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SYNTHESIS AND INHIBITORY ACTIVITY OF ACYL-PEPTIDYL-PYRROLIDINE DERIVATIVES TOWARD POST-PROLINE CLEAVING ENZYME; A STUDY OF SUBSITE SPECIFICITY

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Several pyrrolidine derivatives have been synthesized and examined for their inhibitory activity on post-proline cleaving enzymes from *Flavobacterium meningosepticum* and bovine brain. Almost all the compounds tested in this study inhibited the activity of both enzymes at low IC_{50} values (from nM to μM) but a specificity difference was observed with alkylacyl-peptidyl-pyrrolidine derivatives which strongly inhibited only the bacterial enzyme. The most effective inhibitors have a proline residue on their P_2 sites and a substituted or unsubstituted phenoxybutyryl moiety on their P_3 sites. Thus phenoxybutyryl-prolyl-pyrrolidine is the most effective partial structure of the inhibitors. The best inhibitors found were: 4-(4-benzylphenoxy)butyryl-prolyl-pyrrolidine for bacterial enzyme (IC_{50} 1.4 nM) and 4-phenylbutyryl-thioprolyl-pyrrolidine for bovine brain enzyme (IC_{50} 67 nM). In the passive avoidance test, using amnesic rats experimentally induced with scopolamine, the pyrrolidine derivatives which had potent inhibitory activity toward post-proline cleaving enzymes also showed strong anti-amnesic activities at doses of $1 \sim 5$ mg/kg, i.p.

KEY WORDS: Acyl-Peptidyl-Pyrrolidine derivatives, Post-Proline cleaving enzyme, Anti-amnesic activity.

INTRODUCTION

Post-proline cleaving enzyme (EC 3.4.21.26) was discovered by Walter *et al.*[1] in human uterus as an oxytocin-degrading enzyme and classified as a serine proteinase using active site-directed irreversible inhibitors.[2] The enzyme is highly active in the brain and readily degrades proline-containing low molecular weight oligopeptides such as thyrotropin releasing hormone (TRH),[3] luteinizing hormone releasing hormone (LH-RH),[4] angiotensin II,[5] bradikinine,[6] substance P,[7] and neurotensin.[8] The enzyme also degrades vasopressin which is the oligopeptide speculatively associated with memory as described earlier.[9–16]

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Abbreviations: 4-PhBu, 4-phenylbutyryl; Py, pyrrolidine; Hyp, 4-hydroxy-L-proline residue; aHyp, allo-4-hydroxy-L-proline residue; Thp, L-thioproline residue; Pyr, 3-pyrrolidone-5-carboxylic acid residue; Ind, DL-indoline-2-carboxylic acid residue; Pip, L-pipecolinic acid residue; Acp, 1-amino-1-cyclopentanecarboxylic acid residue; 2-StBu, 4-(2-styrylphenoxy)butyryl; 4-BzlBu, 4-(4-benzylphenoxy)butyryl; 5-IqBu, 4-(5-isoquinolinoxy)butyryl; 3-PyBu, 4-(3-pyridyloxy)butyryl; HOBT, 1-hydroxybenzotriazole hydrate; WSC HCl, 1-ethyl-3-(3-diethylaminopropyl)carbodiimide hydrochloride.

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.[2] On the other hand, benzyloxycarbonyl-prolyl-prolinal is a specific potential transition state inhibitor of the enzyme.[17] Yoshimoto *et al.*[18] and Saito *et al.*[19] reported 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, in particular acyl-peptidyl-prolinal derivatives, have some effect in the fixation of memory. This suggests the potential use of post-proline cleaving enzyme activity inhibitors as anti-amnesic agents for preventing and/or curing amnesia.

Recently, another type of inhibitor which was different from aldehyde or chloromethylketone derivatives, was proposed by Tsuru *et al.*[20] They reported that a thioproline and/or thiazolidine derivative showed as strong inhibitory activity as prolinal derivatives towards post-proline cleaving enzyme and suggested that the compounds were involved in memory consolidation and the learning process.

The purpose of this study was to synthesize a new type of pyrrolidine derivative as potential post-proline cleaving enzyme inhibitors, and to clarify the relationship between structure and inhibitory activity *in vitro*. In particular, $P_2(S_2)$ and $P_3(S_3)$ subsite specificity was examined at this stage.

MATERIALS AND METHODS

Materials

Post-proline cleaving enzyme of bovine brain was a generous gift from Dr. Tsuru, Nagasaki University, and bacterial enzyme and Z-Gly-Pro-*p*-nitroanilide were purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Amino acids and coupling reagents were purchased from Kokusan Chemical Works, Ltd., Tokyo, and other chemicals used in this study were all reagent grade and purchased from Nacarai Tesque, Kyoto.

Enzyme Assay, Measurement of IC_{50}

Enzyme assays for inhibitors of post-proline cleaving enzyme were carried out by the method of Yoshimoto *et al.*[20-22] and Saito *et al.*[19] The *Flavobacterium* 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 1 h. To this solution, 0.125 ml of 2.5 mM Z-Gly-Pro-*p*-nitroanilide was added, and the release of *p*-nitroaniline was measured at 25°C and 410 nm. The IC_{50} value was estimated from the inhibitor concentration vs. activity curve.[21] The mean activity value from duplicate measurements at each concentration were used in this study. 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 1 h.[20] Measurements 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, pH 7.0, was preincubated at 30°C for 15 min in a total volume of 0.2 ml (0.2 units/ml). To the solution, also preincubated (30°C, 15 min) 2.5 ml of 50 mM phosphate buffer with 0.005% Triton X-100, pH 7.0, each containing Z-Gly-Pro-*p*-nitroanilide as a substrate in 40% dioxane ([S] = 1.44, 1.20, 0.96, 0.72, and 0.48 mM) and inhibitors were added and the enzyme reaction was initiated. Immediately after the addition, the reaction velocity was measured at 30°C and 410 nm. The inhibitory constant, K_i was calculated graphically by the methods of Dixon,[23] and Lineweaver and Burk.[2]

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 Kubota *et al.*,[38] Yoshimoto *et al.*,[18] and Saito *et al.*[15] 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 6h 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 1h 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 administrated 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 administrated but to which only scopolamine and physiological saline were administrated intraperitoneally) and the treated group (rats to which both the test compound and scopolamine were administrated). In this study "amnesic" rats were defined as those rats whose latency of step-down in the retention test was less than 300 sec.

Synthesis of Acyl-Peptidyl-Pyrrolidine Derivatives

All the amino acids used here were of L-configuration, unless otherwise stated. Compounds **1–8**, **10**, **12**, **14**, **16**, **17**, **19**, **20**, **22–24**, **26–29**, **31–50**, and **54–66** were synthesized from respective N-acyl amino acids and pyrrolidine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC · HCl) and 1-hydroxybenzotriazole hydrate (HOBT).[25,26] Compounds **18**, **25**, **30** and **51–53** were synthesized from the respective acids and corresponding pyrrolidine amides by the same manner as above. Hydrogenolysis of compounds **10**, **12**, **14** and **16** gave compounds **9**, **11**, **13** and **15** respectively.[27–30]

N-Acyl Amino Acids

4-phenylbutyryl-glycine (4-PhBu-Gly-OH) was synthesized as follows. 4-phenylbutyryl chloride (1.83 g, 10 mM) was added to an ice cooled solution of glycine (750 mg, 10 mM) in 1N NaOH (20 ml). The mixture

was stirred overnight and washed with EtOAc. The aqueous layer was acidified with conc. HCl and extracted with EtOAc. The aqueous layer was washed with a small portion of brine, and dried over Na_2SO_4. The solvent was evaporated *in vacuo* to give 4-phBu-Gly-OH (2.0 g, 91%). Synthesized in a similar manner were: 4-PhBu-Ala-OH (52%), 4-PhBu-Val-OH (40%), 4-PhBu-Leu-OH (35%), 4-PhBu-Nle-OH (50%), 4-PhBu-Met-OH (78%), 4-PhBu-Phe-OH (45%), 4-Phe-Thr-OH (62%), 4-PhBu-Ser(OBzl)-OH (36%), 4-PhBu-Tyr(OBzl)-OH (32%), 4-PhBu-Lys(Z)-OH (28%), 4-PhBu-Glu(OBzl)-OH (31%), 4-PhBu-Hyp-OH (30%), 4-PhBu-thioproline (4-PhBu-Thp-OH, 86%), DL-l-(4-PhBu)indoline-2-carboxylic acid (4-PhBu-Ind-OH, 26%), l-(4-PhBu)pipecolinic acid (4-PhBu-Pip-OH, 93%), l-(4-PhBu)amino-1-cyclopentanecarboxylic acid (4-PhBu-Acp-OH, 60%) oleoyl-Val-OH (45%), oleoyl-Leu-OH (38%), oleoyl-Nle-OH (33%), oleoyl-Phe-OH (48%), oleoyl-Tyr(OBzl)-OH (26%), oleoyl-Pro-OH (31%), caproyl-Pro-OH (75%), caprylyl-Pro-OH (79%), lauroyl-Pro-OH (34%), myristoyl-Pro-OH (65%), palmitoyl-Pro-OH (38%), and linoleoyl-Pro-OH (40%).

