concluded that the memory effects of these metabolites were mediated by central rather than peripheral receptors,[7] and that these binding sites were present in the hilus of the hippocampal formation and other organs in the brain.[8]

Post-proline cleaving enzyme (EC.3.4.21.26) was discovered by Walter *et al.*[9] and classified as a serine proteinase using active-site directed, irreversible inhibitors.[10] The enzyme is highly active in the brain and readily degrades proline-containing low molecular weight oligopeptides such as thyrotropin releasing hormone (TRH),[11] luteinizing hormone releasing hormone (LH-RH),[12] angiotensin II,[13] bradykinin,[14] substance P,[15] and neurotensin.[16] The enzyme also degrades vasopressin which is the oligopeptide speculatively associated with memory as described above.

Low molecular weight inhibitors of post-proline cleaving enzyme have been widely studied. Chloromethylketone derivatives of acyl-proline or acyl-peptidyl-proline inhibited the enzyme by alkylation of the active-site with pseudo-first order rate kinetics.[10] On the other hand, carbobenzoxy-prolyl-prolinal (prolinal is a residue in which the carboxylic acid group of C-terminal proline is reduced to a formyl group) is a specific potential transition state inhibitor of the enzyme.[17] Tsuru and Yoshimoto found that compounds capable of inhibiting the post-proline cleaving enzyme activity were effective for preventing experimental amnesia caused in rats by scopolamine, and inferred that post-proline cleaving enzyme inhibitors have some relation to the fixation of memory.[18] This suggests the potential use of post-proline cleaving enzyme inhibitors as nootropic agents for preventing and/or curing amnesia.

The purpose of this study was to synthesize various acyl-peptidyl-prolinal derivatives as potential post-proline cleaving enzyme inhibitors, and to clarify both the relationships between structure and inhibitory activity *in vitro* and betwen inhibitory activity and preventing the effect of experimental amnesia in rats *in vivo*.

MATERIALS AND METHODS

Animals

Male Wister SCL rats weighing 121 ± 22 g were supplied by Charls River Japan Inc., Osaka, and were housed in a cage for a period of at least one week under standardized conditions of temperature (23 ± 2°C), relative humidity (60 ± 5%), automatically controlled light cycle (8:00–20:00) and free access to tap water and normal laboratory diet.

Chemicals

Amino acids, protected amino acids, and coupling reagent were purchased from Kokusan Chemical Works, Ltd., Tokyo, and other chemicals used in this study were all reagent grade and purchased from Nacalai Tesque, Kyoto.

Synthetic Inhibitors

Various acyl-peptidyl-prolinal derivatives were synthesized and tested as potential inhibitors of post-proline cleaving enzyme, and some of them were also tested as antiamnesic agents *in vivo*. The acyl-peptidyl-prolinal derivatives which have the general structure (**III** in Scheme I) were synthesized in the usual manner as follows[20] (see Scheme I). Carbobenzoxy-amino acid and proline methyl ester hydrochloride were coupled by N-ethyl-N′,N′-dimethylaminopropyl carbodiimide hydrochloride (WSCD·HCl) with triethylamine in anhydrous dichloromethane to obtain the carbobenzoxy-peptidyl-proline methyl ester after purification by silica gel column chromatography in high yield. The protecting carbobenzoxy group of carbobenzoxy-peptidyl-proline methyl ester was removed by catalytic reduction with a small amount of 5% palladium on carbon under hydrogen in ethanol to obtain the amino terminal free peptidyl-proline methyl ester quantitatively. Amino terminal free peptidyl-proline methyl ester thus obtained was acylated by the corresponding acylchloride with triethylamine in anhydrous tetrahydrofuran to give acyl-peptidyl-proline methyl ester (**I**) in reasonable yield. The ester group of the acyl-peptidyl-proline methyl ester was then reduced to the alcohol using sodium borohydride in tert.-butanol with 20% absolute methanol under reflux to give the acyl-peptidyl-prolinol (**II**) in 40 ~ 60% yield. Acyl-peptidyl-prolinol was oxidized to the aldehyde by sulfur trioxide-pyridine complex with triethylamine in anhydrous dimethylsulfoxide to give the desired compound acyl-peptidyl-prolinal (**III**) in 40 ~ 60% yield. Analytical data for the target prolinal derivatives are presented in Table I, and other spectroscopic data are as follows.

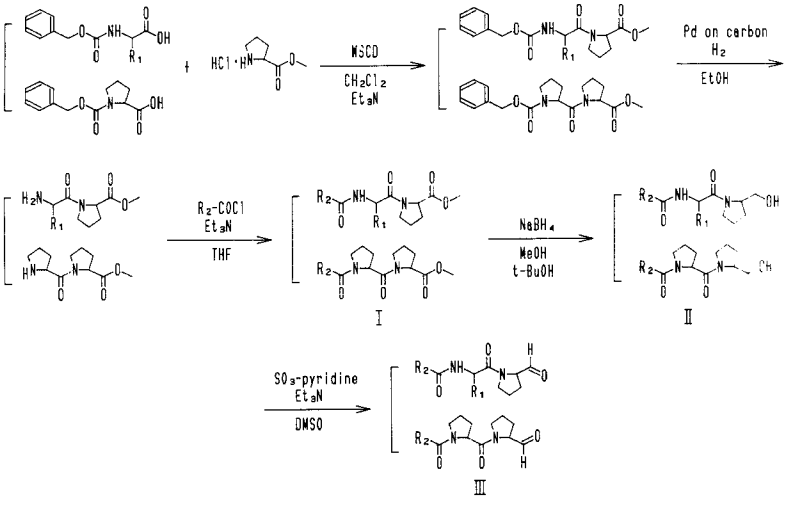

TABLE I(a)
Structure and Elemental Analysis Data for the Compounds Tested
$R\text{-}(CH_2)_n\text{-}CO\text{-}X\text{-}Pro\text{-}H$

