Note

The Synthesis of Some Tripeptide Derivatives Related to Gramicidin S and Their Identification in Its Partial Hydrolysates by Gas Chromatography

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The synthesis of some tripeptide derivatives related to gramicidin S has been reported by L. R. M. Synge *et al.*[1] We have also previously[2] described the synthesis of Z- and Tfa-derivatives of those dipeptides and the identification of the dipeptide fragments in the partial hydrolysates of gramicidin S by gas chromatography (GLC). As an extension of this procedure for sequence confirmation of an oligopetide we were interested to determine whether the fragments of the tripeptide sequences occurring in its partial hydrolysates could be effectively deduced in a similar manner. This paper describes the synthesis of some reasonable tripeptide derivatives and a study of the partial hydrolysis of gramicidin S.

I. Synthesis of some tripeptide derivatives related to the sequence of gramicidin S. The N-elongation strategy was applied to the synthesis of Z-tripeptide derivatives of the sequences, L-Orn-L-Leu-L-Phe (**2**), L-Orn-L-Leu-D-Phe (**4**), L-Leu-D-Phe-L-Pro (**6**) and D-Phe-L-Pro-L-Val (**8**), and the fragment condensation strategy was used for the synthesis of the sequences, L-Val-L-Orn-L-Leu (**1**) and L-Pro-L-Val-L-Orn (**9**), in order to use the Z-group throughly as an N-protecting group. For the fragment coupling the DCCD/HOBt-method was adopted so that racemization during coupling was minimized.[3]

1 was prepared by condensing Z-L-Val-δ-Z-L-Orn-OH[1] with H-L-Leu-OMe by the DCCD/HOBt-method. **2** and **4** were obtained by condensing α, δ-diZ-L-Orn-OH with H-L-Leu-L-Phe-OMe (HBr) or H-L-Leu-D-Phe-OMe (HBr) by the MCA-method, respectively. **6** was prepared by condensation between Z-L-Leu-OH and H-D-Phe-L-Pro-OMe, and **8** by that between Z-D-Phe-OH and H-L-Pro-L-Val-OMe, by the DCCD/HOBt-method, respectively. **9** was prepared by condensing Z-L-Pro-L-Val-OH[6] with H-δ-Z-L-Orn-OMe (HCl) by the DCCD/HOBt-method. **1**,**2**,**4** and **9** were obtained as crystals, but **6** and **8** as oils.

Tfa-tripeptide derivatives, Tfa-L-Val-δ-Tfa-L-Orn-L-Leu-(**10**), -α,δ-diTfa-L-Orn-L-Leu-L-Phe- (**11**), -α, δ-diTfa-L-Orn-L-Leu-D-Phe- (**12**), -L-Leu-D-Phe-L-Pro- (**13**), -D-Phe-L-Pro-L-Val- (**14**) and -L-Pro-L-Val-δ-Tfa-L-Orn-OMe (**15**) were prepared from the corresponding Z (or diZ)-tripeptide-OMe according to the literature,[5] respectively. **10**,**11**,**12** and **15** were obtained as crystals, but **13** and **14**, however, only as oils.

II. *Identification of the tripeptide fragments in the partial hydrolysates of gramicidin S in the form of Tfa-methyl ester derivatives by GLC.* Respective t_R-values of the Tfa-tripeptide derivatives were determined by GLC, as shown in Table I. L-Leu-D-Phe-L-Pro (**13**) and L-Pro-L-Val-L-Orn (**15**) could not be separated from each other, when they were co-injected.

Gramicidin S was hydrolyzed partially in conc. hydrochloric acid at 50°C for 5 h, 1, 2, and 4 days.[e,f,7] Each product was converted to the respective Tfa-methyl ester derivative, as reported in the lit.[4,2] The resulting neutral fractions (designated as GS-$5h_{50}$, GS-$1d_{50}$ etc, respectively, corresponding to different hydrolysis times and the temperature) were subjected to GLC analysis and the peaks on gas chromatograms were assigned to di-[2] and tripeptide sequences by comparison with their q_i -values, as shown in Table II. These peaks could be separated more effectively by applying temperature programming conditions, as shown in Fig. 1 and Table III.

As seen from Table III or Fig. 1, peaks, Nos. 1, 2, 3, 5, 10, 13, 14, and 15, could be assigned to the respective di-and tripeptide sequences. Peaks, Nos. 4,6,7,8,9,11 and 12, could not be assigned to

TABLE I. q_i-VALUES OF THE SYNTHESIZED TRIPEPTIDE DERIVATIVES IN GLC[a]

Tfa-Tripeptide methyl ester of	q_i[b]	
	Separate	Mixed
1. L-Val-L-Orn-L-Leu (**10**)	0.52	0.53
2. D-Phe-L-Pro-L-Val (**14**)	1.00	1.00
3. L-Leu-D-Phe-L-Pro (**13**)	1.12	1.10
4. L-Pro-L-Val-L-Orn (**15**)	1.13	1.10
5. L-Orn-L-Leu-L-Phe (**11**)	2.03	—[c]
6. L-Orn-L-Leu-D-Phe (**12**)	2.14	2.01

[a] Oven temperature 240°C, isothermal. Carrier gas N_2, 0.6 Kg/cm² at inlet.