4-(2-styrylphenoxy)butyryl-valine (2-StBu-Val-OH) was synthesized as follows. A solution of 4-(2-styrylphenoxy)butyric acid (1.0 g, 3.54 mM), H-Val-OEt hydrochloride (640 mg, 3.54 mM), WSC·HCl (680 mg, 3.54 mM), and Et_3N (360 mg, 3.54 mM) in CH_2Cl_2 (30 ml) was stirred overnight at room temperature. The mixture was extracted with EtOAc, and the organic layer was successively washed with 10% aqueous citric acid, 5% aqueous $NaHCO_3$, and brine and then dried over $MgSO_4$. The solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using $CHCl_3$ as an eluting solvent to give 2-StBu-Val-OEt (1.4 g). 2-StBu-Val-OEt was hydrolyzed by aqueous NaOH to give 2-StBu-Val-OH (1.2 g, total yield 89%). Synthesized in a similar manner were: 2-StBu-Leu-OH (88%), 2-StBu-Phe-OH (88%), 2-StBu-Pro-OH (32%), 4-(4-benzylphenoxy)butyryl-Val-OH (4-BzlBu-Val-OH, 89%), 4-BzlBu-Leu-OH (86%), 4-BzlBu-Phe-OH (89%), 4-BzlBu-Pro-OH (77%), 4-(5-isoquinolinoxy)-butyryl-Val-OH (5-IqBu-Val-OH, 80%), 5-IqBu-Leu-OH, (60%), 5-IqBu-Nle-OH (86%), 5-IqBu-Phe-OH (90%), 5-IqBu-Pro-OH (90%), 4-(3-pyridyloxy)butyryl-Val-OH (3-PyBu-Val-OH, 90%), 3-PyBu-Leu-OH (52%), 3-PyBu-Nle-OH (62%), 3-PyBu-Phe-OH (84%), 3-PyBu-Pro-OH (67%), 4-phenoxybutyryl-Pro-OH (47%), 4-(2-benzylphenoxy)butyryl-Pro-OH (84%), 4-(2′-chalconoxy)butyryl-Pro-OH (43%), 4-(2-chalconoxy)butyryl-Pro-OH (34%), 4-(2-phenethylphenoxy)butyryl-Pro-OH (45%), 4-(2-benzoylphenoxy)butyryl-Pro-OH (40%) and 4-(2-allylphenoxy)butyryl-Pro-OH (33%).

1-{N-(4-Phenylbutyryl)-Pro}-pyrrolidine (4-PhBu-Pro-Py, 17)

4-PhBu-Pro (510 mg, 2.0 mM), and pyrrolidine (140 mg, 2.0 mM) were dissolved in anhydrous dimethylformamide (10 ml). To this solution, WSC·HCl (370 mg, 1.95 mM), and HOBT (290 mg, 2.0 mM) were added. The mixture was stirred at room temperature overnight and evaporated *in vacuo* under 45°C. The residue was dissolved in EtOAc, and the organic layer was successively washed with 10% aqueous citric acid, 5% aqueous $NaHCO_3$, and brine, and then dried over Na_2SO_4. The solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using $CHCl_3$-MeOH (100:0 – 97:3) as an eluting solvent to give **17** (400 mg, 65%). Synthesized in a similar manner were: 4-PhBu-Gly-Py (**1**, 58%), 4-PhBu-Ala-Py (**2**, 52%), 4-PhBu-Val-Py (**3**, 75%), 4-PhBu-Leu-Py (**4**, 70%), 4-PhBu-Nle-Py (**5**, 68%), 4-PhBu-Met-Py (**6**, 53%), 4-PhBu-Phe-Py (**7**, 56%), 4-PhBu-Thr-Py (**8**, 50%), 4-PhBu-Ser(OBzl)-Py (**10**, 46%), 4-PhBu-Tyr(OBzl)-Py (**12**, 48%), 4-PhBu-Lys(Z)-Py (**14**, 43%), 4-PhBu-Glu(OBzl)-Py (**16**, 38%), 4-PhBu-Hyp-Py (**19**, 79%), 4-PhBu-Thp-Py (**20**, 45%), 4-PhBu-Ind-Py (**22**, 39%), 4-PhBu-Pip-Py (**23**, 27%), 4-PhBu-Acp-Py (**24**, 20%), 2-StBu-Val-Py (**26**, 28%), 2-StBu-Leu-Py (**27**, 70%), 2-StBu-Phe-Py (**28**, 80%), 2-StBu-Pro-Py (**29**, 74%), 4-BzlBu-Val-Py (**31**, 95%), 4-BzlBu-Leu-Py (**32**, 64%), 4-BzlBu-Phe-Py (**33**, 87%), 4-BzlBu-Pro-Py (**34**, 30%), oleoyl-Val-Py (**35**, 40%), oleoyl-Leu-Py (**36**, 45%), oleoyl-Nle-Py (**37**, 46%), oleoyl-Phe-Py (**38**, 38%), oleoyl-Tyr(OBzl)-Py (**39**, 26%), oleoyl-Pro-Py (**40**, 25%), 5-IqBu-Val-Py (**41**, 70%), 5-IqBu-Leu-Py (**42**, 60%), 5-IqBu-Nle-Py (**43**, 70%), 5-IqBu-Phe-Py (**44**, 75%), 5-IqBu-Pro-Py (**45**, 12%), 3-PyBu-Val-Py (**46**, 90%), 3-PyBu-Leu-Py (**47**, 52%), 3-PyBu-Nle-Py (**48**, 80%), 3-PyBu-Phe-Py (**49**, 64%), 3-PyBu-Pro-Py (**50**, 20%), caproyl-Pro-Py (**54**, 98%), caprylyl-Pro-Py (**55**, 63%), lauroyl-Pro-Py (**56**, 54%), myristoyl-Pro-Py (**57**, 42%), palmitoyl-Pro-Py (**58**, 35%), linoleoyl-Pro-Py (**59**, 20%), 4-phenoxybutyryl-Pro-Py (**60**, 53%), 4-(2-benzylphenoxy)butyryl-Pro-Py (**61**, 70%), 4-(2′-chalconoxy)butyryl-Pro-Py (**62**, 42%), 4-(2-chalconoxy)butyryl-Pro-Py (**63**, 57%), 4-(2-phenethylphenoxy)butyryl-Pro-Py (**64**, 71%), 4-(2-benzoylphenoxy)butyryl-Pro-Py (**65**, 71%), and 4-(2-allylphenoxy)butyryl-Pro-Py (**66**, 69%).

*1-{1-(4-Phenylbutyryl)-3-Pyrrolidone-5-Carbonyl}-Pyrrolidine (4-PhBu-Pyr-Py, **21**)*

A solution of sulfur trioxide-pyridine complex[31] (830 mg, 5.2 mM) in anhydrous dimethyl sulfoxide (2 ml) was added to a stirred solution of 4-PhBu-Hyp-Py (**19**, 570 mg, 1.7 mM), and Et_3N (0.72 ml, 5.2 mM) in anhydrous dimethyl sulfoxide (6 ml) at room temperature. The reaction mixture was stirred for 15 min and poured into ice-water. The mixture was extracted with EtOAc and the organic layer was successively washed with 10% aqueous citric acid, saturated aqueous $NaHCO_3$, and brine, and then dried over Na_2SO_4. The solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using $CHCl_3$–MeOH (99:1) as an eluting solvent to give **21** (340 mg, 60%).

*1-{N-(2-Styrylphenoxyacetyl)-Pro}-Pyrrolidine (2-Styrylphenoxyacetyl-Pro-Py, **51**)*

2-Styrylphenoxyacetic acid (250 mg, 1.0 mM) and Pro-Py (170 mg, 1.0 mg) were dissolved in anhydrous dimethylformamide (20 ml). To this solution, WSC HCl (190 mg, 1.0 mM), and HOBT (160 mg, 1.0 mM) were added. The mixture was stirred at room temperature overnight and worked up in the usual way. The residue was purified by column chromatography on silica gel using $CHCl_3$ as an eluting solvent to give **51** (250 mg, 62%). Synthesized in a similar manner were: 4-PhBu-aHyp-Py (**18**, 70%), 2-StBu-Ala-Py (**25**, 60%), 4-BzlBu-Ala-Py (**30**, 50%), 3-(2-Styrylphenoxy)propionyl-Pro-Py (**52**, 61%), and 5-(2-styrylphenoxy)pentanoyl-Pro-Py (**53**, 20%). Analytical data for pyrrolidine derivatives are presented in Table I. The ^{1}H-NMR data (TMS, $CDCl_3$) are as follows.

