BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 51 (12), 3665—3666 (1978)

Synthesis of *cyclo*(-L-Leucyl-L-lysyl-glycyl-)$_2$ and Its Hydrolysis by Trypsin

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(Received July 10, 1978)

Synopsis. A protected cyclic hexapeptide, *cyclo*(-L-Leu-L-Lys(ε-benzyloxycarbonyl)-Gly-)$_2$ (**6**), was synthesized by doubling cyclization of the corresponding tripeptide azide. Compound **6** was converted by hydrogenolysis into a cyclic hexapeptide, *cyclo*(-L-Leu-L-Lys-Gly-)$_2$, which was hydrolyzed by trypsin to a tripeptide, H-Gly-L-Leu-L-Lys-OH.

A few studies have been carried out on the enzymatic hydrolysis of cyclic peptides. Three cyclic hexapeptides, *cyclo*(-Gly$_5$-L-Lys-),[1] *cyclo*(-Gly$_2$-L-Lys-)$_2$,[2] and *cyclo*(-Gly$_2$-L-Phe$_2$-Gly-L-Lys-),[3] all of which contain the -Gly-L-Lys-Gly- sequence, were hydrolyzed by trypsin at the L-Lys-Gly bond. Izumiya *et al.*[4] observed that the rate of tryptic hydrolysis of H-L-Leu-L-Lys-NH_2 is higher than that of H-Gly-L-Lys-NH_2. It thus seemed of interest to synthesize *cyclo*(-L-Leu-L-Lys-Gly-)$_2$ (**7**) and see if it is hydrolyzed by trypsin faster than *cyclo*(-Gly$_2$-L-Lys-)$_2$.

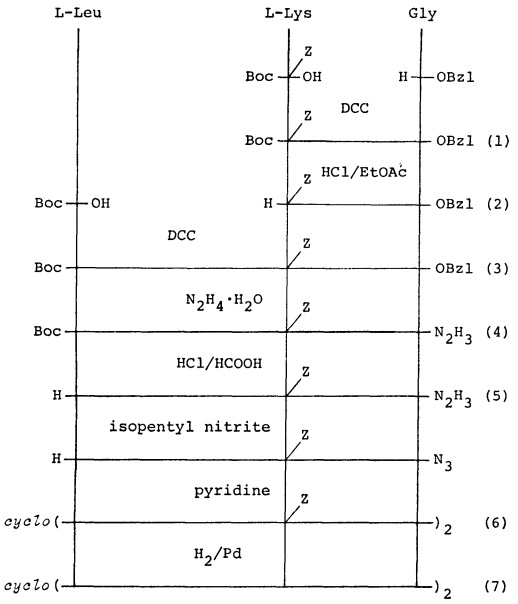

Fig. 1. Synthesis of cyclic hexapeptides, **6** and **7**.

The synthetic route[5] is shown in Fig. 1. A key intermediate, *cyclo*(-L-Leu-L-Lys(Z)-Gly-)$_2$ (**6**) was obtained in 30% yield by the cyclization of the tripeptide azide in pyridine (concentration, 5×10^{-3} M) at 0 °C. Compound **6** was hydrogenolyzed to give the final product **7** as crystalline dihydrochloride. The dimeric nature of **7** was confirmed by FD-mass spectrometry; M^+ 597 was observed, the calculated molecular weight corresponding to the cyclic hexapeptide being 596.7. The homogeneity of **7** was confirmed by TLC and paper chromatography.

Compound **7** was hydrolyzed by trypsin, giving a peptide (R_f^3 0.48) in Tris buffer at pH 7.8. No other spot except for the peptide with R_f^3 0.48 could be observed on a paper chromatogram during the course of incubation of **7**. The peptide with R_f^3 0.48 was assigned to a tripeptide, H-Gly-L-Leu-L-Lys-OH (**11**), by comparison with an authentic **11**, which was synthesized by the hydrogenation of Z-Gly-L-Leu-L-Lys(Z)-OBzl (**10**). Compound **10** was prepared from Z-Gly-OH and H-L-Leu-L-Lys(Z)-OBzl (**9**) which was derived from Boc-L-Leu-L-Lys(Z)-OBzl (**8**). The result of tryptic digestion is similar to that obtained for *cyclo*(-Gly$_2$-L-Lys-)$_2$.[2] This indicates that the rate-determining step is the conversion of **7** into a linear hexapeptide H-Gly-L-Leu-L-Lys-Gly-L-Leu-L-Lys-OH, which is cleaved to **11** immediately. It was observed, however, that tryptic hydrolysis of **7** proceeds slowly as compared to *cyclo*(-Gly$_2$-L-Lys-)$_2$. Steric effect of the bulky side chain in leucyl residues in the rigid backbone of **7** seems to hinder tryptic digestion.

