Tokyo College of Pharmacy
Kashiwagi, Shinjuku-ku, Tokyo

SABURO ISHIWATA
KEIICHI ITAKURA

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Studies on the Synthesis of Proinsulin. I. Synthesis of Partially Protected Tritriacontapeptide related to the Connecting Peptide Fragment of Porcine Proinsulin[1]

Recently, Chance and coworkers[2] have proposed an amino acid sequence of the connecting peptide fragment of porcine proinsulin. This polypeptide chain connects the carboxy-terminus of the B chain to the amino-terminus of the A chain of insulin molecule.

B chain ⟶ Arg-Arg-Glu-Ala-Gln-Asn-Pro-Gln-Ala-Gly-Ala-Val-Glu-Leu-Gly-Gly-Gly-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Leu-Gly-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-Lys-Arg ⟶ A chain
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

Amino Acid Sequence of Connecting Peptide Portion of Porcine Proinsulin

The present communication describes the synthesis of partially protected tritriacontapeptide related to the connecting peptide fragment of porcine proinsulin.

Thus, we subdivided the connecting peptide chain into four fragments of proper size and synthesized the protected peptides, Z-Arg(NO_2)-Arg(H^+)-Glu-Ala-Gln-Asn-Pro-Gln-Ala-Gly-NHNH-BOC (I), Z-Ala-Val-Glu(OBu^t)-Leu-Gly-Gly-Gly-Leu-Gly-NHNH-BOC (II), Z-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu(OBu^t)-Gly-NHNH-BOC (III) and Z-Pro-Pro-Gln-Lys(F)-Arg-(H^+)OH (IV), as intermediates for the construction of the entire sequence. This approach is similar to that on the synthesis of fragments of ribonuclease T_1.[3]

The absence of sulfur-containing amino acid residues in the connecting peptide led us to use Z group as α-amino protecting group of the intermediates, which was removed readily by catalytic hydrogenolysis. Preparation of these peptide fragments was conducted mostly by stepwise elongation starting from the C-terminus with the desired Z-amino acid N-hydroxysuccinimide[4] or 2,4,5-trichlorophenyl[5] esters. To avoid the complexity of increasing coupling stages, some intermediates of these peptides were prepared by fragment condensation using Z-tetra-, Z-tri- or Z-dipeptide azides and in certain instances by Anderson's mixed anhydride procedure[6] with Z-dipeptides terminating with glycine. The ε-amino group of lysine was pro-

1) The amino acids except glycine are L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochemistry*, **5**, 2485 (1966). The following abbreviations are used: F=formyl; NO_2=nitro; TFA=trifluoroacetic acid; DMF=dimethylformamide; AP-M=aminopeptidase M. Solvent systems used for thin-layer chromatography are as follows: Rf^{I}, Partridge system (S.M. Partridge, *Biochem. J.*, 42, 238 (1948)); Rf^{II}, 1-butanol-pyridine-acetic acid-water (30:20:6:24). Rf^{III} value refers to the system of 1-butanol-pyridine-acetic acid-water (30:20:6:24) on paper chromatography.

2) R.E. Chance, R.M. Ellis and W.W. Bromer, *Science*, **161**, 165 (1968).
3) N. Yanaihara, C. Yanaihara, G. Dupuis, J. Beacham, R. Camble and K. Hofmann, *J. Am. Chem. Soc.*, **91**, 2184 (1969).
4) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
5) J. Pless and R.A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963).
6) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **88**, 1338 (1966).

tected by formyl function, since the recent reports[7] describe smooth removal of this group with hydrazine derivatives. For protection of glutamic acid side chain we used *tert*-butyl ester,[8] which was cleaved readily from peptides with TFA prior to fragment condensation.

The intermediates in the synthesis of these peptide fragments were proved to be homogeneous by thin-layer chromatography, amino acid analyses of the acid hydrolysates and enzymatic digests and elemental analysis.

Data for the characterization of the four peptide fragments (I, II, III and IV) are summarized in Table I and II. AP-M digestion[9] was carried out on the hydrogenated compounds of I, II and III which were treated with TFA.

Table I. Physical Properties and Elemental Analyses of Subunits

Compound	$[\alpha]_D^{20}$ a)	Rf^{I}	Rf^{II}	mp (°C)	Formula	Analysis (%) Calcd. C	H	N	Found C	H	N
I.	−20.7° (c=1.09)	0.35	0.65	—	$C_{59}H_{94}O_{23}N_{22}\cdot 2H_2O$ b)	46.8	6.5	20.3	46.5	6.8	20.5
II.	−16.6° (c=2.15)	0.90	0.90	242—243 (decomp.)	$C_{50}H_{81}O_{15}N_{11}$	55.8	7.6	14.3	55.5	7.7	14.2
III.	−24.0° (c=2.10)	0.88	0.89	250—251 (decomp.)	$C_{55}H_{90}O_{16}N_{12}$	56.2	7.7	14.3	55.8	7.8	13.9
IV.	−31.2° (c=1.04)	0.36	0.64	—	$C_{38}H_{58}O_{12}N_{10}\cdot 2H_2O$ b)	51.7	7.1	15.9	51.8	6.9	16.3

a) DMF was used as solvent. b) Monoacetate.