*4-PhBu-Gly-Py (**1**)*: 1.80–2.38(8H, m), 2.67(2H, m), 3.32–3.56(4H, m), 3.98(2H, m, d, J = 5 Hz), 6.54(1H, broad), 7.22(5H, m). *4-PhBu-Ala-Py (**2**)*: 1.32(3H, d, J = 8 Hz), 1.80–2.31(8H, m), 2.65(2H, m), 3.29–3.68 (4H, m), 4.72(1H, m), 6.50(1H, broad), 7.20(5H, m). *4-PhBu-Val-Py (**3**)*: 0.94(3H, d, J = 7 Hz), 0.98(3H, d, J = 7 Hz), 1.80–2.20(6H, m), 2.20(3H, m), 2.64(2H, m), 3.20–3.80(4H, m), 5.62(1H, dd, J = 7 and 9 Hz), 6.26(1H, d, J = 9 Hz), 7.22(5H, m). *4-PhBu-Leu-Py (**4**)*: 0.94(3H, d, J = 7 Hz), 0.98(3H, d, J = 7 Hz), 1.40–2.40(11H, m), 2.62(2H, m), 3.20–3.30(4H, m), 5.82(1H, m), 6.19(1H. d, J = 9Hz), 7.20(5H, m). *4-PhBu-Nle-Py (**5**)*: 0.88(3H, m), 1.30(4H, m), 1.60–2.40(10H, m), 2.64(2H, m), 3.40(4H, m), 4.74(1H, m), 5.28(1H, d, J = 9 Hz), 7.21(5H, m). *4-PhBu-Met-Py (**6**)*: 1.80–2.80(12H, m), 2.10(3H, s), 3.20–3.80(6H, m), 5.90(1H, m), 6.38(1H, d, J = 9 Hz), 7.22(5H, m). *4-PhBu-Phe-Py (**7**)*: 1.50–2.30(8H, m), 2.60(3H, m), 3.00(2H, m), 3.36(3H, m), 5.82(1H, m), 6.38(1H, d, J = 9 Hz), 7.20–7.30(10H, m). *4-PhBu-Thr-Py (**8**)*: 1.15 and 1.17(total 3H, both d, J = 6 Hz), 1.80–2.34(8H, m), 2.64(2H, m), 3.38-PhBu-4.28(6H, m), 4.60–4.75(1H, m), 6.46 and 6.69 (total 1H, both d, J = 7 Hz). *4-PhBu-Ser-Py (**9**)*: 1.70–2.40(8H, m), 2.67(2H, t, J = 7 Hz), 3.40–3.80(6H, m), 3.80 (1H, s), 4.80(1H, m), 6.78(1H, d, J = 7 Hz), 7.10–7.40(5H, m). *4-PhBu-Ser(OBzl)-Py (**10**)*: 1.70–2.40(8H, m), 2.64(2H, m), 3.20–3.80(6H, m), 4.48(2H, m), 4.96(1H, m), 6.44(1H, d, J = 8 Hz), 7.10–7.40(10H, m). *4-PhBu-Tyr-Py (**11**)*: 1.50–2.40(8H, m), 2.60(2H, t, J = 7 Hz), 2.88(2H, d, J = 8 Hz), 3.40(4H, m), 4.92(1H, q, J = 8 Hz), 6.50(1H, d, J = 8 Hz), 6.70(2H, d, J = 9 Hz), 7.00(2H, d, J = 9 Hz), 7.10–7.40(5H, m), 7.75(1H, s). *4-PhBu-Tyr(OBzl)-Py (**12**)*: 1.40–2.40(8H, m), 2.60(2H, t, J = 8 Hz), 2.92(2H, m), 3.32(4H, m), 4.90(1H, m), 5.01(2H, s), 6.60(1H, d, J = 8 Hz), 6.85(2H, d, J = 9 Hz), 7.10(2H, d, J = 9 Hz), 7.20–7.50(10H, m). *4-PhBu-Lys-Py (**13**)*: 1.20–2.40(14H, m), 2.62(14H, m), 3.30–3.80(4H, m), 4.73(1H, m), 6.40(1H, d, J = 8 Hz), 7.10–7.40(5H, m). *4-PhBu-Lys(Z)-Py (**14**)*: 1.20–2.30(14H, m), 2.62(2H, t, J = 7 Hz), 3.16(2H, m), 3.30–3.80(4H, m), 4.74(1H, m), 4.92(1H, m), 5.08(2H, s), 6.42(1H, d, J = 8 Hz), 7.10–7.40(5H, m). *4-PhBu-Glu-Py (**15**)*: 1.70–2.80(14H, m), 3.30–3.70(4H, m), 4.90(1H, m), 6.44(1H, d, J = 8 Hz), 7.20(5H, m), 7.80(1H, s). *4-PhBu-Glu(OBzl)-Py (**16**)*: 1.70–2.80(14H, m), 3.50(4H, m), 4.82(1H, m), 5.12(2H, s), 6.60(1H, d, J = 8 Hz), 7.10–7.40(10H, m). *4-PhBu-Pro-Py (**17**)*: 1.70–2.47(12H, m), 2.67(2H, m), 3.21–3.90(6H, m), 4.63(1H, m), 7.17(5H, m). *4-PhBu-aHyp-Py (**18**)*: 1.72–2.38(10H, m), 2.68(2H, m), 3.42–3.64(5H, m), 4.10(1H, m), 4.40(1H, m), 4.72(1H, dd, J = 7 and 8 Hz), 6.37(1H, d, J = 12 Hz), 7.21(5H, m). *4-PhBu-Hyp-Py (**19**)*: 1.82–2.26(10H, m), 2.53–2.75(2H, m), 3.30–3.80(6H, m), 4.17(1H, d, J = 4 Hz), 4.66(1H, t, J = 8 Hz), 4.60(1H, m), 7.16(5H, m). *4-PhBu-Thp-Py (**20**)*: 1.80–2.43(8H, m), 2.59–2.47(2H, m), 4.12–4.50(4H, m), 4.47–4.68 (2H, m), 5.04(1H, t, J = 7 Hz), 7.21 (5H, m). *4-PhBu-Pyr-Py (**21**)*: 1.80–2.42(8H, m), 2.56–2.75(4H, m), 3.22–3.66(4H, m), 3.70–4.20 (2H, m), 5.19(1H, dd, J = 3 and 8 Hz), 7.20(5H, m). *4-PhBu-Ind-Py (**22**)*: 1.78–2.16(8H, m), 2.67–3.56(8H, m), 4.86 and 5.28(total 1H, both m), 6.88–7.22(9H, m). *4-PhBu-Pip-Py (**23**)*: 1.68–2.12(12H, m), 2.36(2H, m), 2.68(2H, m), 3.30–3.73(6H, m), 5.30 (1H, m), 7.20(5H, m). *4-PhBu-Acp-Py (**24**)*: 1.77–2.46(16H, m), 2.64(2H, m), 3.46(4H, m), 5.58(1H, broad), 7.20 (5H, m). *2-StBu-Ala-Py (**25**)*: 1.29(3H, d, J = 6 Hz), 1.78–2.54(8H, m), 3.23–3.62(4H, m), 4.07 (2H, t,

TABLE I
Physicochemical properties of acyl-peptidyl-pyrrolidine derivatives. R-(CH_2)n-CO-X-Pyrrolidine

No.	R-	n	X	Molecular Formula	MW.	Refractive Index (26°C)	m.p. (°C)	MS (m/z)	Elemental Analysis Calcd.; upper Found; lower C:	H:	N:
1	Phenyl-	3	Gly	$C_{16}H_{22}N_2O_2$	274.4		93–94	275 CI,$M^+ + 1$	70.04 70.15	8.08 8.22	10.21 10.25
2	Phenyl-	3	Ala	$C_{17}H_{24}N_2O_2$	288.4			288 EI,M^+	70.80 70.58	8.39 8.48	9.71 9.56
3	Phenyl-	3	Val	$C_{19}H_{28}N_2O_2$	316.4	1.52468		316 EI,M^+	72.11 72.07	8.92 8.92	8.85 8.99
4	Phenyl-	3	Leu	$C_{20}H_{30}N_2O_2$	330.5	1.51639		330 EI,M^+	72.69 72.69	9.15 9.04	8.48 8.72
5	Phenyl-	3	Nle	$C_{20}H_{30}N_2O_2$	330.5	1.51925		330 EI,M^+	72.69 72.98	9.15 9.01	8.48 8.61
6	Phenyl-	3	Met	$C_{19}H_{28}N_2O_2S$	349.5	1.52978		349 EI,M^+			
7	Phenyl-	3	Phe	$C_{23}H_{28}N_2O_2$	364.5		76–77	364 EI,M^+	75.79 75.45	7.74 7.75	7.69 7.67
8	Phenyl-	3	Thr	$C_{18}H_{26}N_2O_3$	318.4		83–84	318 EI,M^+	67.90 68.00	8.23 8.32	8.80 8.90
9	Phenyl-	3	Ser	$C_{17}H_{24}N_2O_3$	304.4		108–110	304 EI,M^+	67.08 66.69	7.95 7.95	9.20 9.19
10	Phenyl-	3	Ser (OBzl)	$C_{24}H_{30}N_2O_3$	394.5			394 EI,M^+	73.07 73.18	7.66 7.85	7.10 7.06
11	Phenyl-	3	Tyr	$C_{23}H_{28}N_2O_3$	380.5		150–151	380 EI,M^+	72.61 72.84	7.42 7.53	7.36 7.19
12	Phenyl-	3	Tyr (OBzl)	$C_{30}H_{34}N_2O_3$	470.6	1.57210		470 EI,M^+	76.57 76.72	7.28 7.20	5.95 6.07
13	Phenyl-	3	Lys	$C_{20}H_{31}N_3O_2$	345.5	1.53261		345 EI,M^+	69.53 69.71	9.04 8.77	12.16 12.29