R	n	X	Molecular Formula	MW.	Elemental Analysis					
					Calcd.			Found		
					C	H	N	C	H	N
Phenyl-	3	Ala	$C_{18}H_{24}N_2O_3$	316.4	68.33	7.65	8.85	68.20	7.67	8
Phenyl-	3	Val	$C_{20}H_{28}N_2O_3$	344.5	69.74	8.19	8.13	69.39	8.12	8
Phenyl-	3	Leu	$C_{21}H_{30}N_2O_3$	358.5	70.36	8.43	7.81	70.55	8.39	7
Phenyl-	3	nLeu	$C_{21}H_{30}N_2O_3$	358.5	70.36	8.43	7.81	70.51	8.44	7
Phenyl-	3	Met	$C_{20}H_{28}N_2O_3S$	377.5	63.63	7.48	7.42	63.65	7.50	7
Phenyl-	3	Phe	$C_{24}H_{28}N_2O_3$	392.5	73.44	7.19	7.14	73.54	7.13	7
Phenyl-	3	Pro	$C_{20}H_{26}N_2O_3$	342.4	70.15	7.65	8.18	70.41	7.33	8
Phenyl-	1	Pro	$C_{18}H_{22}N_2O_3$	314.4	68.77	7.05	8.19	69.13	6.70	8
Phenyl-	2	Pro	$C_{19}H_{24}N_2O_3$	328.4	69.49	7.37	8.53	69.60	7.68	8
Phenyl-	4	Pro	$C_{21}H_{28}N_2O_3$	356.5	70.76	7.92	7.86	70.98	7.81	7
2-Styrylphenoxy-	3	Val	$C_{28}H_{34}N_2O_3$	462.6	72.70	7.41	6.06	72.72	7.28	5
2-Styrylphenoxy-	3	Leu	$C_{29}H_{39}N_2O_4$	476.6	73.08	7.61	5.88	73.10	7.49	5
2-Styrylphenoxy-	3	Phe	$C_{32}H_{34}N_2O_4$	510.6	75.27	6.71	5.49	75.32	6.48	5
2-Styrylphenoxy-	3	Pro	$C_{28}H_{32}N_2O_4$	460.6	73.02	7.00	6.08	73.33	6.76	5
4-Benzylphenoxy-	3	Val	$C_{27}H_{34}N_2O_4$	450.6	71.97	7.61	6.22	72.07	7.52	6
4-Benzylphenoxy-	3	Leu	$C_{28}H_{36}N_2O_4$	464.6	72.39	7.81	6.03	72.44	7.70	6
4-Benzylphenoxy-	3	Phe	$C_{31}H_{34}N_2O_4$	498.6	74.67	6.87	5.62	74.78	6.73	5
4-Benzylphenoxy-	3	Pro	$C_{27}H_{32}N_2O_4$	448.6	72.30	7.19	6.25	72.27	6.84	6
5-Isoquinolinoxy-	3	Val	$C_{23}H_{39}N_3O_4$	411.5	67.13	7.10	10.21	66.77	7.26	10
5-Isoquinolinoxy-	3	Leu	$C_{24}H_{31}N_3O_4$	425.5	67.74	7.34	9.87	67.87	6.96	10
5-Isoquinolinoxy-	3	nLeu	$C_{24}H_{31}N_3O_4$	425.5	67.74	7.34	9.87	67.82	7.40	10
5-Isoquinolinoxy-	3	Phe	$C_{27}H_{29}N_3O_4$	459.5	70.57	6.36	9.14	70.52	6.51	9
5-Isoquinolinoxy-	3	Pro	$C_{23}H_{27}N_3O_4$	409.5	67.46	6.65	10.26	67.37	6.61	10

TABLE I (b)
Chemical Structure and Elemental Analysis Data for the Compounds Tested
R-X-Pro-H

No.	R	X	Molecular Formula	MW	Elemental Analysis					
					Calcd.			Found		
					C	H	N	C	H	N
24.	Oleoyl-	Ala	$C_{26}H_{46}N_2O_3$	434.7	71.85	10.67	6.44	72.02	10.67	6.23
25.	Oleoyl-	Val	$C_{28}H_{50}N_2O_3$	462.7	72.68	10.89	6.05	72.74	10.68	5.91
26.	Oleoyl-	Leu	$C_{29}H_{52}N_2O_3$	476.7	73.06	10.99	5.88	73.39	11.11	5.85
27.	Oleoyl-	nLeu	$C_{29}H_{52}N_2O_3$	476.7	73.06	10.99	5.88	73.22	11.24	5.84
28.	Oleoyl-	Phe	$C_{32}H_{50}N_2O_3$	510.8	75.25	9.87	5.48	75.28	9.84	5.47
29.	Oleoyl-	Lys(Z)	$C_{37}H_{59}N_3O_5$	625.9	71.00	9.50	6.71	70.65	9.76	6.63
30.	Oleoyl-	Glu(H)	$C_{28}H_{48}N_2O_4$	476.7	70.55	10.15	5.88	70.45	10.39	5.89
31.	Oleoyl-	Pro	$C_{28}H_{48}N_2O_3$	460.7	73.00	10.50	6.08	72.96	10.70	5.93
32.	Linoleoyl-	Leu	$C_{29}H_{50}N_2O_3$	474.7	73.37	10.62	5.90	73.60	10.55	6.16
33.	Stearoyl-	Leu	$C_{29}H_{54}N_2O_3$	478.8	72.76	11.37	5.85	73.11	10.97	5.99
34.	Palmitoyl-	Leu	$C_{27}H_{50}N_2O_6$	450.7	71.95	11.18	6.22	71.80	11.12	6.20

1. 4-Phenylbutyryl-Ala-Pro-H EI-MS(m/z) = 316 $(M)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 1.40(3H, d, J = 6Hz), 1.88–2.28(8H, m), 2.65(2H, m), 3.56–3.80(2H, m), 4.52(1H, m), 4.78(1H, m), 6.35(1H, d, J = 9Hz), 7.21(5H, s), 9.49(1H, s).

