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The Solid Phase Synthesis of 19-Tyrosine Melittin

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19-Tyrosine melittin, H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Tyr-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2, was synthesized by the solid phase method using with the automatic instrument. It was obtained in 21% yield based on the amount of gultamine initially esterified to the resin. The hemolytic activity of that compound was examined.

Melittin, the main peptide component of honey bee (*Apis mellifica*) venom, is strongly hemolytic and surface active. Its biological activities were extensively studied by Neumann, *et al.*[2] and its amino acid sequence was determined by Habermann and Jentsch.[3] As shown in Fig. 1, a relative long hydrophobic peptide part is attached to a short sequence of hydrophilic basic amino acid residues. The pharmacological and biochemical properties of melittin depend on its surface active character which results from its primary structure.

H-Gly-L-Ile-Gly-L-Ala-L-Val-L-Leu-L-Lys-L-Val-L-Leu-L-Thr-L-Thr-Gly-L-Leu-L-Pro-L-Ala-
L-Leu-L-Ile-L-Ser-L-*Trp*-L-Ile-L-Lys-L-Arg-L-Lys-L-Arg-L-Gln-L-Gln-NH_2

(I): melittin

Boc-Gly-L-Ile-Gly-L-Ala-L-Val-L-Leu-L-Lys(ε-Z)-L-Val-L-Leu-L-Thr(OBz)-L-Thr(OBz)-Gly-L-Leu-L-Pro-L-Ala-
L-Leu-L-Ile-L-Ser(OBz)-L-*Tyr*(OBz)-L-Ile-L-Lys(ε-Z)-L-Arg(NO_2)-L-Lys(ε-Z)-L-Arg(NO_2)-L-Gln-L-Gln-P

(II): P=oxymethylated resin

Bz=benzyl, Z=benzyloxycarbonyl Boc=*t*-butyloxycarbonyl

(III): P=NH_2

Fig. 1. Structures of Melittin and of Synthetic Intermediates

Melittin and its fragment peptides have been synthesized by the solution method and investigated their hemolytic and surface active character by Schröder and Lubke.[4a,b] Re-

1) Location: *1-1, Katahira-2-chome, Sendai.*

2) W. Neumann, E. Habermann, and G. Amend, *Naturwissenschaften*, **39**, 286 (1952); E. Habermann and W. Neumann, *Biochem. Z.*, **328**, 465 (1957); E. Habermann, *Ergeb. Phisiol., Biol. Chem. Exp. Pharmakol.*, **60**, 220 (1968).

3) E. Habermann and J. Jentsch, *Z. Physiol. Chem.*, **348**, 37 (1967).

4) *a*) E. Schröder and K. Lübke, "Peptides," ed. by H.C. Bayermann, A. van de Linde, and W. Maasen van de Brink, North-Holland Publishing, Amsterdam, 1967, p. 271; *b*) Private communication.

cently, various peptides have been synthesized rapidly and in good yield with the Merrifield solid phase method.[5a,b,c] We attempted to synthesize melittin by the Merrifield method. However, melittin contains a tryptophan residue which is degraded by a treatment with 1N hydrochloric acid in acetic acid while removing a N-protecting group.[6] Therefore, it is easier for the synthesis to replace tryptophan with another amino acid. Since it is of interest to synthesize some peptides which have subtle differences in their primary structures for comparing their biological properties with those of native ones, we replaced the tryptophan residue of melittin with tyrosine.

Boc-L-Gln-P
↓ CF_3CO_2H, CH_3CO_2H, EtOH, DMF
↓ Et_3N/DMF, DMF
↓ Boc-L-Gln-ONp/DMF, DMF
Boc-L-Gln-L-Gln-P
↓ CF_3CO_2H, CH_3CO_2H, EtOH, DMF
↓ Et_3N/DMF, DMF
↓ Boc-NO_2-L-Arg/DMF, DCC/DMF
Boc-NO_2-L-Arg-L-Gln-L-Gln-P
↓ HCl/CH_3CO_2H, CH_3CO_2H, EtOH, DMF
↓ Et_3N/DMF, DMF
↓ Boc-ε-Z-L-Lys/CH_2Cl_2, DCC/CH_2Cl_2
Boc-ε-Z-Lys-NO_2-L-Arg-L-Gln-L-Gln-P
↓ repeated above steps twenty two more times
Hexacopeptide-polymer (II)
↓ NH_3/MeOH
↓ HF/anisole
19-Tyrosine-mellitin

Fig. 2. Synthesis of 19-Tyrosine Melittin
P=copolymer, ONp=*p*-nitrophenoxy.