14	Phenyl-	3	Lys (2)	$C_{28}H_{37}N_3O_4$	479.6	1.54480		479 EI,M$^+$	70.12 70.48	7.78 7.77	8.76 8.66
15	Phenyl-	3	Glu	$C_{19}H_{26}N_2O_4$	346.9	1.51620		346 EI,M$^+$			
16	Phenyl-	3	Glu (OBzl)	$C_{26}H_{32}N_2O_4$	436.5	1.53082		436 EI,M$^+$			
17	Phenyl-	3	Pro	$C_{19}H_{26}N_2O_2$	314.4	1.48292		314 EI,M$^+$	72.58 72.77	8.33 8.11	8.91 9.04
18	Phenyl-	3	aHyp	$C_{19}H_{26}N_2O_3$	330.4	1.53962		330 EI,M$^+$	69.07 69.07	7.93 8.05	8.48 8.52
19	Phenyl-	3	Hyp	$C_{19}H_{26}N_2O_3$	330.4	1.54558		330 EI,M$^+$	69.07 69.02	7.93 7.89	8.48 8.56
20	Phenyl-	3	Thp	$C_{18}H_{24}N_2O_2S$	332.5			333 CI,M$^+$ + 1	65.04 65.02	7.28 7.33	8.43 8.50
21	Phenyl	3	Pyr	$C_{19}H_{24}N_2O_3$	328.4	1.54060		329 CI,M$^+$ + 1	69.49 68.92	7.37 7.43	8.53 8.56
22	Phenyl-	3	Ind	$C_{23}H_{26}N_2O_2$	362.5		124–125	362 EI,M$^+$	76.21 76.10	7.23 7.28	7.73 7.86
23	Phenyl-	3	Pip	$C_{20}H_{28}N_2O_2$	328.5	1.54390		328 EI,M$^+$	73.14 72.87	8.59 8.66	8.53 8.47
24	Phenyl-	3	Acp	$C_{20}H_{28}N_2O_2$	328.5		179–180	328 EI,M$^+$	73.14 72.93	8.59 8.64	8.53 8.51
25	2-Styrylphenoxy-	3	Ala	$C_{25}H_{30}N_2O_3$	406.5	1.61700		406 EI,M$^+$			
26	2-Styrylphenoxy-	3	Val	$C_{27}H_{34}N_2O_3$	434.6		153-154	434 EI,M$^+$	74.62 74.74	7.89 7.96	6.45 6.49
27	2-Styrylphenoxy-	3	Leu	$C_{28}H_{36}N_2O_3$	448.6	1.59048		449 CI,M$^+$ + 1			
28	2-Styrylphenoxy-	3	Phe	$C_{31}H_{34}N_2O_3$	482.6			483 CI,M$^+$ + 1	77.15 77.09	7.10 7.18	5.80 5.72
29	2-Styrylphenoxy-	3	Pro	$C_{27}H_{32}N_2O_3$	432.6			432 EI,M$^+$			

TABLE I (*continued*)
Physicochemical properties of acyl-peptidyl-pyrrolidine derivatives. R-(CH_2)n-CO-X-Pyrrolidine

No.	R-	n	X	Molecular Formula	MW.	Refractive Index (26°C)	m.p. (°C)	MS (m/z)	Elemental Analysis Calcd.; upper Found; lower C:	H:	N:
30	4-Benzylphenoxy-	3	Ala	$C_{24}H_{23}N_2O_3$	394.5		89–90	394 EI,M^+	73.07 72.92	7.66 7.71	7.10 7.19
31	4-Benzylphenoxy-	3	Val	$C_{26}H_{34}N_2O_3$	422.6		105–106	422 EI,M^+	73.90 73.80	8.11 8.15	6.63 6.82
32	4-Benzylphenoxy-	3	Leu	$C_{27}H_{26}N_2O_3$	436.6	1.53699		436 EI,M^+	74.28 74.44	8.31 8.41	6.42 6.53
33	4-Benzylphenoxy-	3	Phe	$C_{30}H_{34}N_2O_3$	470.6	1.57880		470 EI,M^+	76.57 76.35	7.28 7.27	5.95 6.00
34	4-Benzylphenoxy-	3	Pro	$C_{26}H_{32}N_2O_3$	420.5	1.56638		420 EI,M^+	74.26 73.83	7.67 7.76	6.66 6.80
35	1-Decenyl-	7	Val	$C_{27}H_{50}N_2O_2$	434.7	1.48240		434 EI,M^+	74.60 74.63	11.59 11.82	6.44 6.44
36	1-Decenyl-	7	Leu	$C_{28}H_{52}N_2O_2$	448.7	1.47948		448 EI,M^+	74.95 74.72	11.68 11.47	6.24 6.51
37	1-Decenyl-	7	Nle	$C_{28}H_{52}N_2O_2$	448.7	1.48009		448 EI,M^+	74.95 75.05	11.68 11.53	6.24 6.11
38	1-Decenyl-	7	Phe	$C_{31}H_{50}N_2O_2$	482.7	1.50400		482 EI,M^+	77.13 77.23	10.44 10.30	5.80 5.66
39	1-Decenyl-	7	Tyr (OBzl)	$C_{38}H_{56}N_2O_3$	588.9	1.52925		588 E1,M^+	77.75 77.72	9.58 9.65	4.76 4.51
40	1-Decenyl-	7	Pro	$C_{27}H_{48}N_2O_2$	432.7	1.49385		432 E1,M^+	74.95 75.06	11.18 11.04	6.47 6.37
41	5-isoquinolinoxy-	3	Val	$C_{22}H_{29}N_3O_3$	383.5	1.56268		383 E1,M^+			

42	5-Isoquinolinoxy-	3	Leu	$C_{23}H_{31}N_3O_3$	397.5	1.55161	397 EI,M^+			
43	5-Isoquinolinoxy-	3	Nle	$C_{23}H_{31}N_3O_3$	397.5	1.55799	397 EI,M^+			
44	5-Isoquinolinoxy-	3	Phe	$C_{26}H_{29}N_3O_3$	431.5		431 EI,M^+			
45	5-Isoquinolinoxy-	3	Pro	$C_{22}H_{27}N_3O_3$	381.5		381 EI,M^+	69.27 69.39	7.13 6.85	11.02 10.96
46	3-Pyridyloxy-	3	Val	$C_{18}H_{27}N_3O_3$	333.4	1.52704	333 EI,M^+			
47	3-Pyridyloxy-	3	Leu	$C_{19}H_{29}N_3O_3$	347.5		347 EI,M^+			
48	3-Pyridyloxy-	3	Nle	$C_{19}H_{29}N_3O_3$	347.5	1.52260	347 EI,M^+			
49	3-Pyridyloxy-	3	Phe	$C_{22}H_{27}N_3O_3$	381.5	1.55839	381 EI,M^+			
50	3-Pyridyloxy-	3	Pro	$C_{18}H_{25}N_3O_3$	331.4	1.50200	331 EI,M^+	65.24 65.35	7.60 7.46	12.68 12.60
51	2-Styrylphenoxy-	1	Pro	$C_{25}H_{28}N_2O_3$	404.5		404 EI,M^+			
52	2-Styrylphenoxy-	2	Pro	$C_{26}H_{30}N_2O_3$	418.5		418 EI,M^+	74.61 74.39	7.22 7.30	6.69 6.78
53	2-Styrylphenoxy-	4	Pro	$C_{28}H_{34}N_2O_3$	446.6		447 CI,$M^+ + 1$	75.31 75.38	7.67 7.36	6.27 6.13
54	Methyl-	4	Pro	$C_{15}H_{26}N_2O_2$	266.4	1.49150	266 EI,M^+			
55	Methyl-	6	Pro	$C_{17}H_{32}N_2O_2$	294.4	1.49135	294 EI,M^+	69.35 69.26	10.27 10.21	9.51 9.49

TABLE I (*continued*)
Physicochemical properties of acyl-peptidyl-pyrrolidine derivatives. R-(CH_2)n-CO-X-Pyrrolidine

No.	R-	n	X	Molecular Formula	MW.	Refractive Index (26°C)	m.p. (°C)	MS (m/z)	Elemental Analysis Calcd.; upper Found; lower C:	H:	N:
56	Methyl-	10	Pro	$C_{21}H_{38}N_2O_2$	350.5	1.48403		350 EI,M^+			
57	Methyl-	12	Pro	$C_{23}H_{42}N_2O_2$	378.6			378 EI,M^+	72.97 73.29	11.18 11.09	7.40 7.41
58	Methyl-	14	Pro	$C_{25}H_{46}N_2O_2$	406.6	1.48300		407 EI,$M^+ + 1$	73.84 73.84	11.40 11.30	6.89 6.84
59	1,4-Decadienyl-	7	Pro	$C_{27}H_{45}N_2O_2$	430.7	1.50299		430 EI,M^+			
60	Phenoxy-	3	Pro	$C_{19}H_{26}N_2O_3$	330.4			330 EI,M^+	69.07 69.03	7.93 7.80	8.48 8.46
61	2-Benzylphenoxy-	3	Pro	$C_{26}H_{32}N_2O_3$	420.5			421 CI,$M^+ + 1$	74.26 73.93	7.67 7.70	6.66 6.62
62	2'-Chalconoxy-	3	Pro	$C_{28}H_{32}N_2O_4$	460.6			461 CI,$M^+ + 1$	73.02 73.25	7.00 6.91	6.08 6.01
63	2-Chalconoxy-	3	Pro	$C_{28}H_{32}N_2O_4$	460.6			461 CI,$M^+ + 1$	73.02 73.10	7.00 6.97	6.08 5.99
64	2-Phenethylphenoxy-	3	Pro	$C_{27}H_{32}N_2O_3$	434.6			434 EI,M^+			
65	2-Benzoylphenoxy-	3	Pro	$C_{26}H_{34}N_2O_4$	434.5			434 EI,M^+			
66	2-Allylphenoxy-	3	Pro	$C_{22}H_{30}N_2O_3$	370.5			371 CI,$M^+ + 1$	71.32 71.40	8.16 8.13	7.56 7.58