Table II. Amino Acid Analyses of Subunits

Compound	Amino acid ratios: In Acid hydrolysate a) (6N HCl, 110°, 24 hr)							In AP-M digest of deblocked product						
I.	Arg+Orn b) 1.81		Glu 2.99	Ala 1.99	Asp 1.02	Pro 0.95	Gly 1.06	Arg 1.78	Glu 0.96	Ala 2.15	Gln 1.54	Gly 1.11	Asp c) 0.00	Pro c) 0.00
II.	Ala 1.01	Val 1.01	Glu 0.91	Leu 2.00	Gly 4.08			Ala 0.99	Val 1.03	Glu 0.97	Leu 1.97	Gly 3.99		
III.	Gly 2.04	Leu 3.01	Glu 1.91	Ala 2.04				Gly 1.92	Leu 3.09	Gln 1.00	Glu 1.03	Ala 1.99		
IV.	Pro 2.15	Glu 1.02	Lys 0.89	Arg 0.94	NH_3 1.02									

a) In the cases of I, II, and III, ammonia was not estimated because of the presence of BOC-hydrazide function in the molecules.
b) I contains one nitroarginine residue which was partially converted to ornithine.
c) Similar result was obtained in the case of histidylprolyl linkage.[10]

Chart 1 indicates outline of the method for construction of tritriacontapeptide.

The starting pentapeptide, H-Pro-Pro-Gln-Lys(F)-Arg(H^+)-OH (V) $[[\alpha]_D^{20}$ −108.0° (c=0.50, 10% AcOH); amino acid ratios in acid hydrolysate Pro 1.97, Glu 0.95, Arg 1.07, Lys

7) H. Yajima, Y. Okada, Y. Kinomura and H. Minami, *J. Am. Chem. Soc.*, **90**, 527 (1968); H. Yajima, K. Kawasaki, Y. Okada, H. Minami, K. Kubo and I. Yamashita, *Chem. Pharm. Bull.* (Tokyo), **16**, 919 (1968); R. Geiger and W. Siedel, *Chem. Ber.*, **101**, 3386 (1968).
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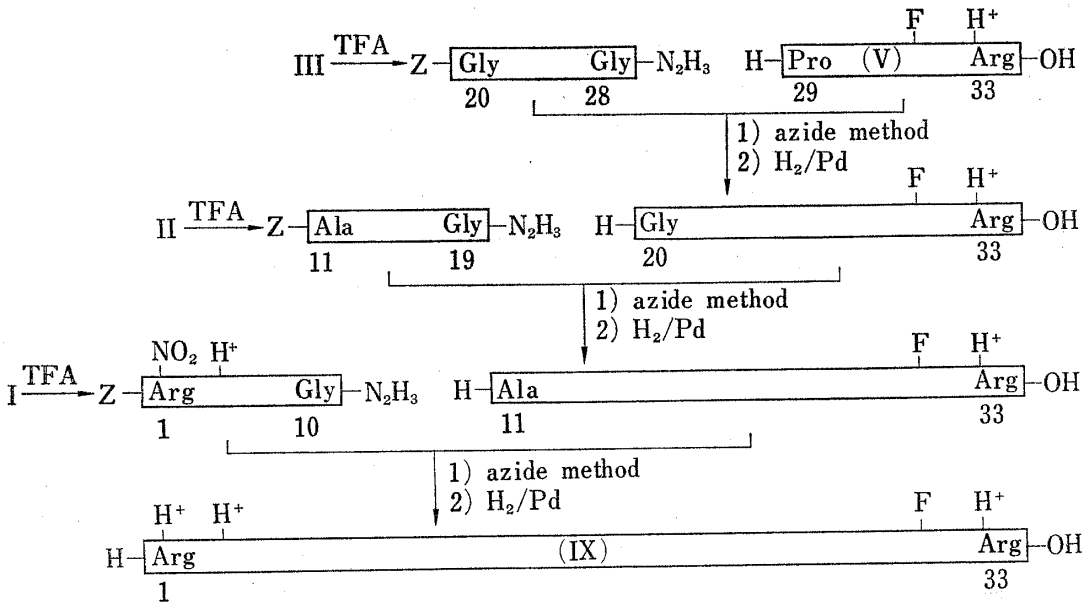

Chart 1

1.02, NH_3 0.92; Rf^{I} 0.07, Rf^{II} 0.29, Rf^{III} 0.28] was prepared by hydrogenolysis of IV. Removal of the BOC or OBu^t protecting group of I, II and III was carried out with anhydrous TFA at room temperature. The ensuing hydrazides were converted to the corresponding azides with isoamylnitrite in DMF containing three equimoles of 6N HCl in dioxane at −10——15°. Condensation of the azides with the desired amino components was performed at 0—5° for 24—48 hr.