[b] $q_i = t_{R_i}/t_{R_2}$ (standard t_R of **14**=8.6 min).

[c] Omitted in this test because this diastereomer could not be detected in the hydrolysates of gramicidin S.

Abbreviations: DCCD, dicyclohexyl carbodiimide; HOBt, 1-hydroxy benzotriazole; MCA, mixed carbonic anhydride; Tfa, trifluoroacetyl; THF, tetrahydrofuran; Z, carbobenzoxy.

TABLE II. q_i-VALUES OF THE PEAKS IN GLC-ANALYSIS[a] OF HYDROLYSATES OF GRAMICIDIN S AND THEIR ASSIGNMENT TO THE TRIPEPTIDE SEQUENCES

Peak No.	q_i[b]				Assignment to the tripeptides
	GS-5h$_{50}$[c]	GS-1d$_{50}$[c]	GS-2d$_{50}$[c]	GS-4d$_{50}$[c]	
1.	0.53	0.53	*0.48*	*0.53*	L-Val-L-Orn-L-Leu (**10**)
2.	0.60	*0.60*	0.59	0.61	
3.	0.80	*0.81*	*0.72*	0.81	
4.	*0.89*	*0.89*	0.84	*0.89*	
5.	1.00	1.00	1.00	1.00	D-Phe-L-Pro-L-Val (**14**)
6.	1.10	1.10	1.20	1.26	L-Leu-D-Phe-L-Pro (**13**) a/o L-Pro-L-Val-L-Orn (**15**)
7.	*1.44*	*1.46*	*1.40*	*1.46*	
8.	*1.92*	*1.96*	*1.79*	*1.91*	L-Orn-L-Leu-D-Phe (**12**)

[a] Oven temperature 240°C, isothermal. Carrier gas N_2, 0.8 Kg/cm^2 at inlet.
[b] $q_i = t_{R_i}/t_{R_5}$ (standard t_R of **14** = 7.30 min). The peak values in italic type were comparatively small in each fractogram.
[c] Designation of hydrolysates, see Text.

TABLE III. q_i-Values OF THE PEAKS IN GLC-ANALYSIS[a] OF HYDROLYSATES OF GRAMICIDIN S (GS-1d$_{50}$)[b] AND THEIR ASSIGNMENT TO THE DI- AND TRIPEPTIDE SEQUENCES

Peak No.	t_R (min)	Assignment	t_R (min) of the Synthesized compound
1	2.2	L-Pro-L-Val	2.2
2	5.8	L-Val-L-Orn a/o L-Orn-L-Leu	5.8
3	7.1	L-Leu-D-Phe	7.0
5	10.5	D-Phe-L-Pro	10.6
10	27.7	L-Val-L-Orn-L-Leu	27.7
13	33.9	D-Phe-L-Pro-L-Val	34.3
14	36.6	L-Leu-D-Phe-L-Pro a/o L-Pro-L-Val-L-Orn	37.3
15	54[c]	L-Orn-L-Leu-D-Phe	57[c]

[a] Temperature programming conditions: isothermal at 190°C for 0~15 min, then 4°C/min to 230°C and again isothermal at this temperature.
[b] Designation of hydrolysates, see Text.
[c] A broad peak.

any expected ones. These peaks appeared invariably and constantly in the hydrolysates so far as obtained under the conditions described above. They can be apparently attributed to the presence of peptide fragments formed by the rearrangement of the original sequence of gramicidin S during the course of hydrolysis.[cf.9]

EXPERIMENTAL PROCEDURES*

All melting points were uncorrected. Gas chromatography: a Hitachi K-53, 5% OV-17, 1 m×3 mm column and FID-detector, oven temp. see Tables and Fig. ^{1}H-NMR-spectra: a Hitachi R 24 with TMS as an internal standard. TLC: Merck silica gel G/Wako silica gel (1:1), developed with CMA (chloroform (760 ml)/MeOH (940 ml)/acetic acid (24 ml)), and visualized with A (0.05% acridine solution in MeOH) under UV light after heating at 100°C, or with I_2 vapour.

