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Synthesis of Peptidic Inhibitors of the Collagenase from *Achromobacter iophagus*

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Several new peptides of the general structure Pro–Leu–X–Pro have been synthesized, by the well-known procedures with proline in L- or D-form and with either glycine or sarcosine in the position of X-. These peptides act as competitive inhibitors of collagenase from *Achromobacter iophagus*.

Keywords——collagenase; *Achromobacter iophagus*; competitive inhibitors; synthetic peptides; synthetic substrates

Bacterial collagenases cleave in native collagen and in synthetic substrates preferentially bonds at the amino terminal of a glycine residue.[2)] The collagenase from *Clostridium histolyticum* was widely studied in this respect. It was established, that this enzyme has preferential affinity for substrates of general formula Pro-R-Gly-Pro, where R is the residue of a neutral amino acid.[2–8)] The collagenase from *Achromobacter iophagus* which was recently obtained in our Laboratory in pure state[9,10)] differs somewhat from the *Clostridium* collagenase in the action both on collagen and on synthetic substrates. In collagen it prefers the structures of formula Pro-R-Gly-Ala[11)] and it cleaves the synthetic substrate Z-Gly-Pro-Leu-Gly-Ala-D-Arg, which is not cleaved at all by the *Clostridium* enzyme[12)]

Both collagenases Zn-metalloenzymes in nature are inhibited in a non competitive way by reagents which remove the metal from their active site, like SH-compounds or EDTA. As regards inhibitors which are structurally analogous to the peptide substrates, Nagai, *et al.*[8)] have studied the effect of several synthetic peptides of the type Pro-R_1-R_2-Pro on the activity of crude *Clostridium* collagenase. They reported some to be competitive inhibitors and others to be non competitive inhibitors.

In the present work we aimed at synthetizing competitive peptide inhibitors of bacterial collagenases which could be employed either in active site labeling reactions or as ligands in affinity chromatography. This paper will be limited to the chemical part of our investigations. The biochemical study of the inhibitors is published elsewhere[13)].

Two kinds of changes have been introduced in the structure Pro-R-Gly-Pro: either a replacement of an L-amino acid by its D-isomer or a substitution of the hydrogen in the peptide bond R-Gly, susceptible to enzymatic cleavage by a methyl group. Consequently oligopeptides were synthesized which contained D-proline instead of L-proline or Sarcosine instead

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of glycine. All the peptides were found to be competitive inhibitors of *Achromobacter* collagenase. Among them the tetrapeptide L-Pro-Leu-Sar-L-Pro turned out to be the most efficient.

Peptide synthesis was carried out using three procedures: 1/Phosphazo intermediates,[14] 2/ or anhydrides with phosphoroxychloride,[15] 3/ activation of the active carboxylic group by 1-hydroxybenzotriazol as nucleophilic derivative,[16] followed by dicyclohexylcarbodiimide (DCC) coupling.

Inhibitory Activities of Peptides

The examined synthetic peptides were all found to be competitive inhibitors for the hydrolysis of the synthetic substrate CBz-Gly-Pro-Leu-Gly-Pro[7] by the *Achromobacter* collagenase.[13] Compound V is the most efficient inhibitor, K_i being 2.2 mmol.

Results and Discussion

The main physicochemical charactereristics of the protected peptides prepared as intermediates are summarized in the Table. The Z-derivatives[17] were cleaved using 10% Pd charcoal as a catalyst[14] and Boc derivatives using 4 N HCl in dioxan according to Schwyzer's method.[18] The protected peptides were obtained by applying conventional procedures as depicted in the Scheme. While Z-derivatives can be utilized either with the phosphazo or the DCC methods the use of the Boc derivatives is limited to the DCC method. Z-Pro-Leu-Sar-Pro-OH was synthesized by the phosphazo method as follows: coupling of Z-Leu-OH with Sar-OEt. HCl in the presence of PCl_3 and pyridine led to Z-Leu-Sar-OEt. The ester was saponified[14] and subsequent coupling with Pro-OMe. HCl by the same method, gave the tripeptide Z-Leu-Sar-Pro-OMe was submitted to hydrogenolysis before being coupled