2. 4-Phenylbutyryl-Val-Pro-1H EI-MS(m/z) = 345 $(M + H)^+$; ^{1}H-MNR (from TMS, $CDCl_3$) 0.95(3H, d, J = 7Hz), 1.02(3H, d, J = 7Hz), 1.82–2.27(9H, m), 2.61(2H, m), 3.52–3.83(2H, m), 4.44(1H, m), 4.62(1H, dd, J = 7Hz, J = 9Hz), 6.63(1H, d, J = 9Hz), 7.18(5H, s), 9.46(1H, s).

3. 4-Phenylbutyryl-Leu-Pro-H EI-MS(m/z) = 358 $(M)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.95(3H, d, J = 6Hz), 1.01(3H, d, J = 6Hz), 1.57(3H, m), 1.85–2.41(8H, m), 2.62(2H, m), 3.45–3.83(2H, m), 4.48(1H, m), 4.82(1H, m), 6.40(1H, d, J = 9Hz), 7.18(5H, s), 9.45(1H, s).

4-Phenylbutyryl-nLeu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.88(3H, m), 1.00–1.60(6H, m), 1.68–2.38(8H, m), 2.13(2H, m), 3.25–3.82(2H, m), 4.53–4.98(2H, m), 6.83(1H, d, J = 8Hz), 7.16(5H, s), 9.35(1H, s).

5. 4-Phenylbutyryl-Met-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.67–2.32(10H, m), 2.32–2.83(4H, m), 3.10(3H, s), 3.40–3.90(2H, m), 4.03–5.26(2H, m), 7.19(5H, s), 9.38(1H, s).

6. 4-Phenylbutyryl-Phe-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.71–2.02(6H, m), 2.15(2H, m), 2.55(2H, m), 3.06(2H, m), 3.56–3.86(2H, m), 4.20(1H, m), 5.02(1H, m), 7.18(5H, s), 7.25(5H, s), 9.19(1H, s).

7. 4-Phenylbutyryl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.88–2.43(12H, m), 2.63(2H, m), 3.28–3.82(4H, m), 4.57(2H, m), 7.13(5H, s), 9.49(1H, s).

8. Phenylacetyl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.72–2.43(8H, m), 3.27–3.95(4H, m), 3.67(2H, s), 4.63(2H, m), 7.22(5H, s), 9.42(1H, s).

9. 3-Phenylpropionyl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.66–2.32(8H, m), 2.42–3.13(4H, m), 2.83(2H, m), 3.20–3.92(4H, m), 4.52(2H, m), 7.14(5H, s), 9.49(1H, s).

10. 5-Phenylvaleryl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.51–2.46(14H, m), 2.46–2.83(2H, m), 3.20–3.92(4H, m), 4.52(24, m), 7.21(5H, s), 9.49(1H, s).

11. 4-(2-Styrylphenoxy)butyryl-Val-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.92(3H, d, J = 7Hz), 0.99(3H, d, J = 7Hz), 1.90–2.56(9H, m), 3.50(2H, m), 4.06(2H, m), 4.60(2H, m), 6.84–7.58(12H, m), 9.43(1H, s).

12. 4-(2-Styrylphenoxy)butyryl-Leu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.89(3H, d, J = 6Hz), 0.95(3H, d, J = 6Hz), 1.40–2.64(11H, m), 3.48(2H, m), 4.07(2H, m), 4.30(1H, m), 4.83(1H, m), 6.46(1H, m), 6.84–7.62(11H, m), 9.37(1H, s).

13. 4-(2-Styrylphenoxy)butyryl-Phe-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.40(4H, m), 2.18(2H, m), 2.42(2H, m), 2.98(2H, m), 3.50(2H, m), 4.04(2H, m), 4.24(1H, m), 4.96(1H, m), 6.84–7.62(17H, m), 9.48(1H, s).

14. 4-(2-Styrylphenoxy)butyryl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.64–2.30(10H, m), 2.54(2H, m), 3.20–3.88(4H, m), 4.70(2H, m), 4.28–4.57(2H, m), 6.84–7.58(11H, m), 9.49(1H, s).

15. 4-(4-Benzylphenoxy)butyryl-Val-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.06(6H, d, J = 7Hz), 1.80–2.66(9H, m), 3.47(2H, m), 3.90(2H, m), 3.95(2H, m), 4.60(2H, m), 6.73–7.11(4H, m), 7.18(5H, s), 9.46(1H, s).

16. 4-(4-Benzylphenoxy)butyryl-Leu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.92(6H, m), 1.50–2.16(9H, m), 2.38(2H, m), 3.47(2H, m), 3.88(2H, m), 3.92(2H, m), 4.30(1H, m), 4.80(1H, m), 6.72–7.10(4H, m), 7.18(5H, s), 9.49(1H, s).

17. 4-(4-Benzylphenoxy)butyryl-Phe-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.64–2.18(6H, m), 2.20(2H, m), 3.03(2H, m), 3.51(2H, m), 3.91(1H, m), 3.94(2H, m), 4.20(1H, m) 4.96(1H, m), 6.63–7.12(4H, m), 7.24(10H, s), 9.39(1H, s).

18. 4-(4-Benzylphenoxy)butyryl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.94–2.14(10H, m), 2.50(2H, m), 3.52(4H, m), 3.87(2H, s), 3.94(2H, m), 4.20–4.66(2H, m), 6.74–7.10(4H, m), 7.20(5H, s), 9.49(1H, s).

