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Studies on Peptides. LXVIII.[1,2] Synthesis of the Tritriacontapeptide Amide corresponding to the Entire Amino Acid Sequence of the Desulfated Form of Porcine Cholecystokinin-Pancreozymin (CCK-PZ)

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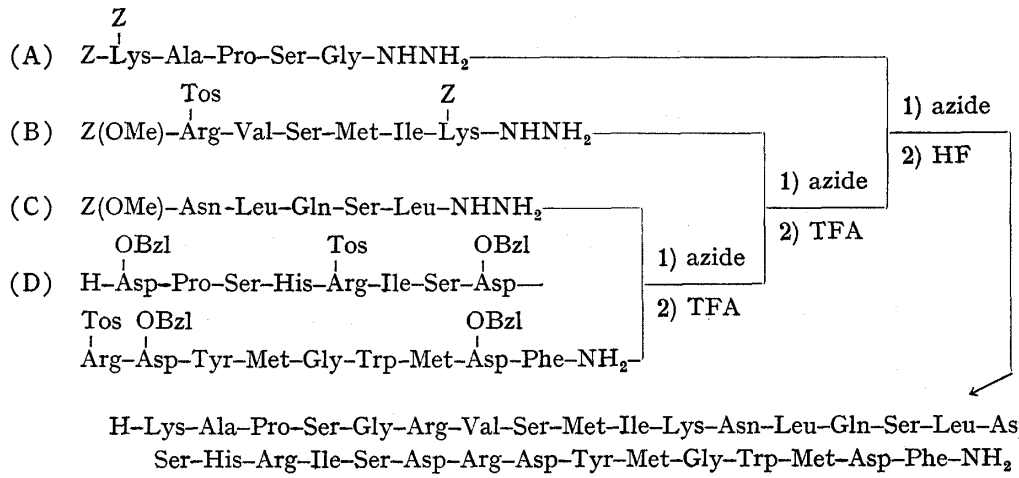
The tritriacontapeptide amide, [27-Tyr]-CCK-PZ, was synthesized by successive azide condensation of 4 peptide fragments, (1—5), (6—11), (12—16), and (17—33), followed by exposure to hydrogen fluoride to remove all protecting groups. Though the CCK-PZ activity of synthetic peptide was only 1/250 of the standard from the natural source, it was smoothly labeled with radioactive iodine. Chain length activity relationship of CCK-PZ was also discussed.

In the preceding two papers,[1,4] we have described the syntheses of two segments of porcine cholecystokinin-pancreozymin (CCK–PZ)[5]; the one was the protected heptadecapeptide amide which covered the C-terminal portion of CCK–PZ and the latter the N-terminal hexadecapeptide. The entire chain length of CCK–PZ with 33 amino acid residues could be built up directly by the DCC condensation[6] of these two fragments with an aid of excellent racemization suppressors, such as N-hydroxysuccinimide[7] or N-hydroxybenzotriazole.[8] However, we decided to elongate the peptide chain from the heptadecapeptide amide to the tritriacontapeptide by assembling suitable peptide fragments by the azide procedure[9] exclusively, since risk of racemization is much less in the azide procedure than the former.

Figure 1 illustrates our synthetic route to the tritriacontapeptide amide (I) corresponding to the entire amino acid sequence of the desulfated form of CCK–PZ, termed as [27-Tyr]-CCK–PZ. Starting with the heptadecapeptide amide (D)[4] as an amino component, three peptide fragments were successively condensed for this purpose. These three fragments, Z–Lys(Z)–Ala–Pro–Ser–Gly–$NHNH_2$ (A), Z(OMe)–Arg(Tos)–Val–Ser–Met–Ile–Lys(Z)–$NHNH_2$ (B) and Z(OMe)–Asn–Leu–Gln–Ser–Leu–$NHNH_2$ (C), were newly synthesized with available small peptide fragments for our previous synthesis of the N-terminal hexadecapeptide.[1] Among these, the N-terminal pentapeptide hydrazide (A, positions 1—5) is the unit applied