Experimental

TLC was carried out on Merck silica gel G with the following systems: R_f^1, $CHCl_3$-MeOH (5: 1); R_f^2, *n*-BuOH-AcOH-pyridine-H_2O (4: 1: 1: 2). The ratio in parentheses is given in terms of vol. Paper chromatography was performed on Toyo Roshi No. 52 paper with the solvent system: R_f^3, the same solvent as used for R_f^2. Mass spectrum was taken on a Hitachi mass spectrometer Model JMS-01SG 2 equipped with combined sources: the sample was charged by emitter dipping technique; anode heating current, 18 mA. Crystalline trypsin (Nutritional Biochemicals, Cleveland, U.S.A.) was used without further purification.

Boc-L-Lys(Z)-Gly-OBzl (1). DCC (2.06 g, 10 mmol) was added to a solution of dicyclohexylamine salt[2] (5.62 g, 10 mmol) of Boc-L-Lys(Z)-OH and H-Gly-OBzl·TosOH (3.37 g, 10 mmol) in CH_2Cl_2 (20 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. After removal of *N,N'*-dicyclohexylurea, the filtrate was evaporated and the residue was diluted with EtOAc. The solution was successively washed with 0.5 M citric acid, 0.5 M $NaHCO_3$, and water, and then dried (Na_2SO_4). After evaporation under reduced pressure, the residue was solidified by the addition of ether. It was recrystallized from EtOAc-ether-petroleum ether; yield, 4.48 g (85%); R_f^1 0.86. Physical constants and results of elemental analyses of all crystalline compounds are given in Table 1.

H-L-Lys(Z)-Gly-OBzl·HCl (2·HCl). Compound **1** (1.32 g, 2.5 mmol) was dissolved in 2.57 M HCl in EtOAc (19.4 ml, 50 mmol), and the solution was left to stand at room temperature for 1 h and then evaporated. The resulting crystals were collected with the aid of ether; yield, 1.13 g (96%); R_f^1 0.50.

Boc-L-Leu-L-Lys(Z)-Gly-OBzl (3). This was prepared from Boc-L-Leu-OH and **2**·HCl as described for **1**;

Table 1. Analytical data of synthetic peptides

Compound	Mp (°C)	$[\alpha]_D^{20}$	Formula	Found (%)			Calcd (%)		
				C	H	N	C	H	N
1	72—74	−13.5° a)	$C_{28}H_{37}O_7N_3$	63.51	7.00	8.08	63.74	7.07	7.97
2·HCl	142—146		$C_{23}H_{30}O_5N_3Cl\cdot 1/2H_2O$	58.17	6.55	9.14	58.40	6.61	8.88
3	115—119	−29.0° a)	$C_{34}H_{48}O_8N_4$	63.54	7.48	8.80	63.73	7.55	8.74
4	78—84	−21.4° a)	$C_{27}H_{44}O_7N_6$	57.29	7.95	14.53	57.43	7.85	14.88
6	247—248(dec)	−101° b)	$C_{44}H_{64}O_{14}N_8$	60.89	7.47	12.60	61.09	7.46	12.96
7·2HCl	195—197(dec)	−120° b)	$C_{28}H_{54}O_6N_8Cl_2\cdot 3/2H_2O$	48.09	8.11	15.73	48.23	8.24	16.08
8	110—111	−29.5° a)	$C_{32}H_{45}O_7N_3$	65.36	7.78	7.29	65.84	7.77	7.20
10	96—98	−35.8° a)	$C_{37}H_{46}O_8N_4$	65.59	6.90	8.25	65.86	6.87	8.30

a) MeOH (*c* 2) used. b) AcOH (*c* 0.2) used.

yield, 82%; R_f^1 0.72.

*Boc-L-Leu-L-Lys(Z)-Gly-N_2H_3 (**4**).* A solution of **3** (640 mg, 1 mmol) and $N_2H_4\cdot H_2O$ (0.95 ml, 20 mmol) in DMF (10 ml) was left to stand for 3 days at 38 °C, and then evaporated. The residue was dissolved in $CHCl_3$ (50 ml), washed with water, and dried (Na_2SO_4). After removal of the solvent, the residue was solidified by the addition of ether. Recrystallization from MeOH–ether–petroleum ether yielded 468 mg (83%); R_f^1 0.60.