19. 4-(5-Isoquinolinoxy)butyryl-Val-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.88(3H, d, J = 7Hz), 0.95(3H, d, J = 7Hz), 1.74–2.62(9H, m), 3.42–4.74(6H, m), 6.28(1H, d, J = 7Hz), 6.96(1H, dd, J = 2Hz, J = 6Hz), 7.44–7.50(2H, m), 7.98(1H, d, J = 6Hz), 8.49(1H, d, J = 6Hz), 9.16(1H, s), 9.39(1H, d, J = 2Hz).

20. 4-(5-Isoquinolinoxy)butyryl-Leu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.88(3H, d, J = 7Hz), 0.92(3H, d, J = 7Hz), 1.44–2.60(11H, m), 3.40–4.96(6H, m), 6.38(1H, d, J = 7Hz), 6.98(1H, dd, J = 2Hz, J = 6Hz), 7.44–7.52(2H, m), 7.98(1H, d, J = 6Hz), 8.51(1H, d, J = 6Hz), 9.19(1H, s), 9.41(1H, d, J = 2Hz).

21. 4-(5-Isoquinolinoxy)butyryl-nLeu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.84(3H, d, J = 7Hz), 1.25–2.62(14H, m), 3.38–4.92(6H, m), 6.49(1H, d, J = 7Hz), 6.98(1H, dd, J = 2Hz, J = 6Hz), 7.46–7.50(2H, m), 7.99(1H, d, J = 6Hz), 8.51(1H, d, J = 6Hz), 9.18(1H, s), 9.41(1H, d, J = 2Hz).

22. 4-(5-Isoquinolinoxy)butyryl-Phe-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.70–2.60(8H, m), 3.02(2H, m), 3.28–3.68(3H, m), 4.00(1H, m), 6.46(1H, d, J = 7Hz), 6.98(1H, dd, J = 2Hz, J = 6Hz), 7.44–7.52(2H, m), 8.00(1H, d, J = 6Hz), 8.51(1H, d, J = 6Hz), 9.18(1H, s), 9.38(1H, d, J = 2Hz).

23. 4-(5-Isoquinolinoxy)butyryl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 1.76–2.40(10H, m), 2.62(2H, m), 3.20–3.88(4H, m), 4.20(2H, m), 4.18–4.70(2H, m), 6.98(1H, dd, J = 2Hz, J = 6Hz), 7.44–7.50(2H, m), 7.97(1H, d, J = 6Hz), 8.49(1H, d, J = 6Hz), 9.16(1H, s), 9.50(1H, d, J = 2Hz).

24. Oleoyl-Ala-Pro-H EI-MS(m/z) = 434 $(M)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80(3H, m), 1.18–2.19(35H, m), 3.50(2H, m), 4.43(1H, m), 4.66(1H, m), 5.25(2H, m), 6.64(1H, d, J = 9Hz), 9.40(1H, d, J = 2Hz).

25. Oleoyl-Val-Pro-H EI-MS(m/z) = 463 $(M + H)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80–1.10(9H, m), 1.10–2.30(3H, m), 3.72(2H, m), 4.54(1H, m), 4.66(1H, dd, J = 9Hz, J = 7Hz), 5.34(2H, m), 6.08(1H, d, J = 8Hz), 9.53(1H, d, J = 2Hz).

26. Oleoyl-Leu-Pro-H EI-MS(m/z) = 447 $(M + H)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80–1.10(9H, m), 1.20–2.30(35H, m), 3.70(2H, m), 4.52(1H, m), 4.85(1H, m), 5.34(2H, m), 6.19(1H, d, J = 8Hz), 9.50(1H, d, J = 2Hz).

27. Oleoyl-nLeu-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.84(6H, m), 1.00–2.30(38H, m), 3.70(2H, m), 4.50(1H, m), 4.77(1H, m), 5.30(2H, m), 6.40(1H, d, J = 8Hz), 9.47(1H, d, J = 2Hz).

28. Oleoyl-Phe-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.88(3H, m), 1.00–2.40(32H, m), 3.08(2H, m), 3.60(2H, m), 4.38(1H, m), 5.00(1H, m), 5.32(2H, m), 6.24(1H, d, J = 8Hz), 7.26(5H, s), 9.32(1H, d, J = 2Hz).

29. Oleoyl-Lys(Z)-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.88(3H, m), 1.00–2.40(38H, m), 3.20(2H, m), 4.54(1H, m), 4.82(2H, m), 5.08(2H, s), 5.32(2H, m), 6.26(1H, d, J = 8Hz), 7.33(5H, s), 9.48(1H, d, J = 5Hz).

30. Oleoyl-Glu(H)-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.86(3H, m), 1.10–2.60(36H, m), 3.70(2H, m), 4.60(1H, m), 4.84(1H, m), 5.32(2H, m), 6.34(1H, d, J = 8Hz), 9.50(1H, d, J = 1Hz), 9.78(1H, d, J = 2Hz).

31. Oleoyl-Pro-Pro-H ^{1}H-NMR (from TMS, $CDCl_3$) 0.86(3H, m), 1.00—2.40(36H, m), 3.60(4H, m), 4.60(2H, m), 5.32(2H, m), 9.50(1H, d, J = 2Hz).

32. Linoleoyl-Leu-Pro-H EI-MS(m/z) = 475 $(M + H)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80–1.10(9H, m), 1.10–2.30(29H, m), 2.77(2H, m), 3.50(1H, m), 3.83(1H, m),

4.56(1H, m), 4.86(1H, m), 5.35(4H, m), 6.02(1H, d, J = 8Hz), 9.50(1H, d, J = 2Hz).