1. Z-L-Val-δ-Z-L-Orn-L-Leu-OMe (**1**) (DCCD/HOBt-method[8]): HOBt (0.056 g, 1 eq) and DCCD (0.092 g, 1 eq) were added at 0°C with stirring to a solution of Z-L-Val-δ-Z-L-Orn-OH (**2**)[1] (0.20 g, 0.4 mM) in THF (1.2 ml). Stirring was continued for 1.5 h at 0°C and then 1.5 h at room temperature. The dicyclohexyl-urea, which had precipitated out, was filtered off. A solution of H-L-Leu-OMe·HCl (0.073 g, 0.4 mM) and Et_3N (0.06 ml, 1 eq) in chloroform (1.2 ml) was mixed in with stirring at 0°C. Stirring was continued again for 1.5 h at 0°C and then 1 h at room temperature. The solvent was

* All crystalline compounds obtained here showed expected analytical values.

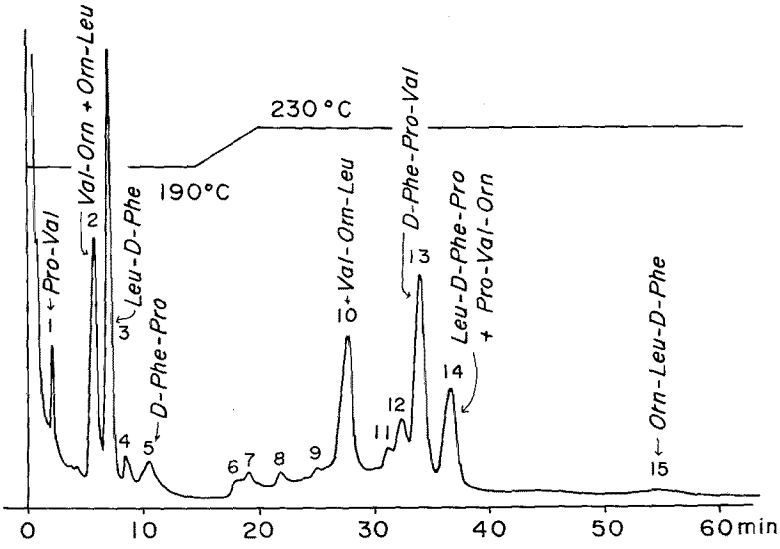

FIG. 1. Cas Chromatogram of Mixtures of Tfa-Peptide Methyl Esters Obtained by 1-Day Acid Hydrolysis (with 12 N HCl at 50°C) of Gramicidin S.

removed in vacuo. The residue was dissolved in AcOEt, and washed successively with 2 N HCl solution, $NaHCO_3$ solution, NaCl solution and water. The precipitate (urea-compound) was filtered off again. The filtrate was dried over Na_2SO_4 and concentrated to dryness *in vacuo*. The residual oil was solidified by scrubbing with *n*-hexane. 0.215 g (86%). mp 178~181°C. TLC: CMA/A, Rf=0.76, a single spot. A part of the solid substance was recrystallized from 2-propanol. mp 187~189°C. $[\alpha]_D^{20}$ −42.2 (c=0.5, MeOH).

2. α, δ-diZ-L-Orn-L-Leu-L-Phe-OMe (**2**) (MCA-method): Z-L-Leu-L-Phe-OMe (1.28 g, 3 mM) was treated with 25% HBr/AcOH (3.75 ml) for 1 h at room temperature. Anhydrous ether was mixed in and the separated oil was washed twice with ether by decantation. This oil (H-L-Leu-L-Phe-OMe·HBr) was condensed with α, δ-di-Z-L-Orn-OH[1] (mp 111~114°C; Lit.[1] mp 112~114°C) (1.20 g, 3 mM) by using ethoxycarbonyl chloride as a coupling agent in AcOEt as usual.[cf.8] After the reaction, the mixture was washed successively in the same way as described for **1**. The organic layer was turbid, and a floating precipitate was found at the surface between the organic and water layers. It was collected by suction. 0.54 g (27%). mp 183~186°C. TLC: CMA/A, Rf=0.75, a single spot. After recrystallization from 2-propanol, mp 179~180°C. $[\alpha]_D^{20}$ −4.2 (c=1, pyridine). (The filtrate was dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was recrystallized from MeOH/water twice. The pure compound was not, however, obtained.)

