ful method for structural study of biogenetic polysaccharides which are available only in a minute quantity. It may be possible to use this procedure for the determination of oligosaccharides containing a mixture of pentoses, hexosamines, and hexoses.

Acknowledgement We are grateful to Prof. A. Tatematu for making the GC-MS available to use and for his encouragement, and to H. Sakurai for valuable discussion.

Faculty of Pharmacy,
Meijo University,
Yagoto, Tenpaku-ku, Nagoya

AKIRA KAMEI
HIDEO YOSHIZUMI
SHUZO AKASHI
KOSHIRO KAGABE

Received November 29, 1975

[Chem. Pharm. Bull. 24(5)1110—1113(1976)]

UDC 547.466.1.04 : 546.15-17.04

Synthesis of [27-Tyr]-Cholecystokinin-Pancreozymin (CCK-PZ)[1]

In a conventional manner, the tritriacontapeptide amide corresponding to the entire amino acid sequence of the desulfated form of porcine cholecystokinin-pancreozymin (CCKPZ) was synthesized by applying protecting groups removable by hydrogen fluoride. Synthetic peptide, though it is not too biological active, was smoothly and efficiently converted to the labeled compound with ^{125}I (specific activity 200—250 μci/μg).

In 1971, Mutt and Jorpes[2] elucidated the complete amino acid sequence of cholecystokinin-pancreozymin (CCK-PZ). The C-terminal dodecapeptide amide[3] and the N-terminal octapeptide[4] were synthesized for the structural confirmation. Radioimmunoassay of CCK-PZ has been encountered the difficulty because of the unavailability of pure CCK-PZ for labeling with radioactive iodine.

We wish to report the synthesis of tritriacontapeptide amide corresponding to the entire amino acid sequence of desulfated CCK-PZ, termed as [27-Tyr]-CCK-PZ (I), which seems to be the most ideal tracer for radioimmunoassay of this important upper intestinal hormone. As strategies for the synthesis of such a peptide containing Met, Lys and Trp residues, amino acid derivatives bearing protecting groups removable by hydrogen fluoride[5] were employed, *i.e.*, Lys(Z), Arg(Tos), and Asp(OBzl). The α-amino function of intermediates were protected by the Z(OMe) group[6] which is known to be cleaved by treatment with trifluoroacetic acid (TFA) without affecting side chain protecting groups employed. Anisole containing 2% ethanedithiol[7] rather than mercaptoethanol was employed to minimize destruction of the Trp residue

1) Peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, OBzl=benzyl ester, ONP=p-nitrophenyl ester, ODNP=2,4-dinitrophenyl ester, OPCP=pentachlorophenyl ester, PCP–O–TCA=pentachlorophenyl trichloroacetate, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide.
2) V. Mutt and J.E. Jorpes, *Eur. J. Biochem.*, **6**, 156 (1968); *idem*, *Biochem. J.*, **125**, 57p (1971).
3) M.A. Ondetti, J. Pluscec, E.F. Sabo, J.T. Sheehan, and N. Williams, *J. Am. Chem. Soc.*, **92**, 195 (1970).
4) M. Bodanszky, N. Chaturvedi, D. Hudson, and M. Itoh, *J. Org. Chem.*, **37**, 2303 (1972).
5) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
6) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962); H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970).
7) J.J. Sharp, A.B. Robinson, and M.D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).

during the various TFA deblocking steps as demonstrated successfully in the synthesis of gastric inhibitory polypeptide.[8]

In order to construct the entire amino acid sequence of (I) (Fig.), the C-terminal heptadecapeptide amide (II) served as an amino component, with which three protected hydrazides, (III), (IV), and (V), were successively condensed by the azide procedure[9] to minimize racemization. For the synthesis of (II), Z(OMe)-Trp-Met-Asp(OBzl)-Phe-NH_2 (mp 188—190°, $[\alpha]_D^{24}$ −20.3° in DMF. *Anal.* Calcd. for $C_{45}H_{50}ON_{69}S$: C, 63.51; H, 5.92; N, 9.88. Found: C, 63.42; H, 5.78; N, 9.87.) was prepared stepwisely by the active ester procedure[10] starting with H-Phe-

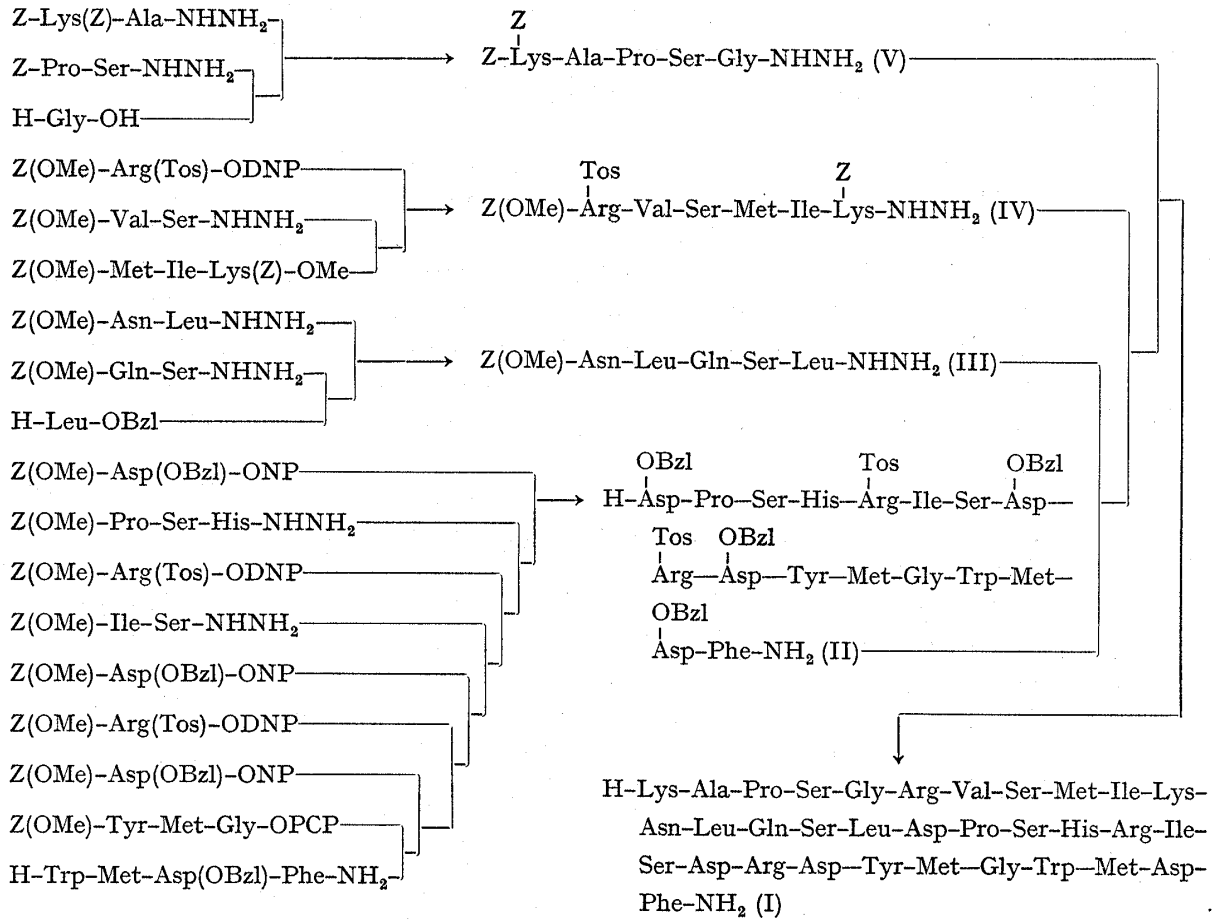

Fig. 1. Synthetic Route to [27-Tyr]-Cholecystokinin-Pancreozymin

NH_2. This, after removal of the α-protecting group, was condensed with Z(OMe)-Tyr-Met-Gly-OH (mp 108—110°, $[\alpha]_D^{24}$ −2.9° in MeOH. *Anal.* Calcd. for $C_{25}H_{31}O_8N_3S \cdot 1/2H_2O$: C, 55.34; H, 5.94; N, 7.75. Found: C, 55.49; H, 5.82; N, 7.59.) by the PCP-O-TCA procedure.[11] Next, chain elongation of the resulting heptapeptide amide to (II) had to be performed mostly in the stepwise manner, because of the presence of three Asp(OBzl) residue (position 17, 24, and 26). They were introduced by the *p*-nitrophenyl ester procedure[10] and two residues of Arg(Tos) by the 2,4-dinitrophenyl ester procedure.[12] Z(OMe)-Ile-Ser-$NHNH_2$ (mp 234—236°, $[\alpha]_D^{24}$ +8.6° in dimethyl formamide (DMF). *Anal.* Calcd. for $C_{18}H_{28}O_6N_4S$: C, 54.53; H, 7.12;

8) H. Yajima, H. Ogawa, M. Kubota, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, H. Adachi, H. Imura, and T. Taminato, *J. Am. Chem. Soc.*, **97**, 5593 (1975).
9) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
10) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
11) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
12) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.

N, 14.13. Found: C, 54.31; H, 6.89; N, 13.85.) and Z(OMe)-Pro-Ser-His-$NHNH_2$ (mp 180—185°, $[\alpha]_D^{24}$ −6.8° in DMF. *Anal.* Calcd. for $C_{23}H_{31}O_7N_7 \cdot H_2O$: C, 51.58; H, 6.21; N, 18.31. Found: C, 51.23; H, 6.04; N, 18.08.) were introduced by the azide procedure. These relatively small acylating agents used excess could be readily removed by washing or precipitation after each coupling step. The analytically and chromatographically pure sample of (II) (mp 213—215°, $[\alpha]_D^{23}$ −12.8° in DMF. *Anal.* Calcd. for $C_{143}H_{175}O_{35}N_{27}S_4 \cdot 6H_2O$: C, 55.97; H, 6.14; N, 12.32. Found: C, 55.91; H, 6.21; N, 12.14) was obtained without particular difficulty.

The protected pentapeptide hydrazide, Z(OMe)-Asn-Leu-Gln-Ser-Leu-$NHNH_2$ (mp 262° dec., $[\alpha]_D^{24}$ +19.0° in dimethyl sulfoxide (DMSO). *Anal.* Calcd. for $C_{33}H_{53}O_{11}N_9 \cdot H_2O$: C, 51.48; H, 7.20; N, 16.37. Found: C, 51.68; H, 6.93; N, 16.12.) was synthesized by two successive azide condensation of Z(OMe)-Gln-Ser-$NHNH_2$ and Z(OMe)-Asn-Leu-$NHNH_2$ with H-Leu-OBzl followed by exposure the resulting protected pentapeptide ester to hydrazine hydrate in the usual manner.

In order to synthesize the protected hexapeptide hydrazide, Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-$NHNH_2$ (IV) (mp 242—244°, $[\alpha]_D^{24}$ +1.0° in DMSO. *Anal.* Calcd. for $C_{55}H_{82}O_{14}N_{12}S_2$: C, 55.07; H, 6.89; N, 14.01. Found: C, 54.78; H, 6.84; N, 14.04.), Z(OMe)-Met-OH was condensed with H-Ile-Lys(Z)-OMe by the mixed anhydride procedure[13] and the resulting protected tripeptide ester, after treatment with TFA, was condensed with Z(OMe)-Val-Ser-$NHNH_2$ by the azide procedure to form the protected pentapeptide ester. Next, Z(OMe)-Arg(Tos)-OH was condensed by the 2,4-dinitrophenyl ester procedure as performed earlier and the resulting protected hexapeptide ester was exposed to hydrazine hydrate as usual to form (IV).

The N-terminal pentapeptide hydrazide, Z-Lys-(Z)-Ala-Pro-Ser-Gly-$NHNH_2$ (mp 95—100°, $[\alpha]_D^{24}$ −13.3° in DMSO. *Anal.* Calcd. for $C_{35}H_{48}O_{10}N_8$: C, 54.11; H, 6.75; N, 14.42. Found: C, 54.40; H, 6.70; N, 14.17.) was prepared by two successive azide condensations of Z-Pro-Ser-$NHNH_2$ and Z-Lys(Z)-Ala-$NHNH_2$ with the triethylammonium salt of Gly. The resulting protected pentapeptide was converted to (V) by treatment with diazomethane followed by exposure to hydrazine hydrate.

Four peptide fragments were then condensed by the azide procedure. Each product was purified by column chromatography on Sephadex LH-20 using DMSO as eluent followed by repeated precipitation from DMSO with ethyl acetate. Purity of every intermediate as well as the final protected tritriacontapeptide amide, Z-Lys(Z)-Ala-Pro-Ser-Gly-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-Asn-Leu-Gln-Ser-Leu-Asp(OBzl)-Pro-Ser-His-Arg(Tos)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2 (mp 220—223°, $[\alpha]_D^{23}$ +5.1° in DMSO. *Rf* 0.65 in $CHCl_3$–MeOH–H_2O=8: 3: 1. Amino acid ratios in an acid hydrolysate: Lys 2.29, Ala 1.16, Pro 2.27, Ser 4.79, Gly 2.00, Arg 3.03, Val 1.44, Met 2.56, Ile 2.21, Asp 4.83, Leu 1.77, Glu 1.04, His 0.73, Tyr 0.66, Phe 0.94 (average recovery 85%). *Anal.* Calcd. for $C_{232}H_{316}O_{61}N_{50}S_6 \cdot 14H_2O$: C, 53.32; H, 6.64; N, 13.40. Found: C, 53.67; H, 6.59; N, 13.06.), was confirmed by three criteria; thin-layer chromatography, elemental analysis and acid hydrolysis.

The above protected tritriacontapeptide amide was exposed to hydrogen fluoride at 0° for 60 minutes to remove all protecting groups. Anisole containing 2% ethanedithiol and skatol served as scavengers to avoid alkylation.[14] The resulting deblocked peptide was immediately converted to the corresponding acetate with Amberlite IR-4B (acetate form) and purified by column chromatography on Sephadex G-25 and CM-cellulose. To elute the desired compound, 0.2 M acetic acid was used in the former step and gradient elution with 0.1 M ammonium bicarbonate buffer (pH 7.8) in the latter step. Absorbency at 280 mμ due to Trp served to monitor the chromatographic purification. The synthetic tritriacontapeptide amide thus

13) T. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951).
14) S. Sano and S. Kawanishi, *J. Am. Chem. Soc.*, **97**, 3480 (1975), R.S. Feinberg and R.B. Merrifield, *ibid.*, **97**, 3485 (1975).

purified exhibited a single spot on thin layer chromatography. Its purity was further assessed by amino acid analysis in 3N Tos-OH hydrolysate and aminopeptidase (AP-M)[15] digest (*Rf* 0.44 in *n*-BuOH–AcOH–pyridine–H_2O=4: 1: 1: 2, $[\alpha]_D^{24}$—35.8° in 3% AcOH. Amino acid ratios in 3N Tos-OH hydrolysates and AP-M digests (number in parentheses): Lys 2.10 (1.80), Ala 0.92 (1.15), Pro 2.04 (2.25), Ser 4.50, Gly 2.00 (2.00), Arg 2.70 (2.77), Val 1.28 (1.14), Met 2.54 (2.73), Ile 1.77 (2.39), Leu 1.95 (1.95), Glu 1.00, Asp 5.18 (3.59), His 0.69 (0.85), Tyr 0.70 (0.78), Trp 0.77 (0.65), Phe 0.69 (0.73), Ser+Asn (5.82 calcd. as Ser), Gln (1.19), average recovery 85% and 81% respectively. *Anal.* Calcd. for $C_{166}H_{262}O_{49}N_{50}S_3 \cdot 6CH_3COOH \cdot 15H_2O$: C, 47.84; H, 7.13; N, 15.67. Found: C, 48.06; H, 7.05; N, 15.37.

When secretory response of amylase was examined in isolated and perfused rat pancreas,[16] the relative potency of our synthetic peptide was estimated to be about 1/250 of natural CCK-PZ (GIH Research Unit, 3400 Ivy U/mg, Karolinska Institute, Stokholm, Sweden). Its gall-bladder contractile potency in dogs was also low as seen in its pancreozymin activity. As mentioned by Ondetti, *et al.*[3,17] relative potency of unsulfated C-terminal hepta or octapeptide amide of CCK-PZ was about 1/300 of their Tyr(SO_3H)-derivatives. It seems therefore that the sulfate moiety at the Tyr residue at position 27 contributes immensely for the peptide with full chain length of CCK-PZ to exert a high degree of biological activity.

Synthetic peptide, though it is not too biologically active, was easily and efficiently converted by the method of Hunter and Greenwood[18] to the ^{125}I labeled compound with a relatively high specific activity (200—250 μci/μg, lit.[19] iodonated natural CCK-PZ 30—100 μci/μg). This compound may very well be a useful synthetic compound for the development of radioimmunoassay of CCK-PZ.

Acknowledgement The authors express their sincere appreciation to the grant of Ministry of Education, Science and Culture (grant No 947072) and the support of the Mitsubishi Foundation.

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto
Haruaki Yajima
Yoshiro Mori
Yoshiaki Kiso
Kaname Koyama

School of Medicine, Kyoto University Sakyo-ku, Kyoto
Takayoshi Tobe
Motoichi Setoyama
Hideki Adachi

Veterinary Medicine, Hokkaido University Sapporo
Tomio Kanno
Atsushi Saito

Received January 5, 1976

15) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963).
16) T. Kanno, *J. Physiol.*, **245**, 599 (1975).
17) M.A. Ondetti, B. Rubin, S.L. Engel, J. Plusces, and J.T. Sheehan, *Am. J. Digest. Diseases*, **15**, 149 (1970).
18) W.M. Hunter and F.C. Greenwood, *Nature*, **194**, 495 (1962).
19) R.F. Harvey, L. Dowsett, M. Hartog, and A.E. Read, *Gut.*, **15**, 690 (1974).