J = 6 Hz), 4.70(1H, m), 6.54(1H, d, J = 8 Hz), 6.82–7.62(11H, m). *2-StBu-Val-Py (**26**)*: 1.92(6H, d, J = 7 Hz), 1.63–2.64(9H, m), 3.28–3.72(4H, m), 4.02 (2H, t, J = 6.0 Hz), 4.57(1H, dd, J = 7 Hz and 9 Hz), 6.73–7.61(12H, m). *2-StBu-Leu-Py (**27**)*: 1.87(3H, d, J = 6 Hz), 1.93(3H, d, J = 6 Hz), 1.45–2.62(11H, m), 3.22–3.75(4H, m), 4.00(2H, t, J = 6 Hz), 4.60–4.97(1H, m), 6.70–7.61(12H, m). *2-StBu-Phe-Py (**28**)*: 1.53(4H, m), 2.05–2.55(4H, m), 2.96(2H, d, J = 7 Hz), 3.28(4H, m), 3.95(2H, t, J = 6 Hz), 4.91(1H, m), 6.68–7.62(12H, m), 7.13(5H, m). *2-StBu-Pro-Py (**29**)*: 1.70–2.40(10H, m), 2.60(2H, m), 3.20–3.90(8H, m), 4.10(2H, t, J = 6 Hz), 4.61(1H, m), 6.80–7.63(11H, m). *4-BzlBu-Ala-Py (**30**)*: 1.31(3H, d, J = 6 Hz), 1.87–2.49(10H, m), 3.28–3.68(4H, m), 3.90 (2H, m), 4.72 (1H, m), 6.57(1H, d, J = 6 Hz), 6.76–7.12(4H, m), 7.20(5H, m). *4-BzlBu-Val-Py (**31**)*: 1.84-(6H, d, J = 7 Hz), 1.62–2.60(9H, m), 3.28–3.67(H, m), 3.86 (2H, s), 3.92(2H, t, J = 6 Hz), 4.57(1H, dd, J = 7 Hz and 8 Hz), 6.66–7.11(4H, m), 7.16 (5H, m). *4-BzlBu-Leu-Py (**32**)*: 1.88(3H, d, J = 6 Hz), 1.95(3H, d, J = 6Hz), 1.47–2.61(11H, m), 3.27–3.64(4H, m), 3.85(2H, s), 3.90(2H, t, J = 6 Hz), 4.62–4.98(1H, m), 6.65–7.10 (4H, m), 7.15(5H, m). *4-BzlBu-Phe-Py (**33**)*: 1.57(4H, m), 2.07–2.55(4H, m), 2.99(2H, d, J = 7 Hz), 3.30(4H, m), 3.84(2H, s), 3.87(2H, t, J = 6 Hz), 4.93(1H, m), 6.64–7.48(10H, m), 7.16(5H, m). *4-BzlBu-Pro-Py (**34**)*: 1.69–2.63(12H, m), 3.25–3.75(6H, m), 3.87(2H, s), 3.96 (2H, t, J = 7 Hz), 4.62(1H, m), 6.68–7.12(4H, m), 7.16(5H, m). *Oleoyl-Val-Py (**35**)*: 0.88(3H, m), 0.92(3H, d, J = 7 Hz), 0.96(3H d, J = 7 Hz), 1.10–2.30(33H, m), 3.30–3.90(4H, m), 4.40(1H, dd, J = 7 Hz and 9 Hz), 5.32(2H, m), 6.18(1H, d, J = 9 Hz). *Oleoyl-Leu-Py (**36**)*: 0.80–1.00(9H, m), 1.10–2.30(35H, m), 3.30–3.80(4H, m), 4.82(1H, m), 5.32(2H, m), 6.26(1H, d, J = 8 Hz). *Oleoyl-Nle-Py (**37**)*: 0.88(6H, m), 1.00–2.30(38H, m), 3.30–3.80(4H, m), 4.74(1H, m), 5.32 (2H, m), 6.32(1H, d, J = 8 Hz). *Oleoyl-Phe-Py (**38**)*: 0.87(3H, m), 1.10–2.60(32H, m), 3.00(2H, dd, J = 3 Hz and 8 Hz), 3.34(4H, m), 4.90(1H, m), 5.32(2H, m), 6.34(1H, d, J = 8 Hz), 7.21(5H, m). *Oleoyl-Tyr(OBzl)-Py (**39**)*: 0.88(3H, m), 1.10–2.80(32H, m), 2.92(2H, m), 3.32(4H, m), 4.86(1H, m), 5.02(2H, s), 5.32(2H, m), 6.34(1H, d, J = 8 Hz), 6.85(2H, d, J = 9 Hz), 7.10(2H, d, J = 9 Hz), 7.20–7.50(5H, m). *Oleoyl-Pro-Py (**40**)*: 0.86(3H, m), 1.00–2.40(36H, m), 3.20–4.00(6H, m), 4.64(1H, m), 5.32 (2H, m). *5-IqBu-Val-Py (**41**)*: 0.89(3H, d, J = 6 Hz), 0.95(3H, d, J = 6 Hz), 1.76–2.10(5H, m), 2.22–2.64(4H, m), 3.36–3.77(4H, m), 4.20(2H, t, J = 6 Hz), 4.63(1H, dd. J = 6 Hz and 9 Hz), 6.43(1H, d, J = 9 Hz), 6.98(1H, dd, J = 3 Hz and 6 Hz), 7.50(2H, m), 8.00(1H, d, J = 6 Hz), 8.51(1H, d, J = 6 Hz), 9.19(1H, broad). *5-IqBu-Leu-Py (**42**)*: 0.84(3H, d, J = 7 Hz), 0.95(3H, d, J = 7 Hz), 1.34–2.60(11H, m), 3.28–3.73(4H, m), 4.18(2H, t, J = 6Hz), 4.80(1H, m), 6.36(1H, m), 6.96(1H, dd, J = 3 Hz and 6 Hz), 7.46(2H, m), 7.97(1H, d, J = 6 Hz), 8.50(1H, d, J = 6 Hz), 9.18(1H, broad). *5-IqBu-Nle-Py (**43**)*: 0.83(3H, m), 1.17–2.02(10H, m), 2.17–2.61(4H, m), 3.30–3.71(4H, m), 4.19(2H, d, J = 6Hz), 4.75(1H, m), 6.47(1H, m), 6.97(1H, dd, J = 3Hz and 6Hz), 7.47(2H, m), 8.00(1H, d, J = 6Hz), 8.51(1H, d, J = 6Hz), 9.19(1H, broad). *5-IqBu-Phe-Py (**44**)*: 1.68(4H, m), 2.18–2.67(4H, m), 2.99(2H, d, J = 8Hz), 3.95(4H, m), 4.15(2H, t, J = 6Hz), 4.94(1H, m), 6.83(1H, m), 6.95(1H, dd, J = 3Hz) and 6Hz), 7.21 (5H, s), 7.47(2H, m), 7.98(1H, d, J = 6Hz), 8.51(1H, d, J = 6Hz), 9.19(1H, broad). *5-IqBu-Pro-Py (**45**)*: 1.76–2.70(12H, m), 3.30–3.92(6H, m), 4.20(2H, d, J = 6Hz), 4.66 (1H, m), 6.98(1H, dd, J = 3Hz and 6Hz), 7.46(2H, m), 7.98(1H, d, J = 6Hz), 8.48(1H, d, J = 6Hz), 9.16(1H, broad). *3-PyBu-Val-Py (**46**)*: 0.91(3H, d, J = 7Hz), 0.96(3H, d, J = 7Hz), 1.85–2.53(9H, m), 3.39–3.77(4H, m), 4.05(2H, t, J = 6Hz), 4.61(1H, dd, J = 6Hz and 9Hz), 6.33(1H, d, J = 9Hz), 7.18(2H, m), 8.20(1H, m), 8.28(1H, m). *3-PyBu-Leu-Py (**47**)*: 0.91(3H, d, J = 8Hz), 0.94(3H, d, J = 8Hz), 1.37–2.53(11H, m), 3.30–3.76(4H, m), 4.03(2H, t, J = 6Hz), 4.82(1H, m), 6.35(1H, m), 7.20(2H, m), 8.20(1H, m), 8.29(1H, m). *3-PyBu-Nle-Py (**48**)*: 0.86(3H, m), 1.21–2.54(14H, m), 3.45(4H, m), 4.05(2H, t, J = 6Hz), 4.75(1H, m), 6.49(1H, m), 7.19(2H, m), 8.20(1H, m), 8.29(1H, m). *3-PyBu-Phe-Py (**49**)*: 1.62–1.82(4H, m), 2.04–2.23(2H, m), 2.33–2.49(2H, m), 2.99(2H, m), 3.24(4H, m), 4.00(2H, t, J = 6Hz), 4.93(1H, m), 6.62(1H, m), 7.22(7H, m), 8.20(1H, m), 8.28 (1H, m). *3-PyBu-Pro-Py (**50**)*: 1.79–2.62(12H, m), 3.50–3.86(6H, m), 4.07(2H, t, J = 6Hz), 4.66 (1H, m), 7.20(2H, m), 8.18(1H, m), 8.29(1H, m). *2-Styryl-phenoxyacetyl-Pro-Py (**51**)*: 1.78–2.40(8H, m), 3.20–3.96(6H, m), 4.68 (1H, m), 4.76(2H, s), 6.84–7.64(11H, m). *3-(2-Styrylphenoxy)propionyl-Pro-Py (**52**)*: 1.80–2.32(8H, m), 2.91(2H, m), 3.28–3.93(6H, m), 4.40(2H, m), 4.66(1H, m), 6.92–7.62(11H, m). *5-(2-Styrylphenoxy)pentanoyl-Pro-Py (**53**)*: 1.72–2.96(12H, m), 2.43(2H, m), 3.28–3.88(6H, m), 4.05(2H, m), 4.62(1H, m), 6.84–7.62(11H, m). *Caproyl-Pro-Py (**54**)*: 0.89(3H, m), 1.26–1.36(4H, m), 1.48–2.38(12H, m), 3.29–3.98(6H, m), 4.64(1H, m). *Caprylyl-Pro-Py (**55**)*: 0.88(3H, m), 1.56–2.38(12H, m), 2.29(8H, m), 3.22–3.98(6H, m), 4.64 (1H, m). *Lauroyl-Pro-Py (**56**)*: 0.88(3H, m), 1.26(18H, m), 2.40–3.40(10H, m), 3.20–4.00 (6H, m), 4.66 (1H, m). *Myristoyl-Pro-Py (**57**)*: 0.88(3H, m), 1.26(20H, m), 1.56–2.38(12H, m), 3.30–3.98(8H, m), 4.64(1H, m). *Palmitoyl-Pro-Py (**58**)*: 0.88(3H, m), 1.26(26H, m), 1.40–2.40(10H, m), 3.30–4.00(6H, m), 4.64(1H, m). *Linoleoyl-Pro-Py (**59**)*:

0.86(3H, m), 1.28(16H, m), 1.40–2.40(14H, m), 2.72(2H, m), 3.20–4.00(6H, m), 4.64(1H, m), 5.32(4H, m). *4-Phenoxybutyryl-Pro-Py (**60**)*: 1.83–2.16(12H, m), 3.30–3.78(6H, m), 3.98(2H, t, J = 7Hz), 4.63(1H, m), 6.74–7.37(5H, m). *4-(2-Benzylphenoxy)butyryl-Pro-Py (**61**)*: 1.68–2.53(12H, m), 3.14–3.70(6H, m), 3.96(2H, t, J = 7Hz), 4.59(1H, m), 6.68–7.44(4H, m), 7.17(5H, m). *4-(2'-Chalconoxy)butyryl-Pro-Py (**62**)*: 1.70–2.55(12H, m), 3.20–3.78(6H, m), 4.12 (2H, t, J = 7Hz), 4.52(1H, m), 6.83–7.07(2H, m), 7.18–7.77(9H, m). *4-(2-Chalconoxy)butyryl-Pro-Py (**63**)*: 1.69–2.70(12H, m), 3.20–3.72(6H, m). 4.11 (2H, t, J = 7Hz), 4.60(1H, m), 6.75–7.02(2H, m), 7.16–8.22(9H, m). *4-(2-Phenethylphenoxy)butyryl-Pro-Py (**64**)*: 1.70–2.33(10H, m), 2.55(2H, m), 3.30–3.91(8H, m), 4.02(2H, t, J = 7Hz), 4.68(1H, m), 4.92–5.20(2H, m), 6.00(1H, m), 6.73–7.21(4H, m). *4-(2-Benzoylphenoxy)butyryl-Pro-Py (**65**)*: 1.60–2.40(12H, m), 3.00–3.90(6H, m), 3.96(2H, t, J = 6Hz), 4.54(1H, m), 6.90–7.90(9H, m). *4-(2-Allylphenoxy)butyryl-Pro-Py (**66**)*: 1.70–2.40(10H, m), 2.55(2H, m), 3.32–3.93(8H, m), 4.02(2H, t, J = 7Hz), 4.68(1H, m), 4.90–5.22(2H, m), 6.00(1H, m), 6.70–7.20(4H, m).

RESULTS AND DISCUSSION

The inhibitory constant and the mode of inhibition of three typical inhibitors, 4-PhBu-Pro-Py (**17**), oleoyl-Pro-Py (**40**), and 4-BzlBu-Pro-Py (**34**) were determined toward the bacterial enzyme. These pyrrolidine derivatives inhibited bacterial enzyme competitively with K_i values of 43.0 nM, 4.8 nM, and 4.1 nM, respectively. The details of the mode of binding of the pyrrolidine derivative are unknown, but it seems that the structure of the pyrrolidine ring is suitable (Figure 1) for the enzyme active site. The IC_{50} values of the inhibitors for post-proline cleaving enzymes from *Flavobacterium* and from bovine brain are summarized in Table II. These compounds showed very potent inhibitory activities.

Recently, many 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*,[32–34] lamb kidney[33,35,36] and from ascidian,[33,37] 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 X and Y 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. Z-Val-Pro-H, Z-Pro-Pro-H, Z-pGlu-Pro-H, and Boc-Pro-Pro-H inhibited bovine brain enzyme, and Z-Pro-Pro-H also inhibited *Flavobacterium* and bovine enzyme strongly. Tsuru *et al.*[20] reported that replacement of the proline residue of P_1 site with thiazolidine or thiazolidine aldehyde (thioprolinal) and conversion of the prolinel residue of the P_2 site to a thioproline residue resulted in an increase in the inhibitory activity. The mechanism of inhibition by this thiazolidine derivative is unknown,[20] but the facts suggest a possibility of appearance of a new type of potent inhibitor. Therefore, in this study, we designed and synthesized the Y-X-pyrrolidine type compounds listed in Table II, and compared their inhibitory activities toward *Flavobacterium* and bovine enzymes in order to clarify the subsite specificity of the enzyme.

The N-terminal arylalkyl-acyl moieties of the inhibitors which we examined are 4-phenylbutyryl (**1–24**), 4-(2-styrylphenoxy)butyryl (**25–29**), 4-(4-benzylphenoxy)-butyryl (**30–34**), 4-(5-isoquinolinoxy)butyryl (**41–45**), 4-(3-pyridyloxy)butyryl (**46–50**), 2-styrylphenoxyacetyl (**51**), 3-(2-styrylphenoxy)propionyl (**52**), 5-(2-styryl-phenoxy)pentanoyl (**53**), 4-phenoxybutyryl (**60**), 4-(2-benzylphenoxy)butyryl (**61**),

TABLE II
Inhibitory activity of acyl-peptidyl-pyrrolidine derivatives on post-proline cleaving enzymes of *Flavobacterium* and bovine brain.

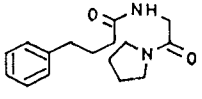
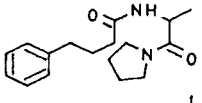
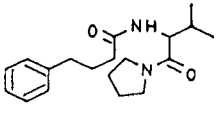
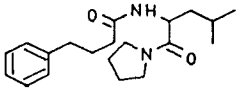
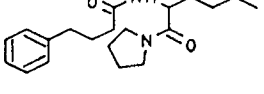
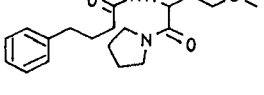
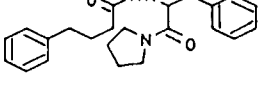
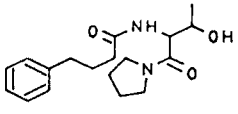
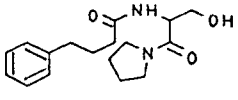
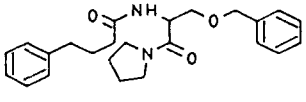
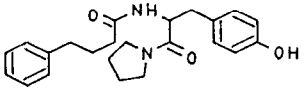
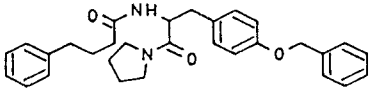
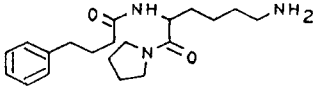
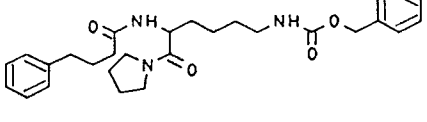
No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium $IC_{50}(\mu M)$	Bovine Brain $IC_{50}(\mu M)$
1.	4-PhBu-Gly-Py		14	22
2.	4-PhBu-Ala-Py		2.0	1.1
3.	4-PhBu-Val-Py		2.7	2.7
4.	4-PhBu-Leu-Py		0.67	4.0
5.	4-PhBu-Nle-Py		0.67	3.2
6.	4-PhBu-Met-Py		3.5	8.4
7.	4-Phbu-Phe-Py		1.2	4.0

TABLE II (continued)

No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium $IC_{50}(\mu M)$	Bovine Brain $IC_{50}(\mu M)$
8.	4-PhBu-Thr-Py		3.2	6.7
9.	4-PhBu-Ser-Py		7.7	9.7
10.	4-PhBu-Ser(OBzl)-Py		2.7	
11.	4-PhBu-Tyr-Py		0.77	2.7
12.	4-PhBu-Tyr(OBzl)-Py		1.3	
13.	4-PhBu-Lys-Py		1.7	2.1
14.	4-PhBu-Lys(Z)-Py		0.62	