33. Stearoyl-Leu-Pro-H EI-MS(m/z) = 479 $(M + H)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80–1.00(9H, m), 1.10–2.30(37H, m), 3.50–3.80(4H, m), 4.40(1H, m), 4.80(1H, m), 6.77(1H, d, J = 8Hz), 9.40(1H, d, J = 2Hz).

34. Palmitoyl-Leu-Pro-H EI-MS(m/z) = 451 $(M + H)^+$; ^{1}H-NMR (from TMS, $CDCl_3$) 0.80–1.10(9H, m), 1.10–2.40(35H, m), 3.40–4.00(2H, m), 4.52(1H, m), 4.88(1H, m), 6.64(1H, d, J = 8Hz), 9.52(1H, d, J = 1Hz).

Enzyme Assay, Measurement of IC_{50}

Enzyme assays for the inhibitors of post-proline cleaving enzyme were carried out by the method of Yoshimoto *et al.*[20,21] The *Flavovacterium* enzyme (0.2 units/ml) was preincubated with or without various concentrations of inhibitors in 0.55 ml of 0.1 M phosphate buffer (pH 7.0) at 25°C for an hour. To this solution, 0.125 ml of 2.5 mM carbobenzoxy-glycyl-proline p-nitroanilide (Z-Gly-Pro-pNA) was added, and the release of p-nitroaniline was measured at 25°C and 410 nm. The inhibitor concentration at which the enzyme activity is half that of the uninhibited value (IC_{50}) was estimated from the inhibitor concentration vs. activity curve.[21] The inhibition of bovine brain enzyme was measured similarly. The enzyme (0.8 units/ml) was preincubated with or without various concentrations of inhibitors in 0.45 ml of 0.1 M phosphate buffer (pH 7.0) containing 10 mM each of EDTA and 2-mercaptoethanol at 35°C for an hour.[20] Measurement of IC_{50} values was performed in the same way as for the bacterial enzyme.

Enzyme Assay, Measurement of K_i value

The bacterial enzyme solution in 50 mM phosphate buffer with 0.005% Triton X-100, pH7.0, was preincubated at 35°C for 15 min in a total volume of 0.2 ml (0.4 units/ml). To the solution, also preincubated (35°C, 15 min) 2.5 ml of 50 mM phosphate buffer with 0.005% Triton X-100, pH 7.0, containing each of Z-Gly-Pro-pNA as a substrate in 40% dioxane ([S] = 0.63, 0.45, and 0.25 mM) and inhibitors were added and the enzyme reaction was initiated. Immediately after the addition, the reaction velocity was measured at 35°C and 410 nm. The inhibition constant, K_i, was calculated graphically by the Dixon method.[23]

Evaluation of the Anti-amnesic Effect

The effects of the test compounds on the acquisition and retention of passive avoidance response were measured as described by Kubota *et al.*[22] and Yoshimoto *et al.*[18] Native male Wistar SLC rats weighing 100 to 200 g were trained in the avoidance box, which was made up of a grid floor (30 × 30 cm) and platform (15 × 15 × 4 cm) at a corner of the box. A rat was placed on the platform and immediately after it stepped down on the floor, an electric shock was delivered through the floor grid, 1.7 mA in strength, using a shock generator/scrambler (SGS-003,BRS/LVE). The rats were deprived of food for 6 h before the training. Immediately after the training trial 3 mg/kg of body weight of scopolamine was injected intraperitoneally (i.p.). Test

compounds were administered intraperitoneally 1 h prior to the training trial and tests were performed 24 and 48 h after the administration of scopolamine. To the control group physiological saline was administered instead of the drug. The number of amnesic rats and of sound rats was counted for each of the control group (rats to which the test compounds were not administered but to which only scopolamine and physiological saline were administered intraperitoneally) and the treated group (rats to which both the test compound and scopolamine were administered).

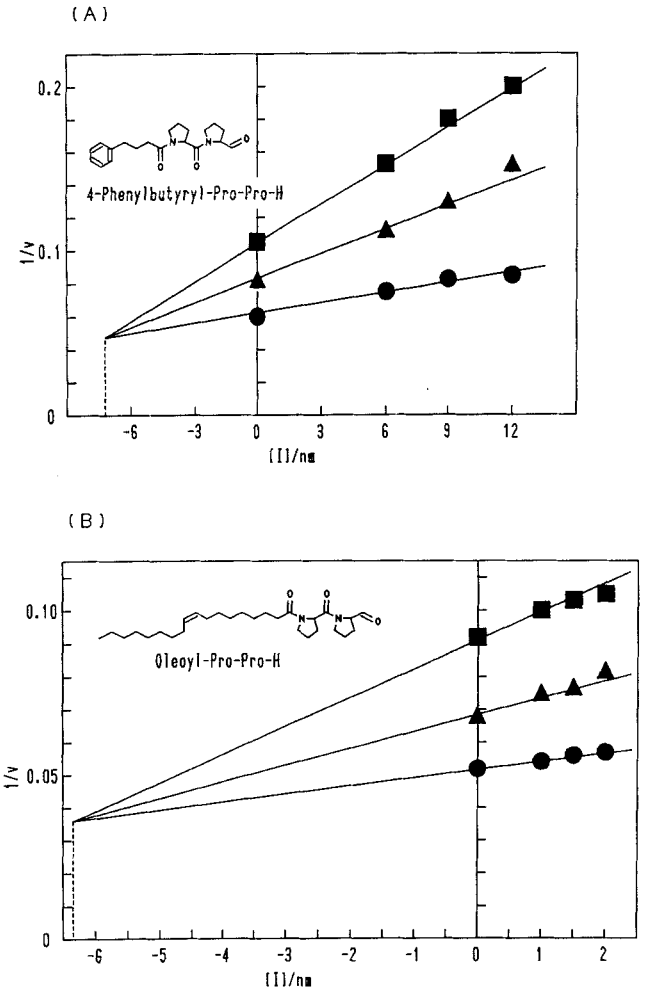

FIGURE 1 Dixon plots of the inhibition of *Flavobacterium* post-proline cleaving enzyme by 4-phenylbutyryl-Pro-Pro-H (A), and by oleoyl-Pro-Pro-H (B). The enzyme was preincubated for 15 min at 35°C and the reaction was initiated by addition of inhibitor and Z-Gly-Pro-pNA as described in Materials and Methods. Inhibitor concentration on abscissa.
(■—■) concentration of substrate is 0.250 mM;
(▲—▲) concentration of substrate is 0.450 mM;
(●—●) concentration of substrate is 0.625 mM.