1) Part LXVII: Y. Mori, K. Koyama, Y. Kiso, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2788 (1976).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, OBzl=benzyl ester, Boc=*tert*-butoxycarbonyl, ODNP=2,4-dinitrophenyl ester, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylformamide.
3) Location: *a) Sakyo-ku, Kyoto, 606, Japan; b) Kumano-cho, Sakyo-ku, Kyoto, 606, Japan; c) Kitaku, Sapporo, 060, Japan.*
4) Y. Mori and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2781 (1976).
5) V. Mutt and J.E. Jorpes, *Eur. J. Biochem.*, **6**, 156 (1968); *idem, Biochem. J.*, **125**, 57 (1971).
6) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
7) F. Weygand, D. Hoffmann, and E. Wunsch, *Z. Naturforsch.*, **21b**, 426 (1966); J.E. Zimmerman and G.W. Anderson, *J. Am. Chem. Soc.*, **89**, 7151 (1967).
8) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
9) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).

previously by Bodanszky, *et al.*[10] for their synthesis of the N-terminal octapeptide, though Z-Lys(Z)-OH, instead of Boc-Lys(Boc)-OH, was adopted in our present synthesis. The pentapeptide hydrazide (C, positions 12—16) is the unit converted directly from the previously synthesized pentapeptide, Z(OMe)-Asn-Leu-Gln-Ser-Leu-OH. Thus amino acid residues located between these two fragments, A and C, were assembled as one building block (B, positions 6—11).



H-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 (I)

Fig. 1. Synthetic route to [27-Tyr]-cholecystokinin-pancreozymin

As mentioned above, the pentapeptide hydrazide (C), Z(OMe)-(CCK-PZ 12—16)-$NHNH_2$, was derived from Z(OMe)-Asn-Leu-Gln-Ser-Leu-OH, *i.e.*, by treatment with diazomethane followed by exposing the resulting methyl ester to hydrazine hydrate. Alternatively, we have also synthesized this hydrazide starting with H-Leu-OBzl as illustrated in Fig. 2. Z-(OMe)-Gln-Ser-OMe, prepared by N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline,[11] was converted to the corresponding hydrazide, which was condensed with H-Leu-OBzl by the azide procedure to give Z(OMe)-Gln-Ser-Leu-OBzl in much better yield than the former experiment.[1] Previously, this unit was prepared in a form of Z-Gln-Ser-Leu-OH.[1] The above protected tripeptide benzyl ester, after removal of the Z(OMe) group[12] by trifluoroacetic acid (TFA), was condensed further with Z(OMe)-Asn-Leu-$NHNH_2$ by the same azide procedure and the resulting protected pentapeptide ester Z(OMe)-Asn-Leu-Gln-Ser-Leu-OBzl, like the corresponding methyl ester mentioned above, was converted to C in the usual hydrazine treatment.

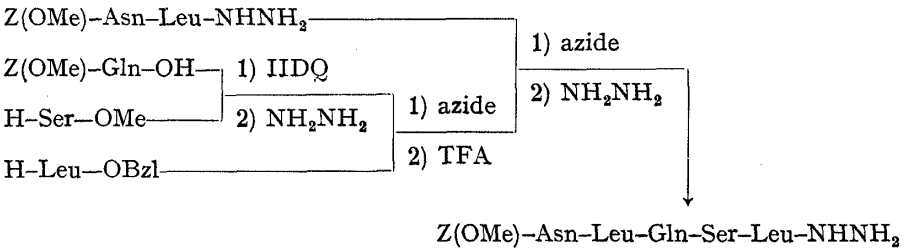


Fig. 2. Synthetic Scheme of the Protected Pentapeptide Hydrazide, Z(OMe)-(CCK-PZ 12—16)-$NHNH_2$

10) M. Bodanszky, N. Chaturvedi, D. Hudson, and M. Itoh, *J. Org. Chem.*, **37**, 2303 (1972).
11) Y. Kiso and H. Yajima, *J. C. S. Chem. Commun.*, **1972**, 942; Y. Kiso, Y. Kai, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).
12) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962), H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970).