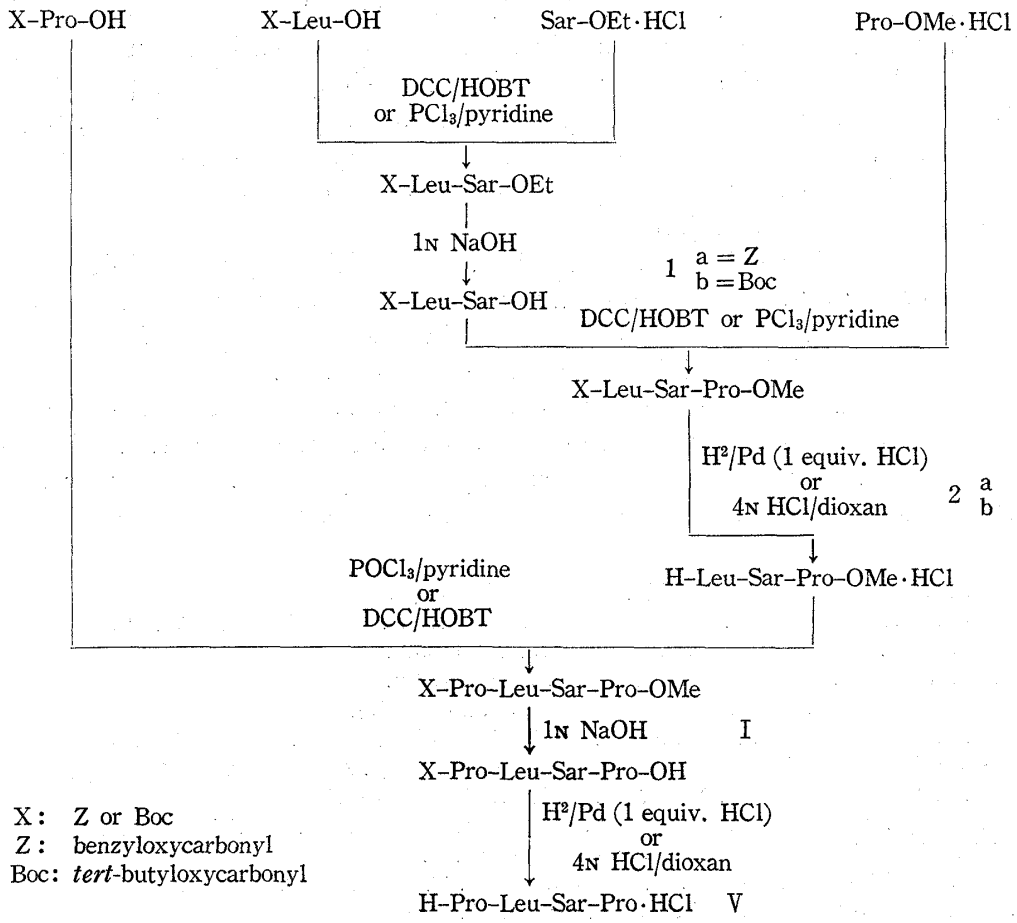

Fig. 1. Synthetic Scheme of Sarcosine Peptides

with Z-Pro-OH, by the anhydride method, in the presence of $POCl_3$,[15] to give the protected tetrapeptide Z-Pro-Leu-Sar-Pro-OMe. After treatment with 1 N NaOH, the tetrapeptide Z-Pro-Leu-Sar-Pro-OH was obtained. Z-D-Pro-Leu-Gly-Pro-OH and Z-Pro-Leu-Gly-D-Pro-OH were prepared similary. The three tetrapeptides can also be prepared as described below. Pro-Leu-Sar-Pro-OH·HCl was synthetized by a modification of König's technique.[16] The condensation of Boc-Leu-Sar-OEt was obtained by DCC coupling of Boc-Leu-OH and Sar-OEt·HCl in the presence of HOBT. In a similar manner Boc-Leu-Sar-Pro-OMe was synthetized from Boc-Leu-Sar-OH, after saponification, by coupling with H-Pro-OMe·HCl. Boc-group was removed by 4 N HCl in dioxan[18] and after crystallization the tripeptide was further coupled with Boc-Pro-OH to give Boc-Pro-Leu-Sar-Pro-OMe which after saponification and deprotection give the expected protected tetrapeptide Pro-Leu-Sar-Pro-OH·HCl.