The synthesis of 19-tyrosine melittin is summerized briefly in Fig. 2. *t*-Butyloxycarbonyl (Boc) group was used for α-amino protecting group. Dicyclohexylcarbodiimide (DCC) was the coupling reagent except for the coupling of glutamine residue. Glutamine was incorporated into the peptide chain by mean of the nitrophenyl ester,[7] since DCC dehydrates acid amide to nitrile. Once glutamine residue has been incorporated into the peptide chain, DCC may be used for subsequent couplings without danger of nitrile formation.[8] Boc-Glutamine was esterified with chloromethylated styrene-2%-divinylbenzene copolymer, and the stepwise synthesis was automatically carried out through twenty five cycles to give the fully protected hexacopeptide resin compound II (Fig. 1). It has been described that incomplete coupling was encountered in extending a chain beyond a glutamine residue, so that trifluoroacetic acid was used as the deprotecting reagent of Boc group of the glutamine residues instesd of 1N hydrochloric acid in acetic acid.[5b]

The cleavage of the protected peptide from the resin was done by ammonolysis of the benzyl likage, which was a peptide anchor to a polymer support,[9] to yield the protected hexacopeptide III. The peptide III was recrystallized from aqueous dimethylformamide to give a pale yellow powder. The deprotection of III was carried out with hydrogen fluoride and anisole by the method of Sakakibara, *et al.*[10] After removal of hydrogen fluoride and anisole, 19-tyrosine melittin IV was isolated and lyophilized. It was shown to be homogeneous on paper chromatography and paper electrophoresis and gave the reasonable amino acid analysis on acid hydrolysis.

Schröder and Lübke measured hemolytic activities of melittin and its synthetic fragments,[4b] and Habermann and Kowallek did those of melittin and its modified peptides with

5) *a*) G.R. Marshall and R.B. Merrifield, *Biochemistry*, **4**, 2394 (1965); *b*) H. Takashima, V. du Vigneaud and R.B. Merrifield, *J. Am. Chem. Soc.*, **90**, 1323 (1968); *c*) B. Gutte and R.B. Merrifield, *ibid.*, **91**, 501 (1969).
6) S. Sano and M. Kurihara, *Seibutsu Butsuri*, **7**, 258 (1967); G.R. Marshall, International Symposium on Hormonal Polypeptides, Milan, 1967.
7) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
8) D.T. Gish, P.G. Katsoyannis, G.P. Hess, and R.J. Steman, *J. Am. Chem. Soc.*, **78**, 5954 (1956).
9) M. Bodanszky and J.T. Sheehan, *Chem. Ind.* (London), 1423 (1964).
10) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).

various reagents.[11] Both of them showed that there was no simple relationship between surface activity and hemolytic activity and that the important thing for hemolytic activity was peptide primary structure. Also, Reman, *et al.* showed the same kind of facts on lysolecithin and lecithin.[12] They described that only a difference of one or two double bonds of fatty acid part of those compounds reduced or diminished their hemolytic activities. Our finding of hemolytic activity order is as follows; melittin I > modified melittin with Koshland reagent V,[13] which was modified on 19-tryptophan>melittin treated with hydrogen fluoride and anisole>19-tyrosine melittin IV. Infrared (IR) spectra of those compounds in deuterium oxide showed unordered form.[14]

Experimental

Boc-L-Glutaminyl Resin——A solution of 2.66 g (10.8 mmoles) of Boc-L-glutamine and 1.09 g (10.8 mmoles) of triethylamine in 14 ml of absolute ethanol and 7 ml of chloroform was added to 10.0 g of chloromethylated copolystyrene-2%-divinylbenzene which contained 1.55 mmoles of chlorine per 1 g of resin.

The reaction mixture was stirred at reflux temperature for 48 hr. The esterified resin was filtered and washed with absolute ethanol three times, water three times and methanol three times. The resin was then dried *in vacuo* over potassium hydroxide pellets; yield, 10.93 g. The amino acid analysis on the acid hydrolysate (dioxane: 12N HCl, 1 : 1, 18 hr, 110°) showed that the product contained 0.608 mmoles of glutamine per g of esterified resin.

Fully protected Hexacopeptide Resin (II)——Boc-L-glutaminyl resin (2.57 g) was placed in the reaction vessel.[15] The following cycle of deprotection, neutralization and coupling was carried out for the introduction of each residue: (1) three times washing with 26 ml portion of glacial acetic acid; (2) cleavage of Boc group by a treatment with 1N hydrochloric acid in acetic acid (30 ml) for 30 min at room temperature; (3) three times washing with 26 ml portions of glacial acetic acid; (4) three times washing with 33 ml portions of absolute ethanol; (5) three times washing with 24 ml portions of dimethylformamide (DMF); (6) neutralization of the hydrochloride with 2.6 ml of trimethylamine in 26 ml of DMF for 10 min; (7) three times washing with 24 ml portions of DMF; (8) three times washing with 28 ml portions of methylene chloride; (9) addition of 6.25—7.80 mmoles of appropriate Boc-amino acid in 20 ml of methylene chloride and mixing for 10 min; (10) addition of 6.24—7.80 mmoles of DCC in 10 ml of methylene chloride followed by a reaction period of 2.5 hr at room temperature; (11) three times washing with 28 ml portions of methylene chloride; (12) three times washing with 33 ml portions of absolute ethanol.

Boc-amino acids with protected side chanis were O-benzyl-L-threonine, O-benzyl-L-serine, ε-benzyloxycarbonyl-L-lysine, O-benzyl-L-tyrosine and nitro-L-arginine. The coupling reaction involving Boc-L-glutamine was carried out with its *p*-nitrophenyl ester. After steps 1—7 of the cycle were carried out, a solution of 6.24 mmoles of the *p*-nitrophenyl ester of Boc-L-glutamine in 20 ml of freshly distilled DMF was added, and the reaction was allowed to proceed for 8 hr.

On coupling of Boc-nitro-L-arginine, step 8 was deleted and DMF was used as the solvent in place of methylene chloride in steps 9—11. On coupling of Boc-L-isoleucine and Boc-L-valine, the coupling reaction was allowed to proceed for 4—5 hr. The cleavage of the Boc group of the glutamine residues was carried out by a treatment with 20 ml of trifluoroacetic acid for 23 min at room temperature. The neutralization of the trifluoroacetate was carried out with 2.6 ml of triethylamine in 26 ml of DMF for 10 min. The hexacopeptide resin was washed three times with 24 ml portions of DMF followed by three times washing with 33 ml of absolute ethanol and drying *in vacuo* over KOH pellets; yield, 4.99 g.

Fully protected Hexacopeptide Amide (III)——Dry ammonia was bubbled into a stirring suspension of 2.5 g of the compound (II) in 110 ml of anhydrous methanol at 0° until the solution was saturated with ammonia. The reaction mixture was stirred for 19.5 hr at 0—4°. The methanol and ammonia were evaporated under a reduced pressure. DMF (110 ml) was added to the dry residue and the resulted suspension was stirred vigorously for 3 hr. The resin was filtered off and washed three times with anhydrous methanol.

The solvent was removed from the combined filtrate on a rotatory evaporator at 30°. The residue was dissolved in 5 ml of DMF and 15 ml of distiiled water was added gradually with stirring. The precipitate was filtered and washed with methanol and dried in a vaccum desiccator over potassium hydroxide pellets;

11) E. Habermann and H. Kowallek, *Z. Physiol. Chem.*, **351**, 884 (1970).
12) F.C. Reman, R.A. Demel, J. de Gier, L. L. M. van Deenen, H. Eibl, and O. Westphal, *Chem. Phys. Lipids*, **3**, 221 (1969).
13) D.E. Koshland, Y.D. Karkhanis, and H.G. Latham, *J. Am. Chem. Soc.*, **86**, 1448 (1964).
14) H. Sui, "Structure and Stability of Biological Macromolecules," ed. by S.N. Timasheff and G.D. Fasman, Marcel Dekker, Inc., N. Y., 1969, pp. 605—613.
15) H. Sugiyama, Y. Miura, and S. Seto, *Yuki Gosei Kagaku Kyokai Shi*, **26**, 1010 (1968).

yield, 936 mg. This presents 32% overall yield based on the amount of glutamine initially esterified on the resin. mp 280°. *Anal.* Calcd. for $C_{186}H_{276}O_{44}N_{40}$: C, 59.14; H, 7.38; N, 14.84. Found: C, 59.14; H, 6.07; N, 14.50.

19-Tyrosine Melittin (IV)——The compound (III) (300 mg) was treated with hydrogen fluoride (*ca.* 15 ml) and anisole (0.3 ml) at 0° for 1 hr. This experiment was carried out with a Diflon reaction system (Toho Kasei Co., Ltd., Osaka). After evaporation of hydrogen fluoride from the reaction mixture, 90 ml of 0.1M acetic acid, 30 ml of ethyl acetate and 100 ml of water were added to the residue. The aqueous layer was washed with two portions of 20 ml ethyl acetate and lyophilized; yield, 140 mg (21%), $[\alpha]_D^{25} = -55.0°$ (*c* 2.0 tris-HCl, pH 8.9). The gel filtration on Sephadex G-25 of IV is shown in Fig. 3. The paper chromatography of IV with *n*-butanol–acetic acid–water–pyridine (15: 3: 12: 10) showed a single spot at *Rf* 0.56 with ninhydrin or Sakaguchi reagents. The paper electrophoresis showed a single spot with Sakaguchi reagent (Fig. 4). Amino acid ratios (hydrolysis condition, 6N HCl, 24 hr, 110°) $Thr_{1.6}Ser_{1.2}Pro_{1.0}$-$Gly_{2.7}Ala_{2.1}Glu_{2.1}Val_{1.6}Ile_{3.1}Leu_{4.1}Tyr_{0.9}Lys_{2.8}Arg_{1.9}$.

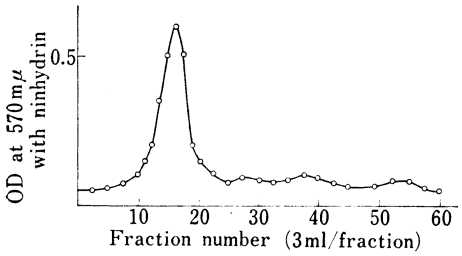

Fig. 3. Gel Filtration of the Synthesized Peptide on Sephadex G-25

The sample was dissolved in 2 ml of 0.1M acetic acid and placed on a 1.6×33 cm column of Sephadex G-25 equilibrated with 0.1M acetic acid, 0.1 M acetic acid was used as an eluent.

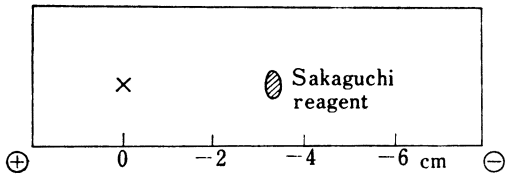

Fig. 4. Paper Electrophoretic Pattern of 19-Tyrosine Melittin (IV)

The electrophoresis was parformed using pH 2.15 buffer (glycine-HCl) on Toyo No. 50 paper at 150 V/38 cm long for 2 hr.

Modified Melittin (V)——Melittin was separated from honey bee venom by the method of Habermann and Jentsch.[3] Melittin (50 mg, 15 μmoles) was dissolved in 4 ml of methanol with stirring. Then, 23 mg of 2-hydroxy-5-nitrobenzyl bromide (100 μmoles) was added to the above solution. After 1 hr stirring at room temperature, the solution was evaporated to dryness with N_2 gas blowing. The residue was dissolved in 0.1 ml of water and applied on Sephadex G-10 column chromatography. After separation, the solution was lyophilized; yield, 51.1 mg. Amino acid ratios (6N HCl, 24 hr, 110°); $Thr_{2.2}Ser_{1.0}Glu_{2.0}Pro_{1.0}$-$Gly_{3.3}Ala_{2.4}Val_{2.0}Ile_{3.1}Leu_{3.9}Lys_{3.1}Arg_{2.4}$.

Hemolytic Test——A mixture of 1 ml of each sample in NaCl (0.85%)-Na_2HPO_4 (0.1M, pH 7.8) (4:1) and of 1 ml of 1% human erythrocyte (which was washed three times with the same buffer solution) suspension in the same buffer solution was incubated for 30 min at 38°. After 8 min 3000 rpm centrifugation, 0.5 ml of the supernatant was diluted with 4.5 ml of water and measured at 415 mμ absorption. Hemolytic activity was difined as a peptide concentration at a half OD value of complete hemolysis.[16] It was checked that hemolytic activity did not depend on an erythrocyte concentration, only depended on a peptide concentration and a period after collecting blood. Hemolytic activities of our compounds were measured on different days, so that I was used as a standard reference. I: I treated with HF and anisole = 1.63: 2.02 μg/ml, I: V = 2.7: 3.2 μg/ml, I: IV = 1.2: 8.4 μg/ml.

IR Spectra——IR spectra were measured with Hitachi EPI-G2 spectrometer in deuterium oxide. All of the compounds I, IV, and V showed amide I band between 1640 and 1645 cm^{-1}.

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16) E. Habermann and K.-G. Reiz, *Biochem. Z.*, **341**, 464 (1965).