In order to prepare the protected hexapeptide hydrazide, (B), Z(OMe)-(CCK-PZ 6—11)-$NHNH_2$, available two peptide fragments,[1] Z(OMe)-Val-Ser-$NHNH_2$ and H-Met-Ile-Lys(Z)-OMe, were first condensed by the azide procedure as illustrated in Fig. 3. The resulting protected pentapeptide ester Z(OMe)-Val-Ser-Met-Ile-Lys(Z)-OMe, after treatment with TFA as usual, was condensed with Z(OMe)-Arg(Tos)-OH by the 2,4-dinitrophenyl ester procedure.[13] This condensation reaction was performed without isolation of the corresponding active ester, because of the great tendency of the intramolecular lactam formation of Arg active esters by heat treatments.[14] Progress of the active ester forming step, as well as the coupling reaction, were monitored by thin-layer chromatography. The resulting hexapeptide ester, Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-OMe, was smoothly converted to the corresponding protected hydrazide (B) by the usual hydrazine treatment.

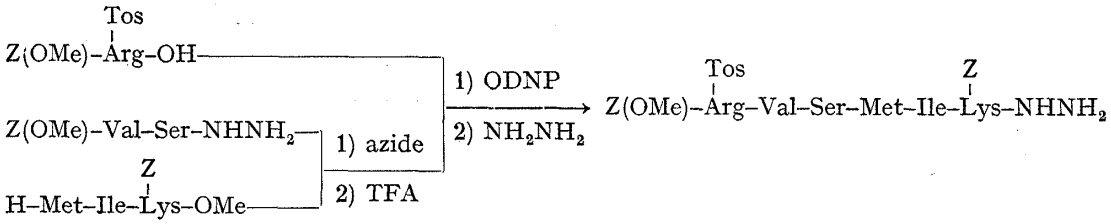


Fig. 3. Synthetic Scheme of the Protected Hexapeptide Hydrazide, Z(OMe)-(CCK-PZ 6—11)-$NHNH_2$

The N-terminal pentapeptide hydrazide (A), Z-(CCK-PZ 1—5)-$NHNH_2$, was synthesized in a different manner from those of Bodanszky, *et al.*[10] as illustrated in Fig. 4. Z-Pro-Ser-$NHNH_2$[15] was condensed with the triethylammonium salt of Gly by the azide procedure. After the reaction, the crude product was once dissolved in a alkaline solution, which after washing with ethyl acetate to remove the rearrangement product of the azide, was acidified with dilute hydrochloric acid. However, no precipitation of the product occurred and extraction of the aqueous solution with ethyl acetate afforded a very small amount of the desired product. Therefore, the aqueous solution was condensed and the residue was applied to a column of silica, which was eluted with methanol to isolate the desired peptide. Z-Pro-Ser-Gly-OH isolated once in such a manner is not soluble freely in water. Thus preparation of this protected tripeptide was unexpectedly laborious. This tripeptide, after hydrogenation, was condensed with an available dipeptide hydrazide, Z-Lys(Z)-Ala-$NHNH_2$[1] by the same azide procedure and the resulting protected pentapeptide, Z-Lys(Z)-Ala-Pro-Ser-Gly-OH was converted to the corresponding hydrazide (A) through the methyl ester in the usual manner.

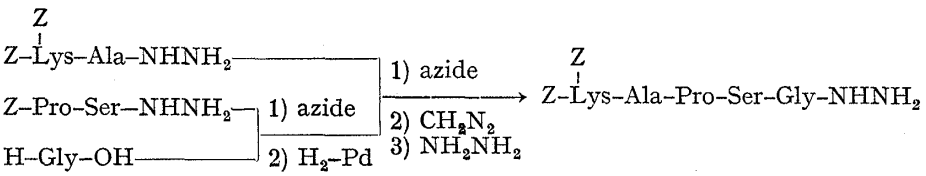


Fig. 4. Synthetic Scheme of the Protected Pentapeptide Hydrazide, Z-(CCK-PZ 1—5)-$NHNH_2$

Three peptide fragments prepared as outlined above were then condensed successively by the azide procedure as shown in Fig. 1. Prior to each coupling reaction, the Z(OMe) group of respective amino components was removed by TFA in the presence of anisole con-

13) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.
14) J. Kovacs and M.Q. Ceprini, *Chem. Ind.*, **1965**, 2100.
15) K. Lubke, E. Schroder, R. Schmeichen, and H. Gibian, *Ann. Chem.*, **679**, 195 (1964).

taining 2% ethanedithiol[16] to minimize destruction of the Trp residue as mentioned previously. It is worth-while mentioning here again that by the use of such a cation scavenger during various TFA treatments, we could carry out the present synthesis without producing any brown color resulted from the destruction of Trp. As also mentioned previously, attempt to estimate the Trp content by hydrolysis with 3N Tos–OH[17] was interfered by the presence of 3 moles of Arg(Tos) which hydrolyzed partially under these conditions. In an amino acid analyzor, these two peaks were overlapped. After each coupling reaction, column chromatography on Sephadex LH-20 was employed to isolate respective products in chromatographically and analytically pure form. The solvent system of DMSO–DMF (50: 10) was employed to elute the desired compounds. A similar solvent system was necessary to perform the every coupling reactions because of the poor solubility of these protected peptide amides. A small amount of mercaptoethanol was added, prior to evaporation of these solvents, to prevent oxidation of the Met residue.

The 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, synthesized after three consequtive azide condensation reactions, was then exposed to hydrogen fluoride[18] at 0° for 60 minutes to remove all protecting groups. Anisole containing 2% ethanedithiol and skatol served as scavengers to avoid alkylation.[19] The resulting deblocked peptide was immediately converted to the corresponding acetate with Amberlite IR-4B and purified by column chromatography on Sephadex G-25 and carboxymethyl (CM)-cellulose. To elute the desired compound, 0.2M acetic acid was used in the former step and gradient elution with 0.1M ammonium bicarbonate buffer (pH 7.8) in the latter step. Most of scavengers were removed in the former purification step. Preliminary, we have tried to elute the desired compound from a column of CM-cellulose by gradient elution with 0.1M ammonium acetate buffer (pH 6.9). However, because of easy removal of ammonium bicarbonate by lyophilization, we decided to take the above stated elution conditions.

The main product thus purified exhibited a single spot on thin-layer chromatography in three different solvent systems. A hydrolysate with 3N Tos–OH contained the constituent amino acids in ratios predicted by theory. The recovery of Trp could be judged as one mole indicating that this acid sensitive amino acid survived under various TFA treatments, when anisole containing 2% ethanedithiol was employed as a cation scavenger. Identification of one mole each of Asn and Gln in the synthetic peptide was also under our particular attention. A hydrolysate of the peptide with aminopeptidase (AP–M)[19] gave a peak overlapped with Ser and Asn in an amino acid analyzor. However, the presence of one mole of Asn could be judged from the difference of recoveries of Asp and Ser in acid and enzymatic hydrolysates. Presence of one mole of Gln was determined without difficulty by this enzymatic hydrolysis, because of the absence of Thr, which interfers its analysis.

The synthetic tritriacontapeptide, [27-Tyr]–CCK–PZ, purity of which was thus assessed, was submitted to biological assays, together with two peptide fragments prepared previously; the N-terminal hexadecapeptide H-(1—16)–OH[1] and the C-terminal heptadecapeptide amide H–(17—33)–NH_2.[4]

When secretory response of amylase was examined in isolated and perfused rat pancreas,[20] the relative potency of our synthetic [27-Tyr]–CCK–PZ was estimated to be about 1/250 of

16) J.J. Sharp, A.B. Robinson, and M.D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).
17) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
18) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Suguhara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
19) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963). AP-M was purchased from The Protein Research Foundation (Osaka), Lot No. 191226.
20) T. Kanno, *J. Physiol.*, **245**, 599 (1975).