Table I. Physicochemical Characteristics of Synthetic Peptides

Compound	mp (°C)	$[\alpha]_D^{25}$, deg	Formula	Amino acid analysis			
				Pro	Leu	Gly	Sar
Z-Pro-Leu-Sar-Pro-OH I	Amorphous	−76 (c=1, DMF)[a]	$C_{27}H_{38}N_4O_7$	2.0	1.0		0.96
Z-D-Pro-Leu-Gly-Pro-OH II	60	−35 (c=1, DMF)	$C_{26}H_{36}N_4O_7$	2.12	1.0	1.0	
Z-Pro-Leu-Gly-D-Pro-OH III	Amorphous	−16 (c=0.4, DMF)	$C_{26}H_{36}N_4O_7$	2.07	0.97	0.9	
Boc-Pro-Leu-Sar-Pro-OH IV	100—101 cryst.	—	$C_{24}H_{40}N_4O_7$	2.0	0.94		0.92
Pro-Leu-Sar-Pro·HCl V	Amorphous	−57 (c=1, DMF)	$C_{19}H_{33}ClN_4O_5$	2.0	1.18		1.19

a) DMF: dimethyl formamide.

Experimental

Melting points were determined in open capillaries and are uncorrected. Amino acid analyses were performed with a Beckman Multichrom B amino acid analyzer. Hydrolysis was for 24 hr at 110° in 6 N HCl under vacuum.[19] Thin-layer chromatography (TLC) was accomplished on Silica gel plates (Kieselgel GE 254, Merck) using: *n*-butanol–water–acetic acid (4: 1: 1) as the solvent. Spots were revealed with Reindel's reagent.[20,21] Optical rotation data were recorded on a Perkin–Elmer Polarimeter 241. Z and Boc–amino acids were prepared as described in the literature.[14,22,23] Amino acids and other derivatives were purchased from Fluka (Schwizerland) or Ega (Germany).

Z-Leu-Sar-OH (1a)——Z–Leu–OH (2.65 g, 10 mmol) was added to a cooled (0°) phosphazo solution prepared from Sar·OEt–HCl (1.54 g, 10 mmol) and PCl_3 (0.68 g, 5 mmol) in absolute pyridine (10 ml). The reaction mixture was stirred at 80° for 3 hr. The oily material which formed subsequently[14] was further purified by chromatography on silicic acid in benzene–ethylacetate (1: 1): yield 1.01 g (28.5%). The dipeptide was saponified by the action of a solution of sodium hydroxide in dioxan–water;[24] 1 equiv. 1 N sodium hydroxyde for 1 equiv. peptide ester (25°, 20 min), the mixture was neutralized with 1 N HCl (1 equiv.). After

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evaporation, the resulting solid was recrystallized from ethylacetate–petroleum ether; yield 0.48 g (51%), mp 135°. *Anal.* Calcd. for $C_{17}H_{29}N_2O_5$: C, 60.74; H, 7.19; N, 8.33. Found: C, 59.98; H, 7.19; N, 9.19.

H-Leu-Sar-Pro-OMe (HCl-2a)——To a chilled at 0° phosphazo solution prepared from Pro–OMe–HCl (1.65 g, 10 mmol) and PCl_3 (0.69 g, 5 mmol) in absolute pyridine (10 ml). Z–Leu–Sar–OH (3.3 g, 10 mmol) was added (80°, 3 hr) and worked up as usual. The tripeptide was isolated as an oily product; yield 1.9 g (24%) which was dissolved in methanol (25 ml), acetic acid (2 ml) and hydrogenated at 60 psi overnight in a Parr hydrogenator in the presence of 10% Pd–charcoal (50 mg). The mixture was filtered through celite and the filtrate was evaporated to dryness under reduced pressure. A white powder formed; yield 0.55 g (76%) which was shown to be homogeneous by TLC.

Z-Pro-Leu-Sar-Pro-OH (I)——The above compound (3.44 g, 11 mmol) and Z–Pro–OH (2.49 g, 10 mmol) triethylamine (TEA) (4.59 ml, 33 mmol) dissolved in absolute tetrahydro furan (THF) (25 ml) were treated, at −15°, with $POCl_3$ (1 ml, 10.1 mmol) in absolute THF (2.5 ml). The resulting solution was stirred at −15° for 1 hr. After addition of water, the THF was evaporated off and the remaining oily product was extracted with ethyl acetate. The extract was washed with concentrated HCl, saturated aqueous sodium bicarbonate, water, and dried over sodium sulfate. The oil separated on evaporation was purified by chromatography on silicic acid in ethyl acetate yield: 1.5 g (25%) and was shown to be homogeneous on TLC. The product saponified according to the general procedure described above gave an amorphous product; yield 0.91 g (62%), homogeneous on TLC, *n*-butanol-4, acetic acid 1, water 1, (BAW) *Rf* 0.80. *Anal.* Calcd. for $C_{27}H_{38}N_4O_7$: C, 61.11; H, 7.22; N, 10.56. Found: C, 61.23; H, 7.51; N, 10.41.