*H-L-Leu-L-Lys(Z)-Gly-N_2H_3·2HCl (**5**·2HCl).* This was prepared from **4** by use of a mixture of 0.0925 M HCl (2.2 eq) in HCOOH and CH_2Cl_2 (1: 1) in place of HCl in EtOAc as described for **2**·HCl; yield, 92%; R_f^1 0.10.

cyclo(*-L-Leu-L-Lys(Z)-Gly-*)$_2$ (**6**). Compound **5**·2HCl (860 mg, 1.6 mmol) was dissolved in DMF (20 ml) containing a few drops of glacial AcOH, and the solution was cooled to −50 °C. To the solution were added 2.7 M HCl in EtOAc (1.78 ml, 4.8 mmol) and isopentyl nitrite (0.237 ml, 1.76 mmol). The solution was stirred for 20 min at −30 °C, and then added dropwise to pyridine (300 ml) at 0 °C. The reaction mixture was stirred for 3 days at 0 °C, and then evaporated. The residue was dissolved in a mixture of MeOH (6 ml) and water (1 ml), and passed successively through columns (each 10.5×1.8 cm) of Dowex 50 (H^+ form) and Dowex 1 (OH^- form). The columns were washed with the same solvent, and the combined effluent (*ca.* 200 ml) was evaporated to dryness. The residue was collected with water. Recrystallization from MeOH–ether yielded 210 mg (30%); R_f^1 0.72, R_f^2 0.87.

cyclo(*-L-Leu-L-Lys-Gly-*)$_2$·*2HCl (**7**·2HCl).* Compound **6** (86.5 mg, 0.1 mmol) was suspended in a mixture of MeOH (1.5 ml) and AcOH (1.5 ml), and hydrogenated in the presence of palladium black. The suspension turned clear with the progress of reaction. In the course of the reaction, the solution showed 3 spots on TLC with R_f^2 values of 0.87 (A), 0.73 (B), and 0.55 (C). Spot A was of the starting material **6**, while spot C was of the final product **7**. Spot B was assumed to be a cyclic hexapeptide in which one of the Z group had been cleaved and another remained. When hydrogenation completed after 4 h, only one spot C was observed on TLC. To this solution was added 2.7 M HCl in EtOAc (0.081 ml, 0.22 mmol) and the filtrate from the catalyst was evaporated to dryness. It was recrystallized from MeOH–EtOAc; yield, 52 mg (75%); R_f^2 0.55, R_f^3 0.66. Found: M^+, 597. Calcd for $C_{28}H_{52}O_6N_8$ (**7**): M, 597.

*Boc-L-Leu-L-Lys(Z)-OBzl (**8**).* Isobutyl chloroformate (0.13 ml, 1 mmol) was added at −10 °C to a chilled solution of Boc-L-Leu-OH (231 mg, 1 mmol) and Et_3N (0.14 ml, 1 mmol) in tetrahydrofuran (2 ml). After 5 min, a chilled mixture of H-L-Lys(Z)-OBzl·TosOH[2] (543 mg, 1 mmol) and Et_3N (0.14 ml, 1 mmol) in $CHCl_3$ (2 ml) was added. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature, and then evaporated. The residual oil was then dissolved in EtOAc, washed successively with 0.5 M citric acid, 0.5 M $NaHCO_3$, and water, and dried (Na_2SO_4). After evaporation, the residue was solidified by the addition of ether. It was recrystallized from EtOAc–ether–petroleum ether; yield, 425 mg (73%); R_f^1 0.85.

*H-L-Leu-L-Lys(Z)-OBzl·HCl (**9**·HCl).* This was prepared from **8** as described for **2**·HCl; yield, 93%; R_f^2 0.75.

*Z-Gly-L-Leu-L-Lys(Z)-OBzl (**10**).* This was prepared from Z-Gly-OH and **9**·HCl as described for **8**; yield, 89%; R_f^1 0.90.

*H-Gly-L-Leu-L-Lys-OH·2HCl (**11**·2HCl).* This was prepared from **10** as described for **7**; yield of hygroscopic crystals, 100%; R_f^2 0.43, R_f^3 0.48.

*Action of Trypsin on **7**.* Tryptic hydrolysis on the substrate **7** was carried out at pH 7.8 and at 30 °C. A solution of **7**·2HCl (6.7 mg, 0.01 mmol) in dimethyl sulfoxide (0.05 ml) was diluted with 0.2 M Tris buffer (0.50 ml) and with water to a total volume of 1 ml and incubated upon addition of trypsin (2 mg). The progress of the reaction was followed by paper chromatography in the course of time. A spot of **11** appeared after 10 min and the spot of **7** diasppeared after 24 h. In a similar experiment, *cyclo*(-Gly_2-L-Lys-)$_2$ was completely hydrolyzed within 4 h.

The authors wish to express their thanks to Mr. Akio Hashimoto, Kitakyushu Municipal Institute of Environmental Health Science, for his help in the measurement of mass spectroscopy.

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5) Abbreviations given by the IUPAC-IUB Commission (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Additional abbreviations: DCC, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide.