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Studies on Peptides. XXVI.[1,2] Application of the Solid Phase Peptide Synthesis to the Screening Test of an Active Fragment proposed for Parathyroid Hormone

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A tentative structure of bovine parathyroid hormone, which regulates calcium and phosphate transport *in vivo*, was reported by Potts, *et al.*[4] in 1965. The proposed structure consists of a single polypeptide chain of 83—84 amino acid residues. It was postulated by the same authors that the C-terminal eicosapeptide (I), prepared by cleavage from parathyroid hormone with dilute acid, would be a biologically active fragment with approximately 30% or less of the potency of the native hormone. However, the definite conclusion will be reserved until the full covalent structure of this hormone is established, since the possibility that an active fragment locates near the N-terminal portion could not be excluded.[5]

H-Tyr-Lys-Glu-Leu-Val-Arg-Lys-Lys-Gly-Trp-His-Ile-Met-Glu-Ser-Phe-Ala-
Val-Leu-Glu(NH_2)-OH I

We undertook the solid phase peptide synthesis of this eicosapeptide by the Merrifield's procedure[6] to obtain a preliminary information about the proposed structure. Production of a pure peptide by this technique is based on the requirement that a quantitative coupling reaction on an insoluble copolymer should be stressed. We felt that even though the product produced by this operation might be quite impure, it may be possible to produce, among other impurities, a peptide which reflects the exact amino acid sequence of this fragment and the activity produced by such a fragment, if any, could be detected.

The C-terminal amino acid, *tert*-butoxycarbonylglutamine[7] was esterified onto chloromethylated copolymer of styrene with 2% divinylbenzene[8] by the standard procedure.[6] The protecting group was removed with 1N HCl in dioxane. The resulting hydrochloride was neutralized with triethylamine. The free base on the resin was allowed to couple with the forecomming amino acid, *tert*-butoxycarbonylleucine[9] by means of dicyclohexylcarbodi-

1) Part XXV: H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **17**, 1962 (1969).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used here are those recommended by IUPAC-IUB Commission on Biochemistry Nomenclature in July, 1965 and July, 1966; *Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967).
3) Location: *Sakyo-ku, Kyoto.*
4) J.T. Potts, Jr., G.D. Aurbach, L.M. Sherwood and A. Sandoval, *Proc. Natl. Acad. Sci. U.S.A.*, **54**, 1743 (1965); J.T. Potts, Jr., G.D. Aurbach and L.M. Sherwood, *Recent Prog. Hormone Res.*, **22**, 101 (1966); see also O.K. Behrens and E.L. Grinnan, *Ann. Rev. Biochem.*, **38**, 83 (1969).
5) J.T. Potts, Jr., H.T. Kentmann, H. Niall, L. Deftos, H.B. Brewer and G.D. Aurbach, *Proceeding of the Montreal Conference*, 1968.
6) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); *idem*, *Biochemistry*, **3**, 1385 (1964); *idem*, *J. Org. Chem.*, **29**, 3100 (1964); *idem*, *Endeavour*, **24**, 3 (1965); *idem*, *Sci. Amer.*, **218**, 56 (1968); *idem*, *Recent Prog. Hormone Res.*, **23**, 451 (1967); R.B. Merrifield and J.M. Stewart, *Nature*, **207**, 522 (1965); *idem*, *Science*, **150**, 178 (1965); R.B. Merrifield, J.M. Stewart and N. Jernberg, *Anal. Chem.*, **38**, 1905 (1966).
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8) Resin was purchased from Fundation for Protein Research, Osaka University.
9) F.C. McKay and N.F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957).

imide (DCC). Addition of the succeeding amino acids one at a time was performed by repeating such cycle until the eicosapeptide stage. Finally, the elongated peptide was cleaved from the resin by hydrogen fluoride.[10,11] This treatment removed also all protecting groups which were necessary to construct the eicosapeptide (I), *i.e.*, the *tert*-butoxycarbonyl group attached at the N-terminal tyrosine, the benzyloxycarbonyl group at the ε-amino function of lysine, the nitro group at the guanidino function of arginine and the benzyl ester at the γ-carboxyl group of glutamic acid.