3. α, δ-diZ-L-Orn-L-Leu-D-Phe-OMe (**4**): 1) Z-L-Leu-D-Phe-OMe (**3**) (MCA-method): Z-L-Leu-OH (1.33 g, 5 mM) was condensed with H-D-Phe-OMe·HCl (1.08 g, 5 mM) in the same way as described for **2**. 1.62 g (76%). mp 105~108°C. TLC: CMA/A, Rf=0.89, a single spot. 2) **4** (MCA-method): **3** (1.28 g, 3 mM) was treated with 25% HBr/AcOH· and washed with anhydrous ether by decantation. The residual oil was condensed with α, δ-diZ-L-Orn-OH[1] (described above in 2.) (1.20 g, 3 mM) in the same way as described for **2**. After the reaction, the neutral fraction was obtained after aqueous acid and alkali work-ups. A floating precipitate was found at the surface between the organic and water layers as in the case of **2**. It was collected by suction. 0.25 g (12%). mp 182~186°C. TLC: CMA/A, Rf=0.70, a single spot. A part of this precipitate was recrystallized from 2-propanol to give a colloidal substance, which was purified on a porcelain plate. Further recrystallization from AcOEt/n-hexane gave a solid substance. mp 184~186°C. $[\alpha]_D^{20}$ −14.2 (c=0.5, pyridine). TLC: CMA/A or I_2, Rf=0.67, a single spot.

4. Z-L-Leu-D-Phe-L-Pro-OMe (**6**) (DCCD/HOBt-method): Z-D-Phe-L-Pro-OMe (**5**) (oil, 0.41 g, 1 mM) was treated with 25% HBr/AcOH and washed twice with anhydrous ether by decantation. The residual oil was condensed with Z-L-Leu-OH (0.28 g, 1 mM) in the same way as described for **1**. After the usual working-up, an oil was obtained as the neutral fraction. 0.19 g (45%). TLC: CMA/A, Rf=0.78.

5. Z-D-Phe-L-Pro-L-Val-OMe (**8**): Z-D-Phe-L-

Pro-OH (7) (an oil, which was prepared from **5** by treating it with 1 N NaOH solution (1.15 eq)[e,f,1] in acetone for 5 days at room temperature.) (0.18 g, 0.45 mM) was condensed with H-L-Val-OMe·HCl (0.08 g, 1 eq) in the same way as described for **2**. After the usual working-up, an oil was obtained as the neutral fraction. 0.21 g (51%).

6. Z-L-Pro-L-Val-δ-Z-L-Orn-OMe (**9**): Z-L-Pro-L-Val-OH (mp 133°C)[6] (0.14 g, 0.4 mM) was condensed with H-δ-Z-L-Orn-OMe·HCl (0.13 g, 0.4 mM) in the same way as described for **1**. The neutral fraction obtained was solidified. 0.18 g (74%). mp 137~139°C. $[\alpha]_D^{20}$ −53.7 (c=2, MeOH). TLC: CMA/A, Rf=0.6, a single spot.

7. Conversion of a Z-tripeptide-OMe to the corresponding Tfa-derivative (**10-15**): by treating a Z-tripeptide-OMe with 25% HBr/AcOH, followed by treatment of the residue with methyl trifluoroacetate in the presence of a slight excess of Et_3N, according to the lit.[5] Tfa-L-Val-δ-Tfa-L-Orn-L-Leu-OMe (**10**): mp 238~242°C. α, δ-diTfa-L-Orn-L-Leu-L-Phe-OMe (**11**): mp 174-177°C. α,δ-diTfa-L-Orn-L-Leu-D-Phe-OMe (**12**): mp 210~214°C. Tfa-L-Leu-D-Phe-L-Pro-OMe (**13**): An oil. NMR ($CDCl_3$) δ: 0.95 (d, isopropyl of Leu) 1.1~2.6 (ring protons of Pro) 3.0 (d, J=8Hz, $-C\underline{H}_2-C_6H_5$) 3.65 (s, $COOCH_3$) 7.15 (C_6H_5). Tfa-D-Phe-L-Pro-L-Val-OMe (**14**): An oil. NMR ($CDCl_3$) δ: 0.85 (d, J=6Hz, isopropyl of Val) 1.1-2.6 (ring protons of Pro) 3.1 (d, J=8Hz, $-C\underline{H}_2C_6H_5$) 3.65 (s, $COOCH_3$) 7.15 (C_6H_5). Tfa-L-Pro-L-Val-δ-Tfa-L-Orn-OMe (**15**): mp 188-190°C.

8. Partial hydrolysis of gramicidin S and conversion of the hydrolysates to Tfa-peptide-OMe: Gramicidin S (100 mg, Comp. Nikken Kagaku, Tokyo) was dissolved in conc. HCl solution and allowed to stand at 50°C in a thermostatically controlled water bath. Aliquots (2 ml each) were taken after different time intervals as shown in Table I. Each one was evaporated to dryness in an evaporating-dish in a water bath. The residue was dissolved in methanolic HCl (0.5 N), heated for 0.5 h under reflux, and evaporated to dryness *in vacuo*. This procedure was repeated once more. The residue was dissolved in MeOH (1 ml) and treated with methyl trifluoroacetate in the presence of Et_3N.[4,2] After the usual working-up, the neutral fraction was obtained. It was concentrated and subjected to gas chromatographic analysis.

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