Z-D-Pro-Leu-Gly-Pro-OH (II)——Z-D-Pro-OH (2.5 g, 10 mmol), Leu–Gly–Pro–OMe[24] (3 g, 10 mmol) and TEA (2.8 ml) were dissolved in THF (50 ml) and cooled to −15° and treated with $POCl_3$ (0.92 ml, 0.92 mmol) in THF for 1 hr and worked up as usual yielded an oil, homogeneous on TLC; yield 2.5 g (50%). This product was saponified using the general procedure described above to give an oil which was purified by chromatography on silicic acid in ethyl acetate: 90; MeOH: 10, yield 0.82 g (47%) mp 60°, homogeneous on TLC, BAW *Rf* 0.71. *Anal.* Calcd. for $C_{26}H_{36}N_4O_7$: C, 60.45; H, 7.02; N, 10.85. Found: C, 60.24; H, 7.39; N, 10.79.

Z-Pro-Leu-Gly-D-Pro-OH (III)——To a chilled phosphazo solution prepared from D-Pro–Me,HCl (1.65 g, 10 mmol) and PCl_3 (0.68 g, 5 mmol) in absolute pyridine (10 ml) Z–Leu–Gly–OH[24] (3.22 g, 10 mmol) was added (80°, 3 hr) and worked up as usual to give an oil; yield 0.61 g (55%). This compound (0.61 g, 1.4 mmol) was hydrogenated in MeOH (12 ml) and acetic acid (1 ml) overnight at 60 psi in the presence of 20 mg of catalyst and worked up in the usual manner to give 0.4 g (99%) of amorphous Leu–Gly–D–Pro–OMe. The latter was treated with Z–Pro–OH (0.32 g, 1.3 mmol), TEA (0.58 ml, 4.2 mmol) in THF (1.5 ml) cooled to −15° and treated with $POCl_3$ (0.12 ml, 1.19 mmol) and worked up as usual to give an oil; yield 0.3 g (40%), homogeneous on TLC, BAW *Rf* 0.72. This product was saponified using the general procedure described above to give an oil which was purified by chromatography on silicic acid in ethyl acetate: 90, MeOH: 10; yield 0.15 g (54%). Homogeneous on TLC, BAW *Rf* 0.67. *Anal.* Calcd. for $C_{26}H_{36}N_4O_7$: C, 60.45; H, 7.02; N, 10.85. Found: C, 60.49; H, 7.60; N, 10.55.

Procedure for the Synthesis of Peptide Using König's Method

Boc-Leu-Sar-OH (1b)——Boc–Leu (2.49 g, 10 mmol) and Sar–OEt·HCl (1.54 g, 10 mmol) with *N*-methylmorpholine (NMM) (1.11 ml, 10 mmol) were coupled in absolute THF (20 ml) using 1-hydroxybenzotriazole (HOBT) (2 g, 10.5 mmol) and (at 0°) DCC (2.18 g, 10.6 mmol) in absolute THF (6.1 ml). The reaction mixture was stored at 0° for 2 hr, followed by 1 hr at room temperature. After cooling at 0°, dicyclohexylurea (DCU) was then filtered off and the filtrate evaporated to dryness (40°). The residue was dissolved in ethyl acetate, washed successively with saturated sodium bicarbonate, an aqueous solution of a mixture of 0.12 M potassium bisulfate and 0.19 M potassium sulfate (1 : 1). After a final washing with water, the organic phase was dried (Na_2SO_4) and evaporated at 40°. The resulting product, from chromatography on alumine activity I, yielded an oil: 1.96 g (60%, ≃5 mmol) was saponified according to the procedure of Schwyzer[18] in dioxan (8 ml) with 1 N NaOH (5.64 ml) for 2 hr at room temperature. After addition of a few drops of 2 N citric acid, the reaction mixture was evaporated to dryness and the residue was taken up in 100 ml ethyl acetate at 0°, treated with 9.29 ml 2 N citric acid. The organic layer was washed several times with water, dried (Na_2SO_4) and evaporated to an oil. It was crystallized from ethyl ether and petroleum ether; yield 1.06 g (59%), mp 106°, BAW *Rf* 0.86. *Anal.* Calcd. for $C_{14}H_{26}N_2O_5$: C, 55.61; H, 8.67; N, 9.27. Found: C, 55.90; H, 8.67; N, 9.44.