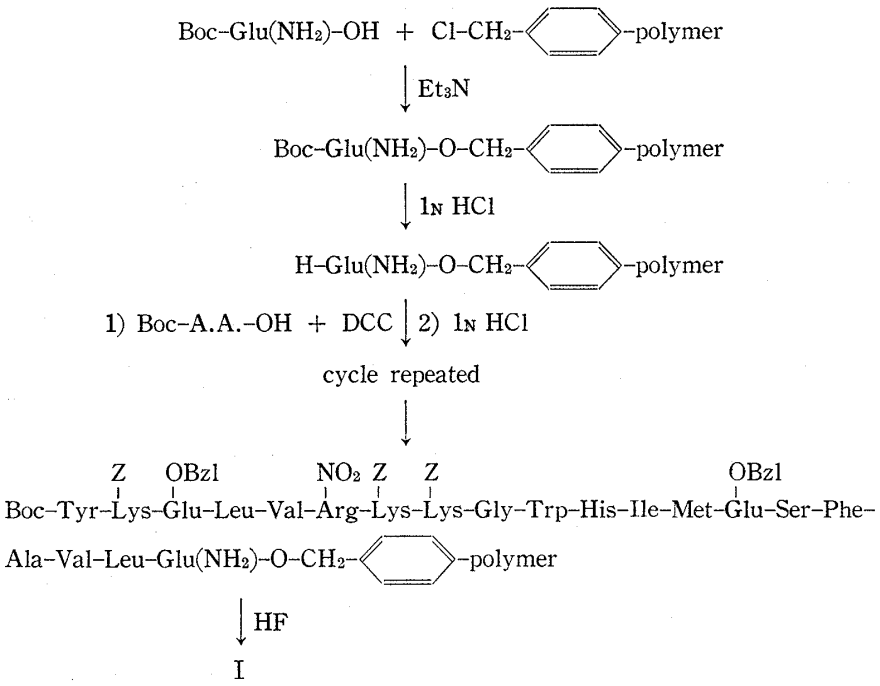

Chart 1. Operation Scheme

We like to make the following remarks with regards to this solid phase synthesis: (i) Leucine and valine were both incorporated very poorly in the standard procedure. The latter case was also pointed out by Gut and Rudinger.[12] Therefore, for the introduction of these two amino acids, the resin was treated twice with fresh *tert*-butoxycarbonyl derivatives plus DCC for 24 hr each. (ii) Fairly good introduction of serine was achieved by the active pentachlorophenyl ester[13] of *tert*-butoxycarbonylserine. The use of the active *p*-nitrophenyl ester for the solid phase peptide synthesis was reported by Bodanszky, *et al.*[14] and Hornle.[15] (iii) According to Gutte and Merrifield[16] and Maas and Steveninck,[17] histidine was introduced without protection of the imidazole nitrogen atom. However the result was quite unsatisfactory in our case. (iv) The entire operation, after introduction of tryptophan, was per-

10) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967); S. Sakakibara and T. Shimonishi, *ibid.*, **38**, 1412 (1965); S. Sakakibara, Y. Kishida, R.N. Nishizawa and Y. Shimonishi, *ibid.*, **41**, 438 (1968); S. Sakakibara, N. Nakamizo, Y. Kishida and S. Yoshimura, *ibid.*, **41**, 1437 (1968).
11) J. Leonard and A.B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).
12) V. Gut and J. Rudinger, "Peptide, 1968," North-Holland Pub. Co., Amsterdam, 1968, p. 185.
13) G. Kupryszewski and M. Formela, *Roczniki Chem.*, **35**, 1933 (1961); J. Kovacs, R. Gianotti and A. Kapoor, *J. Am. Chem. Soc.*, **85**, 2282 (1966); J. Kovacs, M.Q. Ceprini, C.A. Dupraz and G.N. Schmit, *J. Org. Chem.*, **32**, 3696 (1967).
14) M. Bodanszky and J.T. Sheehan, *Chem. Ind.*, **1964**, 1423; **1966**, 1597.
15) S. Hornle, *Z. Physiol. Chem.*, **348**, 1355 (1966).
16) B. Gutt and R.B. Merrifield, *J. Am. Chem. Soc.*, **91**, 501 (1969).
17) M. Maas and J. van Steveninck, *Experientia*, **23**, 407 (1967).