TABLE II
Inhibition of Post-proline Cleaving Enzymes from *Flavobacterium* and Bovine Brain by Acyl Peptidyl Prolinal Derivatives

No.	Chemical Structure	Post-proline cleaving enzyme	
		Flavobacterium IC_{50} (nM)	Bovine brain IC_{50} (nM)
1.	4-Pehnylbutyryl-Ala-Pro-H	57	140
2.	4-Phenylbutyryl-Val-Pro-H	0.87	4.2
3.	4-Phenylbutyryl-Leu-Pro-H	1.2	12
4.	4-Phenylbutyryl-nLeu-Pro-H	1.7	250
5.	4-Phenylbutyryl-Met-Pro-H	2.7	79
6.	4-Phenylbutyryl-Phe-Pro-H	1.1	22
7.	4-Phenylbutyryl-Pro-Pro-H	0.87	8.7
8.	Phenylacetyl-Pro-Pro-H	4.7	4.7
9.	3-Phenylpropionyl-Pro-Pro-H	1.8	4.5
10.	5-Phenylvaleryl-Pro-Peo-H	0.84	4.2
11.	4-(2-Styrylphenoxy)butyryl-Val-Pro-H	0.25	19
12.	4-(2-Styrylphenoxy)butyryl-Leu-Pro-H	6.2	32
13.	4-(2-Styrylphenoxy)butyryl-Phe-Pro-H	0.27	20
14.	4-(2-Styrylphenoxy)butyryl-Pro-Pro-H	0.23	6.5
15.	4-(4-Benzylphenoxy)butyryl-Val-Pro-H	6.7	2
16.	4-(4-Benzylphenoxy)butyryl-Leu-Pro-H	6.5	65
17.	4-(4-Benzylphenoxy)butyryl-Phe-Pro-H	6.2	62
18.	4-(4-Benzylphenoxy)butyryl-Pro-Pro-H	0.20	6.7
19.	4-(5-Isoquinolinoxy)butyryl-Val-Pro-H	3.5	3
20.	4-(5-Isoquinolinoxy)butyryl-Leu-Pro-H	3.5	6
21.	4-(5-Isoquinolinoxy)butyryl-nLeu-Pro-H	3.5	69
22.	4-(5-Isoquinolinoxy)butyryl-Phe-Pro-H	2.3	97
23.	4-(5-Isoquinolinoxy)butyryl-Pro-Pro-H	1.1	7.2
24.	Oleoyl-Ala-Pro-H	52	
25.	Oleoyl-Val-Pro-H	2.2	320
26.	Oleoyl-Leu-Pro-H	2.5	3200
27.	Oleoyl-nLeu-Pro-H	2.2	6200
28.	Oleoyl-Phe-Pro-H	1.2	45000
29.	Oleoyl-Lys(Z)-Pro-H	1.4	6000
30.	Oleoyl-Glu(H)-Pro-H	0.94	190
31.	Oleoyl-Pro-Pro-H	0.97	65
32.	Linoleoyl-Leu-Pro-H	1.9	320
33.	Stearoyl-Leu-Pro-H	3.0	170
34.	Palmitoryl-Leu-Pro-H	2.3	6500

In this study "amnesic" rats were defined to the rats whose latency of step-down in the retention test was less than 300 sec.

RESULTS

The inhibitory constant and the mode of inhibition of two typical inhibitors, 4-phenylbutyryl-Pro-Pro-H (**7**) and oleoyl-Pro-Pro-H (**31**), were measured toward bacterial enzyme. (Figure 1). The K_i values were 7.2 nM and 6.4 nM, respectively. These prolinal derivatives inhibited bacterial enzyme competitively and the extremely low K_i

values were interpreted as meaning that they react stoichiometrically with post-proline cleaving enzyme.

The IC_{50} values of the inhibitors for post-proline cleaving enzymes from *Flavobacterium* and from bovine brain are summarized in Table II. The compounds show very strong inhibitory activities.

Under the present experimental conditions, the retention of the passive avoidance response in control animals was significantly prolonged in the test trials performed 24 h after the training trial. On the other hand, in scopolamine administered rats, the retention of the passive avoidance response was remarkably reduced when compared to that of the control group, and the ratio of induced amnesic rats significantly rose from 33% to 80%, indicating the induction of amnesia (Table III). There are no observable changes in the spontaneous activity and behaviour of scopolamine treated rats. Pretreatment with each of the test compounds strongly affects the retention of the passive avoidance response with 10 μg/kg to 1 mg/kg (i.p.), and the ratio of induced amnesia were remarkably reduced (see Table III).

DISCUSSION

For some serine and cysteine proteinases, substrate analogues such as peptidyl chloromethylketones and peptidyl aldehydes have been used as strong inhibitors. It has been suggested that the mechanism of peptidyl aldehyde inhibitors is direct alkylation of the active site amino acid side chain, either the alcohol of serine or the thiol of cysteine, and the formation of a tetrahedral intermediate-like enzyme-substrate complex. All the prolinal containing compounds described above show very strong inhibitory activity toward post-proline cleaving enzyme which may also be due to the formation of a tetrahedral intermediate.