natural CCK-PZ (GIH Research Unit, 3400 Ivy U/mg, Karolinska Institute, Stockholm, Sweden) and the activity of H-(17—33)-NH_2 was approximately 1/350. The dog gallbladder contractile potency of [27-Tyr]-CCK-PZ and H-(17—33)-NH_2 were also low as seen in their pancreozymin activities. Ondetti, *et al.*[21] observed previously that the cholecystokinin activity of the unsulfated C-terminal hepta or octapeptide amide 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. In these two assay systems, the N-terminal hexadecapeptide, H-(1—16)-OH, was judged as inert in the cholecystokinin activity as expected. However it exhibited the pancreozymin activity of 1/2000. The latter low, but observable response to the amylase release was quite unexpected. This unparallelism in these two biological responses of the synthetic peptide may offer a important cue for future investigations concerning to receptors of the above stated two independent organs, to which CCK-PZ is concerned.

The synthetic [27-Tyr]-CCK-PZ, though it is not too biologically active, was smoothly and efficiently labeled with radioactive iodine (^{125}I) according to the method of Hunter and Greenwood.[22] Attributing to the presence of unmasked Tyr, the specific activity we obtained (200—250 μci/μg) was much higher than that reported in natural CCK-PZ (30—100 μci/μg).[23]

Though a useful compound for the development of radioimmunoassay of CCK-PZ was thus synthesized, selective introduction of the sulfate group at the Tyr residue (position 27) is still the problem unsolved for the total synthesis of CCK-PZ. Synthesis of human gastrin II,[24] one of the gastrointestinal hormones bearing the Tyr(SO_3H) residue, was demonstrated by introduction of the sulfate group to the deblocked heptadecapeptide with sulfur trioxide-pyridine complex at the final stage of the synthesis, though the Met residue was partially modified. This procedure however, can not be applied to [27-Tyr]-CCK-PZ, because of the presence of Ser, which is readily sulfated under this condition and by other means, such as concentrated sulfuric acid or sulfuric acid and DCC. Ondetti, *et al.*[21] introduced this moiety, for example, at the C-terminal decapeptide stage and the dipeptide unit, Ile-Ser, was later attached to this sulfated peptide. The yield in sulfation step was at best 40% and in the chain elongation step *ca.* 60% after diethylaminoethyl (DEAE)-Sephadex purification. Difficulty can be soon realized how to elongate the peptide chain bearing such a acid labile sulfate ester linkage to the full length of CCK-PZ. As expressed by the synthesizer of human gastrin,[24] the problem of developing a really satisfactory synthesis of sulfate esters in peptide series containing Ser, Thr and Met still remains to be solved. Our preliminary tests indicated that H-Tyr(SO_3H)-OH[25] was partially hydrolyzed by TFA and completely by HF. However, this compound is quite stable to CF_3SO_3H and CH_3SO_3H. Experimental results we accumulated through the synthesis of [27-Tyr]-CCK-PZ may offer useful informations necessary to the total synthesis of CCK-PZ which has such unique structural features and biological activities.

Experimental

General experimental methods employed here are essentially the same as those described in the Part LXII[26] of this series. Thin-layer chromatography was performed on silica gel (Kiesel gel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$-MeOH-H_2O (8: 3: 1), Rf_2 $CHCl_3$-MeOH-H_2O (9: 1: 0.5), Rf_3, Rf_4, and Rf_5 *n*-BuOH-AcOH-pyridine-H_2O (4: 1: 1: 2), (30: 6: 20: 24), and (1: 1: 1: 1) respectively.

21) M.A. Ondetti, J. Pluscec, E.F. Sabo, J.T. Sheehan, and N. Williams, *J. Am. Chem. Soc.*, **92**, 195 (1970); M.A. Ondetti, B. Rubin, S.L. Engel, J. Pluscec, and J.T. Sheehan, *Am. J. Digest. Diseases*, **15**, 149 (1970).
22) W.M. Hunter and F.C. Greenwood, *Nature*, **194**, 495 (1962).
23) R.F. Harvey, L. Dowsett, M. Hartog, and A.E. Read, *Gut.*, **15**, 690 (1974).
24) J. Beacham, P.H. Bentley, G.W. Kenner, J.K. MacLeod, J. J. Mendive and R.C. Sheppard, *J. Chem. Soc.*, **1967**, 2520.
25) H.S. Tallan, S.T. Bells, W.H. Stein, and S. Moore, *J. Biol. Chem.*, **217**, 703 (1955).
26) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).