formed in the presence of mercaptoethanol to prevent the oxidative destruction of tryptophan in acidic condition. While we adopted this modification, Marshall[18] reported this effect and Blake and Li[19] applied this modification for the solid phase synthesis of the heptapeptide, H–Met–Glu–His–Phe–Arg–Trp–Gly–OH. However how far this effect can be valid in the solid phase synthesis of tryptophan containing peptides is not known. We cycled nine times the acidic cleavage of the *tert*-butoxycarbonyl group after introduction of the tryptophyl residue. In addition to the use of mercaptoethanol, care was taken to avoid exposing the resin to air by filling nitrogen in the reaction flask. During these operations, some brown color on the resin was detected. However the product liberated from the resin by hydrogen fluoride was yellowish. After leucine aminopeptidase[20] digestion of the partially purified product, it was found that the content of tryptophan was not less than that of histidine, which was incorporated onto the resin in the former cycle. It seems probable that some improvement of the synthesis of tryptophan containing peptides could be obtained by such modification.

The product cleaved from the resin by hydrogen fluoride was passed through a column of Sephadex G-10 for partial purification. The partially purified product produced a long tailing spot with other minor spots on thin-layer chromatography. Though amino acid analysis of an acid hydrolysate revealed the presence of every amino acid involved in this operation, as shown in Table I, their ratios were greately different from the theoretical values. This implies that each amino acid reacted to different degrees. Amino acid contents near the N-terminal portion of the eicosapeptide decreased progressively. It has been stated by Green, *et al.*,[21] Marshall[22] and Wildi, *et al.*[23] that the efficiency of addition to an insoluble peptide polymer decreases after few amino acids. The similar tendency of the solid phase synthesis was also clearly demonstrated by Jolles, *et al.*,[24] that is the efficiency decreased to around 50% or less as we have met in this operation. These results seem to rather provide information which will help to improve the solid method of peptide synthesis and in addition, offer some information in regard to the reproducibility of this procedure.

The crude product and the partially purified material, which may contain the eicosapeptide, were submitted for bioassay according to Munson.[25] The values obtained were both less than one-seventyfifth of that of the natural hormone (Lilly preparation 2500 U/mg) in parathyroidectomized rats. Both samples were judged as biologically inert within a limit of experimental tolerance.

From these results, it may be possible to assume that an active fragment of parathyroid hormone would be still bigger than that proposed initially or locates near the N-terminal portion of this molecule as suggested recently.[5] We wish to mention here that the application of the solid phase peptide synthesis, a rapid and simple procedure, appears to be a useful method of choice to obtain such a preliminary information, besides a tool to prepare pure peptides by stressing every reaction involved.

Experimental

Amino acid analysis was performed on Hitachi Liquid Chromatography, Model 034.

Esterification of Glutamine onto the Resin——Chloromethylated copolymer of 98% styrene with 2%

18) G.R. Marshall, *Advan. Exp. Med. Biol.*, **2**, 48 (1969).
19) J. Blake and C.H. Li, *J. Am. Chem. Soc.*, **90**, 5882 (1968).
20) Partially purified LAP was prepared according to D.H. Spackmann, E.L. Smith and D.M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).
21) B. Green and L.R. Garson, *J. Chem. Soc.* (*C*), **1968**, 401.
22) G.R. Marshall, Abstracts of ACS Meeting, A-4, San Francisco, April 1968.
23) B.S. Wildi and J.H. Johnson, Abstracts of ACS Meeting, A-8, San Francisco, April 1968.
24) P. Jolles and J. Jolles, *Helv. Chim. Acta*, **51**, 980 (1968).
25) P.L. Munson, *Ann. New York Acad. Sci.*, **60**, 776 (1955); "*The Parathyroids*," ed. by R.O. Greep and R.V. Talmage, Charles C. Thomas Springfield, III, 1961, p. 473.

divinylbenzene (6.0 g, chlorine content 1.6 mM/g),[8] *tert*-butoxycarbonylglutamine (4.72 g, 19.2 mM) and triethylamine (2.7 ml) in a mixture of EtOH and chloroform (2:1 v/v, 40 ml) were heated with gentle stirring at 65° for 72 hr. For analysis a part of the resin was removed and washed with EtOH, H_2O and MeOH. The acid hydrolysis of the dried resin gave glutamic acid in 0.25 mM/g (yield 14%).