Boc-Leu-Sar-Pro-OMe (2b)——Boc–Leu–Sar–OH (1.06 g, 3.5 mmol), Pro–OMe. HCl (0.58 g, 3.5 mmol), NMM (0.39 ml, 3.5 mmol), HOBT (0.71 g, 4 mmol) in absolute THF (17 ml) were treated with DCC (0.76 g, 3.7 mmol) in THF (2 ml) and stirred in a similar manner as the preparation of Boc–Leu–Sar and worked up as usual to give an oil; yield 0.78 g (54%), homogeneous on TLC, BAW *Rf* 0.52. It was then treated with 0.72 ml of freshly prepared 4 N HCl in dioxan for 2 hr to remove the Boc-group. Evaporation at 35° gave an oil which was crystallized from ethyl ether (hygroscopic); yield 0.57 g (91%).

Boc-Pro-Leu-Sar-Pro IV——Boc–Pro (0.35 g, 1.64 mmol) Leu–Sar–Pro–OMe·HCl (0.57 g, 1640 mmol), NMM (0.20 ml, 1.640 mmol), HOBT (0.32 g, 2.4 mmol) in absolute THF (3.3 ml) were treated with DCC (0.41 g, 2 mmol) in THF (1 ml) and stirred in a similar manner described above and worked up as usual to give an oil which, after chromatography on silicic acid in ethyl acetate–MeOH (90: 10), crystallized from ethyl acetate; yield 0.50 g (60%); mp 69—70°; BAW *Rf* 0.47. *Anal.* Calcd. for $C_{25}H_{42}N_4O_7$: C, 58.80; H, 8.29; N, 10.97. Found: C, 58.61; H, 8.09; N, 10.79.

This product was saponified according to the method described above to give an oil which was purified by chromatography on silicic acid in ethyl acetate–MeOH (50: 50); yield 0.20 g (40%); mp 100—101°; BAW *Rf* 0.35. *Anal.* Calcd. for $C_{24}H_{40}N_4O_7$: C, 58.04; H, 8.12; N, 11.28. Found: C, 57.84; H, 8.45; N, 11.00.

Pro-Leu-Sar-Pro–HCl V——The above product was then treated with 0.20 ml 4 N HCl in dioxan in the conventional manner to give an amorphous product homogeneous on TLC; yield 0.16 g (94%). *Anal.* Calcd. for $C_{19}H_{33}ClN_4O_5$: C, 52.71; H, 7.63; N, 12.94. Found: C, 53.13; H, 8.45; N, 12.02.

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Effect of Insulin on Serum Calcium Concentration in Rats

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A single intraperitoneal administration of insulin to intact and thyroparathyroidectomized rats caused a significant decrease of serum calcium at 10 mU/100 g body weight of the hormone, while it did not show any drop in serum glucose. Administration of 100 mU/100 g of insulin produced a significant decrease of both calcium and glucose in serum. The time course of serum calcium response to insulin is mimiced that of calcitonin with single injections. The present results indicate that insulin has a hypocalcemic effect in rats.

Keywords——insulin; hypocalcemic effect; calcium; intact rats; thyroparathyroidectomized rats

Introduction

Recently, it has been found that calcium provokes a temporary release of insulin in the perfused rat pancreas.[2)] In contrary, insulin secretion decreases in the absence of extracellular calcium.[3)] Presumably, calcium may be involved in the regulation of insulin release. Since the acute rise in serum calcium was observed in glucose-stimulated insulin secretion,[4)] it is considered that insulin may cause the changes of serum calcium level.

Therefore, the present studies were undertaken to determine whether the serum calcium level is affected by the administration of insulin. We found that the administration of insulin decreased significantly the serum calcium concentration in intact and thyroparathyroidectomized rats.

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