Table III. Physical Properties and Elemental Analyses

Compound	$[\alpha]_D^{20}$ a)	Rf^{I}	Rf^{II}	Formula	Analysis (%) Calcd. C	H	N	Found C	H	N
VI.	−85.1° (c=0.90)	0.35	0.62	$C_{76}H_{122}O_{24}N_{20}\cdot 3H_2O$ b)	52.0	7.4	16.0	52.4	7.4	15.6
VII.	−75.5° (c=0.50)	0.37	0.62	$C_{109}H_{177}O_{35}N_{29}\cdot 4H_2O$ b)	51.8	7.4	16.1	52.0	7.6	15.6
VIII.	−84.9° (c=0.50)	0.32	0.55	$C_{155}H_{253}O_{54}N_{49}\cdot 10H_2O$ c)	48.4	7.2	17.8	48.6	7.4	17.4

a) Solvent used was 50% acetic acid. b) Monoacetate. c) Diacetate.

Table IV. Amino Acid Analyses

Compound	Amino acid ratios in acid hydrolysate (6N HCl, 110°, 48 hr)									
VI.	Gly	Leu	Glu	Ala	Pro	Lys	Arg	NH_3		
	2.08	3.12	2.81	2.04	2.08	0.96	0.89	2.06		
VII.	Ala	Val	Glu	Leu	Gly	Pro	Lys	Arg	NH_3	
	3.05	1.00	3.88	5.06	6.11	1.88	1.00	0.95	2.02	
VIII.	Arg	Glu	Ala	Asp	Pro	Gly	Val	Leu	Lys a)	NH_3
	2.68	6.52	4.94	0.94	2.95	7.30	1.14	5.23	1.22	5.49

a) This high value may be due to the formation of ornithine by partial decomposition of nitroarginine in acid hydrolysis.

The procedure with Dowex 50 developed by Hofmann *et al.*[11] was quite effective for the purification of Z-tetradecapeptide, Z-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-Lys(F)-Arg(H^+)-OH (VI) and Z-tricosapeptide, Z-Ala-Val-Glu-Leu-Gly-Gly-Gly-Leu-Gly-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-Lys(F)-Arg(H^+)-OH (VII), which were obtained in 60 and 63% yields, respectively.

The final azide coupling of Z-decapeptide hydrazide derived from I with partially deblocked tricosapeptide [$[\alpha]_D^{20}$ −88.9° (c=0.45, 50% AcOH); Rf^{I} 0.32, Rf^{II} 0.60, Rf^{III} 0.70] obtained by hydrogenolysis of VII produced Z-tritriacontapeptide, Z-Arg(NO_2)-Arg(H^+)-Glu-Ala-Gln-Asn-Pro-Gln-Ala-Gly-Ala-Val-Glu-Leu-Gly-Gly-Gly-Leu-Gly-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-Lys(F)-Arg(H^+)-OH (VIII) in 53% yield, which was purified with gel filtration on Sephadex G-25 and then column chromatography on CM–cellulose. Analytical data and physical properties of VI, VII and VIII are shown in Table III and IV.

The protected tritriacontapeptide (VIII) was hydrogenated over Pd in 80% acetic acid for 48 hr to remove the Z and NO_2 functions. Purification of the resulting product was carried out by gel filtration on Sephadex G-25 and column chromatography on CM–cellulose using ammonium acetate buffer as eluate. The UV absorption spectrum of this product indicated complete removal of NO_2 group. The data in Table V show that the resulting partially protected tritriacontapeptide, H-Arg(H^+)-Arg(H^+)-Glu-Ala-Gln-Asn-Pro-Gln-Ala-Gly-Ala-Val-Glu-Leu-Gly-Gly-Gly-Leu-Gly-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-Lys(F)-Arg(H^+)-OH (IX) is homogeneous.

Table V. Partially Protected Tritriacontapeptide (IX)

$[\alpha]_D^{20}$ −79.4° (c=0.32, 50% AcOH), Rf^{I} 0.21, Rf^{II} 0.53, Rf^{III} 0.56.

Single component on paper electrophoresis (200 V, 3 hr) at pH 3.6 (Mobility =His×0.76) and pH 6.7 (Mobility=His×0.93).

Amino acid ratios in acid hydrolysate (6N HCl, 110°, 48 hr)

Arg	Glu	Ala	Asp	Pro	Gly	Val	Leu	Lys	NH_3
3.03	6.75	5.09	1.00	3.01	7.14	1.00	5.09	1.03	5.07

Treatment of IX with carboxypeptidase B liberated one equimole of arginine but little amount of lysine. Immunochemical and biological properties of this compound are now under investigation.

Geiger, *et al.*[12] have reported very recently the synthesis by different route of tritriacontapeptide corresponding to the connecting peptide fragment, but no detailed data were given for some intermediates obtained by fragment condensation and the final product.

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Shizuoka College of Pharmacy
160 Oshika, Shizuoka

Noboru Yanaihara
Tadashi Hashimoto
Chizuko Yanaihara
Naoki Sakura

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