Amino Acid Derivatives——*tert*-Butoxycarbonyl derivatives of the following amino acid were prepared:[23,24] glutamine, leucine, valine, phenylalanine, serine, γ-benzylglutamate, methionine, isoleucine, histidine, tryptophan, glycine, N^{ε}-benzyloxycarbonyllysine, N^{G}-nitro-arginine, tyrosine. *tert*-Butoxycarbonylserine was converted to the corresponding pentachlorophenyl ester according to Kovacs, *et al.*[13] mp 158—160°. *Anal.* Calcd. for $C_{14}H_{14}O_5NCl_5$: C, 37.1; H, 3.1; N, 3.1. Found: C, 37.2; H, 3.3; N, 3.2.

Operation——The following cycle was used for condensation of amino acids onto the resin one at a time. 1) Cleavage of the *tert*-butoxycarbonyl group with 1N HCl in dioxane (30 ml for 30 min). 2) Washing with dioxan (30 ml × 3 times). 3) Washing with methylene chloride (30 ml × 3). 4) Neutralization of the hydrochloride with a 10% solution of triethylamine in methylene chloride (30 ml for 10 min). 5) Washing with methylene chloride (30 ml × 3). 6) Addition of the succeeding *tert*-butoxycarbonylamino acid (2 equi-moles) in methylene chloride (25 ml). 7) Addition of DCC (2 equi-moles). Shaking was continued under ice-cooling for one hr and then at room temperature for 24 hr. 8) Washing with methylene chloride (30 ml × 3). 9) Washing with acetic acid to remove dicyclohexylurea (30 ml × 3). 10) Washing with dioxan (30 ml × 3).

For introduction of leucine and valine, the procedures 6, 7 and 8 were repeated twice (with 16.4 mM and 6.2 mM). Dimethylformamide, instead of methylene chloride, was used for *tert*-butoxycarbonyl-N^{G}-nitroarginine and *tert*-butoxycarbonylhistidine in the procedure 6. When *tert*-butoxycarbonylserine pentachlorophenyl ester was employed, the procedure 7 was eliminated. After introduction of tryptophan, solutions containing 2% mercaptoethanol were employed in 1, 2, 3, 8, 9 and 10.

Cleavage of the Elongated Peptide from Its Polymer Support——Starting from 6 g of the chloromethylated resin, the final peptide resin 10.76 g was obtained. The dried peptide resin (4.5 g) was treated with anhydrous hydrogen fluoride (40 ml) in the presence of anisole (8 ml) under ice-cooling for 1 hr. After evaporation of hydrogen fluoride *in vacuo*, the residue was placed over KOH pellets in an evacuated desiccatior

Table I. Amino Acid Ratios in Acid Hydrolysates

	I Hepta-peptide-resin	II Deca-peptide-resin	III Pentadeca peptide-resin	IV Eicosa-peptide-resin	V HF treated	VI Partially purified by Sephadex G-10	Theory
Gln	1.98	2.61	2.88	3.49	3.24	2.99	3
Leu	1.70	1.78	1.71	2.28	2.29	2.19	2
Val	1.23	1.29	1.24	1.66	1.75	1.60	2
Ala	1.00	1.00	1.00	1.00	1.00	1.00	1
Phe	1.01	1.26	1.32	1.28	1.07	1.20	1
Ser	0.83	1.01	0.98	1.00	1.00	1.16	1
Glu		—	—	—	—	—	—
Met		0.85	0.65	0.75	0.55	0.62	1
Ile		0.48	0.48	0.38	0.38	0.39	1
His		0.35	0.35	0.25	0.35	0.42	1
Trp			—	—	—	0.42[a]	1
Gly			0.45	0.48	0.53	0.47	1
Lys			1.59	1.60	2.02	1.58	3
Lys			—	—	—	—	—
Arg			0.46	0.47	0.58	0.49	1
Val							—
Leu							—
Glu							—
Lys							—
Tyr				0.47	0.47	0.38	1

a) Trp in a LAP digest was measured by a basic column using benzylalcohol (2%) in the buffer system. Its mean value was expressed as relative reocvery ratios to those of His, Lys and Arg.

23) E. Schnabel, *Ann. Chem.*, **702**, 188 (1967).
24) F.C. McKay and N.F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957).