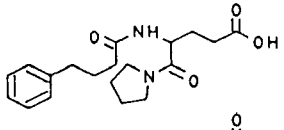
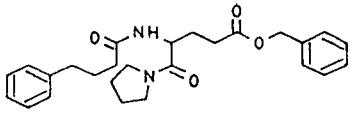
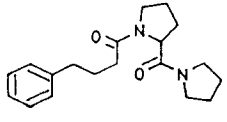
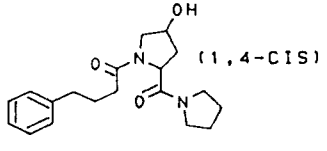
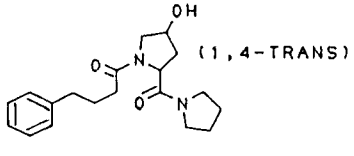
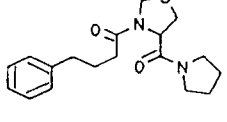
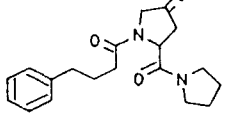
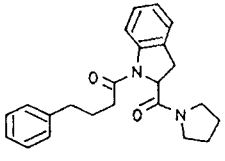
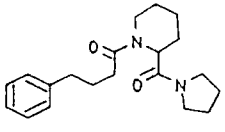
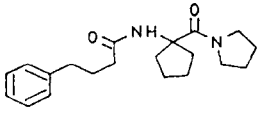
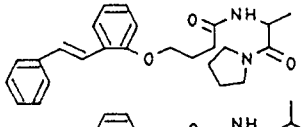
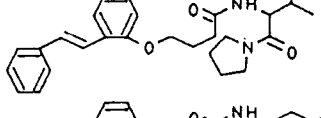
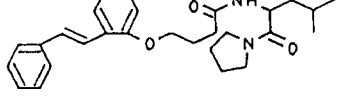
15.	4-PhBu-Glu-Py		1.1	0.84
16.	4-PhBu-Glu(OBzl)-Py		3.5	
17.	4-PhBu-Pro-Py		0.094	0.19
18.	4-PhBu-aHyp-Py		0.45	0.35
19.	4-PhBu-Hyp-Py		0.32	1.8
20.	4-PhBu-Thp-Py		0.89	0.067
21.	4-Phbu-Pyr-Py		1.8	0.89

TABLE II (continued)

No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium IC_{50} (μM)	Bovine Brain IC_{50} (μM)
22.	4-PhBu-Ind-Py		0.62	40
23.	4-PhBu-Pip-Py		0.89	0.89
24.	4-PhBu-Acp-Py		30	180
25.	2-StBu-Ala-Py		0.037	1.5
26.	2-StBu-Val-Py		0.052	10
27.	2-StBu-Leu-Py		0.099	2.3

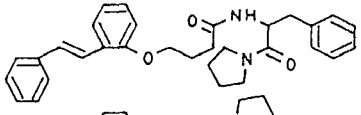
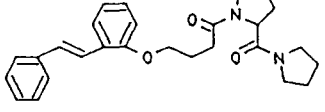
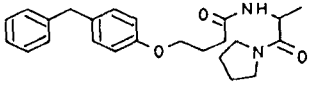
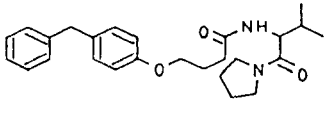
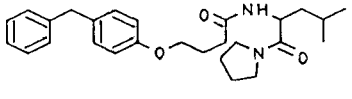
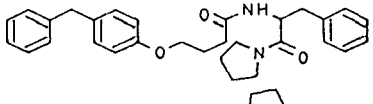
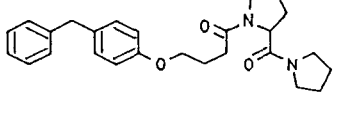
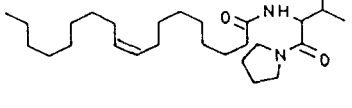
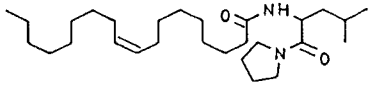
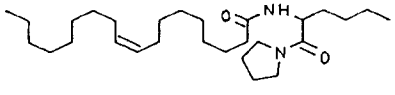
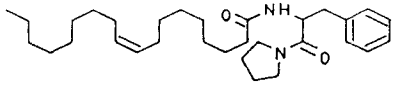
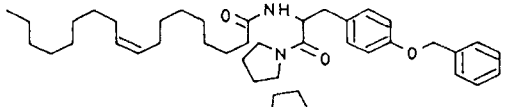
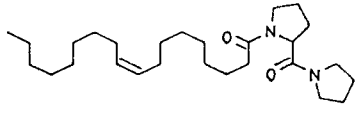
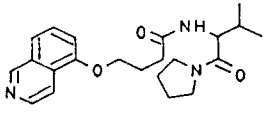
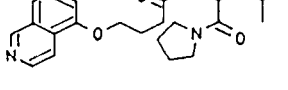
28.	2-StBu-Phe-Py		0.97	
29.	2-StBu-Pro-Py		0.0069	0.14
30.	4-BzlBu-Ala-Py		0.074	2.3
31.	4-BzlBu-Val-Py		0.74	6.9
32.	4-BzlBu-Leu-Py		0.27	24
33.	4-BzlBu-Phe-Py		0.94	
34.	4-BzlBu-Pro-Py		0.0014	0.27
35.	Oleoyl-Val-Py		3.0	170

TABLE II (continued)

No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium $IC_{50}(\mu M)$	Bovine Brain $IC_{50}(\mu M)$
36.	Oleoyl-Leu-Py		0.67	> 200
37.	Oleoyl-Nle-Py		0.23	160
38.	Oleoyl-Phe-Py		6.2	> 200
39.	Oleoyl-Tyr(OBzl)-Py		500	
40.	Oleoyl-Pro-Py		0.069	1.7
41.	5-IqBu-Val-Py		0.12	5.7
42.	5-IqBu-Leu-Py		0.27	11

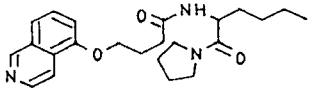
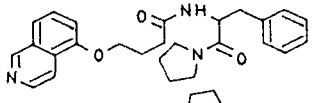
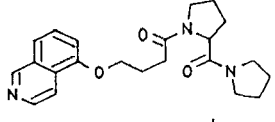
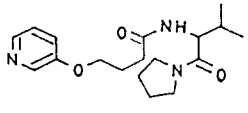
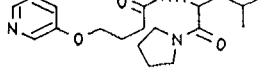
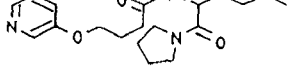
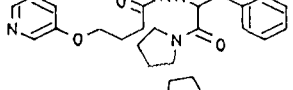
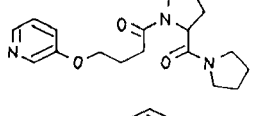
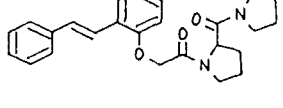
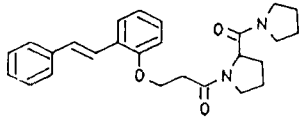
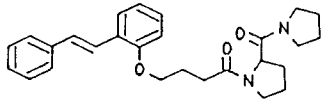
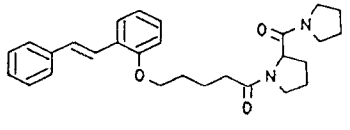
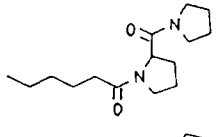
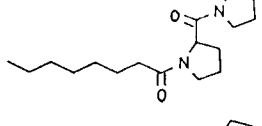
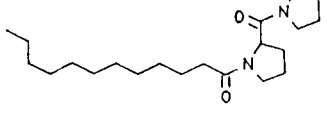
43.	5-IqBu-Nle-Py		0.15	7.4
44.	5-IqBu-Phe-Py		0.35	14
45.	5-IqBu-Pro-Py		0.023	0.40
46.	3-PyBu-Val-Py		1.3	
47.	3-PyBu-Leu-Py		1.3	
48.	3-PyBu-Nle-Py		0.84	
49.	3-PyBu-Phe-Py		2.7	
50.	3-PyBu-Pro-Py		0.13	
51.	2-Styrylphenoxyace-tyl-Pro-Py		0.37	3.7

TABLE II (continued)

No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium $IC_{50}(\mu M)$	Bovine Brain $IC_{50}(\mu M)$
52.	3-(2-Styrylphenoxy) propionyl-Pro-Py		0.011	2.7
29.	4-(2-Styrylphenoxy) butyryl-Pro-Py		0.0069	0.14
53.	5-(2-Styrylphenoxy) pentanoyl-Pro-Py		0.066	0.66
54.	Caproyl-Pro-Py			1.7
55.	Caprylyl-Pro-Py			1.0
56.	Lauroyl-Pro-Py			0.42

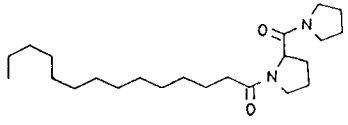
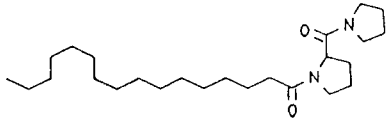
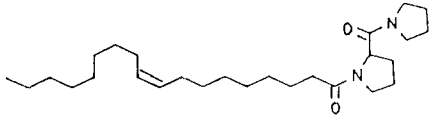
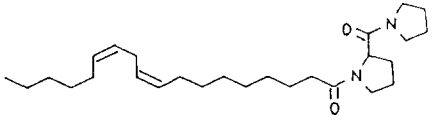
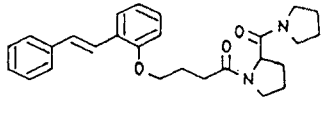
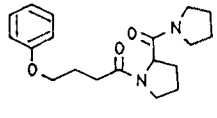
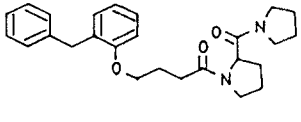
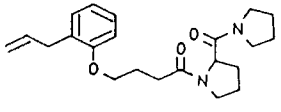
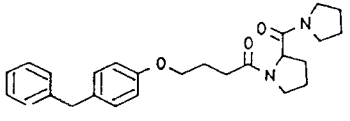
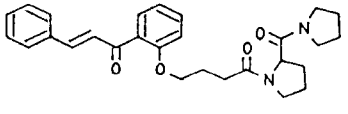
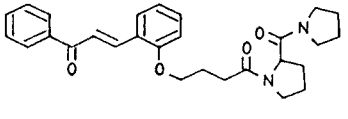
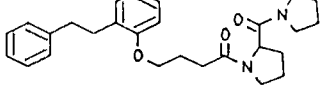
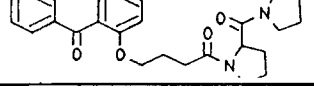
57.	Myristoyl-Pro-Py			4.7
58.	Palmitoyl-Pro-Py			1.8
40.	Oleoyl-Pro-Py			1.7
59.	Linoleoyl-Pro-Py			1.0
29.	4-(2-Styrylphenoxy) butyryl-Pro-Py		0.0069	0.14
60.	4-Phenoxybutyryl-Pro-Py		0.0045	0.45
61.	4-(2-Benzylphenoxy) butyryl-Pro-Py		0.0035	0.21