Recent studies of prolinal derivatives on their inhibitory mechanisms concluded that the prolinal derivatives which have relatively small amino protecting groups like Z- or Boc- moieties inhibited the enzyme noncompetitively.[17,21] However, in our case, 4-phenylbutyryl-Pro-Pro-H and oleoyl-Pro-Pro-H inhibited bacterial enzyme competitively. (Figure 1). The reason for this might be due to the difference of the size of the amino protecting groups or the binding to the enzyme.

Recently, many of the subsite mapping studies have been reported using site specific substrates or inhibitors for post-proline cleaving enzyme from bovine brain,[18,20,21] *Flavobacterium meningosepticum*,[24–26] lamb kidney,[24,27,28] and from ascidian, [25,29] and indicated that at least five subsites were considered to contribute to the interaction between the enzyme and a substrate. One of the most potent post-proline cleaving enzyme inhibitors has a structure of Y-X-Pro-H, where Y is a usual amino protecting group and X is an amino acid residue. Some Y and X groups were examined earlier for designing inhibitors of post-proline cleaving enzyme and it was found that Z-Val-Pro-H and Z-Ile-Pro-H exhibited strong inhibition towards both ascidian and *Flavobacterium* enzymes with IC_{50} values of 10 $\sim$ 30 nM,[25] Z-Val-Pro-H, Z-Pro-Pro-H, Z-pGlu-Pro-H, and Boc-Pro-Pro-H inhibited bovine brain enzyme with K_i values of 0.5 $\sim$ 1.8 nM,[18] and Z-Pro-Pro-H also inhibited *Flavobacterium* and bovine enzymes with low K_i values of 0.2 $\sim$ 0.5 nM.[21] Therefore, in this study, we designed and synthesized the Y-X-Pro-H type compounds listed in Table I, and compared their inhibitory activities toward *Flavobacterium* and bovine enzymes.

The arylalkyl-acyl moieties of the inhibitors which we examined are 4-phenyl-

TABLE III

ventive Action of Post-Proline Cleaving Enzyme Inhibitors toward Scopolamine-Induced Retrograde Amnesia and their Dose-Responses

unds	Drug administered after training (i.p.)	No. of total rats	Training (Means ± S.E.)[a]		Retention test[b]	Amne (%)
			First step-down latency (s.)	No. of descending	No. of amnesic rats/ No. of total rats	
gical	physiological saline	9	6.2 ± 1.7	2.3 ± 0.6	3/9	33
gical	scopolamine (3 mg/kg)	10	2.7 ± 0.6	2.9 ± 0.3	8/10	80
g/kg)	scopolamine (3 mg/kg)	10	3.8 ± 0.8	1.6 ± 0.3	1/10[f]	10
kg)	scopolamine (3 mg/kg)	10	5.2 ± 1.1	3.0 ± 0.8	0/10[g]	0
/kg)	scopolamine (3 mg/kg)	10	3.8 ± 0.8	2.2 ± 0.2	6/10	60
kg)	scopolamine (3 mg/kg)	10	2.4 ± 0.6	2.4 ± 0.6	4/10[e]	40
kg)	scopolamine (3 mg/kg)	10	3.2 ± 0.5	3.2 ± 0.5	0/10[g]	0
g)	scopolamine (3 mg/kg)	10	1.9 ± 0.6	2.2 ± 0.6	1/10[f]	10
g)	scopolamine (3 mg/kg)	10	1.5 ± 0.6	3.0 ± 0.8	3/10[d]	30
kg)	scopolamine (3 mg/kg)	10	2.5 ± 0.4	3.0 ± 0.5	0/10[g]	0
g)	scopolamine (3 mg/kg)	10	2.7 ± 0.9	2.4 ± 0.8	0/10[g]	0
g)	scopolamine (3 mg/kg)	10	1.7 ± 0.6	2.3 ± 0.4	0/10[g]	0
g)	scopolamine (3 mg/kg)	10	3.1 ± 0.7	2.4 ± 0.8	0/10[g]	0
kg)	scopolamine (3 mg/kg)	5	6.3 ± 1.3	2.3 ± 0.5	3/5	60
kg)	scopolamine (3 mg/kg)	5	3.3 ± 0.6	2.3 ± 0.6	3/5	60

	scopolamine (3 mg/kg)	10	3.5 ± 0.6	2.8 ± 0.9	5/10	50
	scopolamine (3 mg/kg)	10	2.1 ± 0.3	2.8 ± 0.5	2/10[e]	20
	scopolamine (3 mg/kg)	10	1.8 ± 0.4	1.6 ± 0.2	1/10[f]	10
	scopolamine (3 mg/kg)	5	9.4 ± 3.0	1.6 ± 0.9	3/5	60
	scopolamine (3 mg/kg)	5	1.7 ± 0.8	2.4 ± 0.6	1/5[d]	20
	scopolamine (3 mg/kg)	5	3.1 ± 0.9	1.8 ± 0.7	0/5[f]	0
	scoplamine 3 mg/kg)	5	2.2 ± 0.8	2.0 ± 0.5	0/5[f]	0
	scopolamine (3 mg/kg)	5	4.7 ± 0.7	2.0 ± 0.9	2/5	40
	scopolamine (3 mg/kg)	10	3.7 ± 0.3	2.1 ± 0.5	0/10[g]	0
	scopolamine (3 mg/kg)	10	2.7 ± 0.5	2.4 ± 0.7	0/10[g]	0
	scopolamine (3 mg/kg)	10	3.2 ± 0.8	1.8 ± 0.7	0/10[g]	0
	scopolamine (3 mg/kg)	10	2.2 ± 0.8	2.4 ± 0.9	0/10[g]	0
	scopolamine (3 mg/kg)	10	4.7 ± 0.9	2.0 ± 0.5	0/10[g]	0
	scopolamine (3 mg/kg)	10	2.2 ± 0.3	3.0 ± 0.2	0/10[g]	0