TABLE II (continued)

No.	Compounds	Chemical Structure	Post-Proline Cleaving Enzyme	
			Flavobacterium $IC_{50}(\mu M)$	Bovine Brain $IC_{50}(\mu M)$
62.	4-(2′-Chalconoxy) butyryl-Pro-Py		0.0025	0.13
63.	4-(2-Chalconoxy) butyryl-Pro-Py		0.0032	0.13
64.	4-(2-Phenethylphen-oxy)butyryl-Pro-Py		0.0067	0.20
65.	4-(2-Benzoylphen-oxy)butyryl-Pro-Py		0.0035	0.17
66.	4-(2-Allylphenoxy) butyryl-Pro-Py		0.0079	0.27
34.	4-(4-Benzylphenoxy) butyryl-Pro-Py		0.0014	0.27

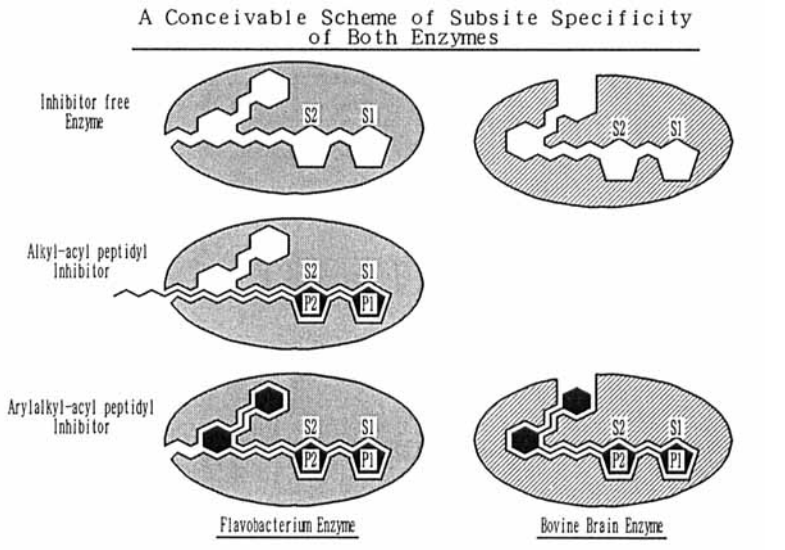


FIGURE 1 A conceivable scheme for subsite specificity of *Flavobacterium* Enzyme (left side) and bovine brain enzyme (right side). The structures of proline residue or pyrrolidine ring are suitable for the active site (S_1) of both enzymes and structure of proline residue is also suitable for the enzyme S_2 sites. Both bacterial and bovine enzyme were inhibited by arylalkyl-acyl inhibitors (bottom) but alkyl-acyl inhibitors inhibited only bacterial enzyme (middle). 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 (in particular, ortho-substituted phenoxybutyryl) moieties for the S_3 subsite.

4-(2′-chalconoxy)butyryl (**62**), 4-(2-chalconoxy)butyryl (**63**), 4-(2-phenethyl-phenoxy)butyryl (**64**), 4-(2-benzoylphenoxy)butyryl (**65**), and 4-(2-allylphenoxy)butyryl (**66**), and the alkyl-acyl moieties are oleoyl (**35–40**), caproyl (**54**), caprylyl (**55**), lauroyl (**56**), myristoyl (**57**), palmitoyl (**58**), and linoleoyl (**59**).

Both bacterial and bovine enzymes were inhibited by arylalkyl-acyyl inhibitors but alkyl-acyl inhibitors (**35–38**, and **40**) 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 (Figure 1). For bacterial enzyme, almost all the bulky and long acyl groups seem to be allowed but the most potent inhibitors tested in this study (**29**, **34**, and **60–66**) which all showed $IC_{50} < 15$ nM for bacterial enzyme and < 500 nM for bovine enzyme, have the same partial structure of a substituted (in particular, ortho-substituted) phenoxybutyryl moiety. (Figure 1). The specificity of the S_3 site was further studied by examining some 2-styrylphenoxy-$(CH_2)_n$-Pro-Py derivatives (**29** and **51–53**) for both enzymes, and some alkyl-acyl-Pro-Py derivatives (**40** and **54–59**) for only bovine enzyme. The arylalkyl-acyl compound which showed the most potent activity toward both enzymes was **29**. The alkyl-acyl compound which had the highest inhibitory activity toward bovine enzyme was **56** (lauroyl), although it was not much more potent than the others. Both compounds which showed the highest activities seem to have similar size acyl moieties. Other arylalkyl-acyl moieties also examined in this study were 4-(5-isoquinolinoxy)butyryl (**41–45**) and 4-(3-pyridyloxy) butyryl (**46–50**). The inhibitory activity of these

TABLE III
The Preventative Action of Post-Proline Cleaving Enzyme Inhibitors toward Scopolamine-Induced Retrograde Amnesia and their Dose-Responses.

Compounds (i.p.)	Drug administered after training (i.p.)	No. of total rats	Training (Means ± S.E.)[a]		Retention test[b]	Amnesia (%)
			First step-down latency (sec.)	No. of descending	No. of amnesic rats/ No. of total rats	
physiological saline	physiological saline	10	2.6 ± 0.8	2.1 ± 0.6	3/10	30
physiological saline	scopolamine (3 mg/kg)	10	1.4 ± 0.6	3.0 ± 0.7	7/10[c]	70
17. (5 mg/kg)	scopolamine (3 mg/kg)	10	2.8 ± 0.5	1.8 ± 0.3	0/10[d]	0
40. (1 mg/kg)	scopolamine (3 mg/kg)	10	1.9 ± 0.6	3.4 ± 0.8	2/10[e]	20

[a]: Total time for the training was 110 sec.
[b]: The step-down latency was measured for 300 sec.
[c]: Significantly different from the physiological saline group ($P < 0.05$, χ^2-analysis).
[d], [e],: Significantly different from the scopolamine group ([d]$P < 0.001$, [e]$P < 0.05$).

heterocyclic compounds is not stronger that that for other arylalkyl-acyl compounds. The reason is unknown at present, but it seems that the S_3 site of the bacterial enzyme prefers a more hydrophobic subsite.

It was reported in recent studies that compounds with valine[37] or proline[18,19-22,32-34] in the P_2 subsite showed higher inhibitory activity than other amino acid residues. In our case, all the compounds containing proline (**17**, **29**, **34**, **40**, **45** and **50**) in the P_2 subsite showed much higher activity than those that did not contain proline residue for bacterial enzyme, and the compounds (**17**, **29**, **34**, **40** and **45**) also showed higher activity for bovine enzyme. An interesting result was obtained for bovine enzyme where compound **20**, which contains a thioproline in its P_2 site, showed the highest inhibitory activity toward the bovine enzyme among all the amino acid residues examined (**1**–**24**). This fact indicates that the P_2 subsite specificities of both enzymes are slightly different from each other. For bovine brain enzyme, we reached the same conclusion as Tsuru *et al.*[20] that thioproline is the most effective P_2 residue for the inhibitor of bovine brain enzyme. Among the other amino acid residues tested in this study for P_2 subsite, hydroxyproline (**18** and **19**), 3-pyrrolidone-5-carboxylic acid (**21**), indoline-2-carboxylic acid (**22**), and pipecolinic acid (**23**) gave rather high activity compared to the compounds with other amino acid residues. The detail of the results of the hydroxyproline derivatives will be discussed elsewhere, but this fact indicates the possibility of a potent inhibitor with a basic structure containing other than proline or thioproline.

Compounds **17** and **40** which have potent inhibitory activity towards post-proline cleaving enzyme, were examined for their anti-amnesic effect on scopolamine induced amnesia rats in the passive avoidance learning test.[38] The intraperitoneally (i.p.) treatment with these compounds **17** and **40** prevented the induction of amnesia at the dose of 5 mg/kg and 1 mg/kg, respectively. (Table III). It can be said that Y-Pro-pyrrolidine derivatives are effective in the retention test. Earlier experiments showed that the mechanism of the nootropic effects are complicated; however, from our results, it appears that at least one mechanism involves the inhibitory activity of the compounds toward the post-proline cleaving enzyme in the brain as reported

previously.[15,18,19] Therefore the post-proline cleaving enzyme is considered to play a part in the regulation of learning and memory consolidation in the brain, and inhibitors of this enzyme are suggested as possible candidates for nootropic agents. The detail of the nootropic effect of the pyrrolidine derivatives will be reported elsewhere.

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