a time for the training, 110 sec. (**7** and **14**); 100 sec. (**1**, **2**, **3**, **4**, **25**, **26**, **27**, **28**, **31**, **33**, and **34**)
step-down latency was measured for 300 s.
ificantly different from the physiological saline group ($P < 0.05$, χ^2-analysis)
[g] Significantly different from the scopolamine group ([d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.005$, [g]$P < 0.001$)

butyryl (compds. **1** to **7**), phenylacetyl (compd. **8**), 3-phenylpropionyl (compd. **9**), 5-phenylvaleryl (compd. **10**), 4-(2-styrylphenoxy)butyryl (compds. **11** to **14**), 4-(4-benzylphenoxy)butyryl (compds. **15** to **18**), and 4-(5-isoquinolinoxy)butyryl (compds. **19** to **23**), and the alkyl-acyl moieties are oleoyl (compds. **24** to **31**), linoleoyl (compd. **32**), stearoyl (compd. **33**), and palmitoyl (compd. **34**). Both bacterial and bovine enzymes were inhibited by arylalkyl-acyl substrates but alkyl-acyl substrates inhibited only the bacterial enzyme. This fact indicates that the amino-terminal side subsite specificity of bovine enzyme (S_3, S_4 and so on) is more strict than that of the bacterial enzyme, and that bovine enzyme seems to require bulky, arylalkyl moieties for its S_3 subsite. For bacterial enzyme, almost all the bulky and long acyl groups seem to be allowed and no clear S_3 and more amino-terminal side specificity was found. The specificity of the S_3 site was further studied by examining some phenyl-$(CH_2)_n$-Pro-H compounds (compds. **7** to **10**) and some alkyl-acyl derivatives (compds. **26**, **32** to **34**). The compounds which had the highest activity were the pairs of **10** (n = 4; 5-phenylvaleryl) and **32** (linoleoyl), and **10** and **33** (steroryl) for bacterial enzyme and bovine enzyme respectively, although their activity was not much greater than the others.

P_2 subsite specificity studies were also carried out for various acyl moieties. According to recent studies, the compounds with valine[29] or proline[18,20–25] in the P_2 subsite showed higher inhibitory activity rather than other amino acid residues. In our case, all the proline containing compounds (**7**, **14**, **18**, and **23**) in the P_2 subsite have much higher activity for the bacterial enzyme. On the contrary, for bovine enzyme, some valine containing compounds (**2**, **15** and **19**) showed the highest activity. These facts indicate that proline and valine are suitable for the S_2 site of both enzymes but there are some differences around the S_2 sites. The same result was also obtained by Yoshimoto *et al.*[21] who concluded that the two enzymes have some different structure around the S_2 and/or S_3 subsite. One of the interesting phenomena observed in the alkyl-acyl derivatives is that the compound **30** which has Glu(H) for its P_2 site (Glu(H) is a residue in which the side chain carboxylic acid moiety of glutamic acid is reduced to a formyl moiety) showed highest activity toward bacterial enzyme, and it also showed as strong an activity as **31** for bovine enzyme. The reason for the specificity of Glu(H) is not clear from this study but the whole structure of Glu(H) might be suitable for the S_2 site and/or the side chain formyl group has special functions for both enzymes.

Various prolinal derivatives with potent inhibitory activity towards post-proline cleaving enzyme were examined for their anti-amnesic effect of scopolamine-induced amnesic rats in the passive avoidance learning test (Table III). Pretreatment with compounds **3**, **4**, **7**, **26** ~ **28**, and **31**, which have potent *in vitro* inhibitory activities towards the enzymes, prevented the induction of amnesia by scopolamine in the passive avoidance learning test at the doses of 10 ~ 25 μg/kg. It can be stated that Y-Pro-Pro-H derivatives were effective in the retention test but no clear difference was observed among the compounds with different P_2 structures. Some recent studies discussed the correlation between the *in vitro* inhibitory activities towards post-proline cleaving enzyme and various *in vivo* methods of anti-amnesic effects on animals such as passive avoidance learning in rats,[18] active avoidance response in mice,[30] one trial passive-active avoidance response in mice,[30] and Y-maze learning in mice.[31] They concluded that the prolinal derivatives which have potent inhibitory activity showed strong anti-amnesic activities. In this study, however, we found that compounds which inhibited only bacterial enzyme showed as strong an anti-amnesic activity as those which inhibited both enzymes. We also found that the compounds,

2 and **26**, showed the maximum effective dose, the so called bell-shape dose dependency. The reason and mechanism for all these facts can not presently be explained but at least the anti-amnesic activity of the compounds is due not only to enzyme inhibitory activity but also to other factors such as membrane permeability, hydrophobicity and inhibitory activity towards other related enzymes.

From the results of dose dependency and other experiments described above, it seems that the mechanism of the anti-amnesic effects of these compounds is complicated. However, the main mechanism is probably, as reported before,[7,18] due to the inhibitory activity of the compounds towards the post-proline cleaving enzyme in the brain which is thought to cleave a special peptide [H-Cys^1-OH pGlu^4-Asn^5-Cys^6-Pro^7-Arg^8-OH] acting directly on memory and related processes.[7] Therefore the post-proline cleaving enzyme is thought to play an important role in the regulation of learning and memory consolidation in brain and inhibitors of this enzyme are suggested as possible candidates for an anti-amnesic drug.

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