Z(OMe)-Gln-Ser-OMe——IIDQ (14.80 g) was added to a mixture of Z(OMe)-Gln-OH (15.50 g) and H-Ser-OMe (prepared from 7.80 g of the hydrochloride with 7 ml of Et_3N) in dimethyl formamide (DMF) (200 ml) and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed batchwisely with 5% citric acid, 5% sodium bicarbonate and H_2O and then precipitated from DMF with MeOH; yield 16.07 g (78%), mp 207—209°, $[\alpha]_D^{18}$ +2.2° (c=0.7, DMSO), Rf_1 0.33. *Anal.* Calcd. for $C_{18}H_{25}O_8N_3$: C, 52.55; H, 6.13; N, 10.21. Found: C, 52.32; H, 6.30; N, 10.24.

Z(OMe)-Gln-Ser-NHNH$_2$——To a solution of Z(OMe)-Gln-Ser-OMe (10.29 g) in MeOH–DMF (100 ml—40 ml), 90% hydrazine hydrate (8 ml) was added. The gelatinous mass formed on standing overnight was collected and washed with MeOH; yield 7.66 g (74%), mp 237—240°, $[\alpha]_D^{18}$ −2.0° (c=0.7, DMSO), Rf_1 0.41. *Anal.* Calcd. for $C_{17}H_{25}O_7N_5$: C, 49.63; H, 6.13; N, 17.02. Found: C, 49.54; H, 5.97; N, 16.82.

Z(OMe)-Gln-Ser-Leu-OBzl——To a solution of Z(OMe)-Gln-Ser-NHNH$_2$ (6.17 g) in DMF (80 ml), 2.03N HCl–DMF (15 ml) and isoamylnitrite (2.01 ml) were added under cooling with ice-NaCl. After stirring for 5 min, the solution was neutralized with Et_3N (4.2 ml) and then combined with a solution of H-Leu-OBzl (prepared from 7.88 g of the hydrochloride with 4.9 ml of Et_3N) in DMF (70 ml). The mixture was stirred at 4° for 72 hr, the solvent was evaporated and the residue was treated with H_2O. The resulting powder was washed batchwisely with 5% citric acid and precipitated twice from DMF with MeOH; yield 6.48 g (71%), mp 175—179°, $[\alpha]_D^{18}$ −28.3° (c=1.0, DMSO), Rf_1 0.66. *Anal.* Calcd. for $C_{30}H_{40}O_9N_4 \cdot 1/2H_2O$: C, 59.10; H, 6.78; N, 9.19. Found: C, 59.13; H, 6.87; N, 9.20.

Z(OMe)-Asn-Leu-Gln-Ser-Leu-OBzl——Z(OMe)-Gln-Ser-Leu-OBzl (4.57 g) was treated with TFA (8 ml) in the presence of anisole (4 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (40 ml) containing Et_3N (2.1 ml). To this solution, the azide (prepared from 3.10 g of Z(OMe)-Asn-Leu-NHNH$_2$ with 2.03N HCl–DMF, 1.07 ml of isoamylnitrite and 2.24 ml of Et_3N) in DMF (60 ml) was added and the mixture was stirred at 4° for 72 hr. The solvent was evaporated *in vacuo*, the residue was treated with AcOEt to give a fine powder, which was washed batchwisely with 5% citric acid and H_2O and then precipitated from DMF with MeOH; yield 3.40 g (55%), mp 231—233°, $[\alpha]_D^{18}$ −24.2° (c=1.1, DMSO), Rf_1 0.41. *Anal.* Calcd. for $C_{40}H_{57}O_{12}N_7$: C, 58.03; H, 6.94; N, 11.84. Found: C, 57.82; H, 7.01; N, 11.71.

Z(OMe)-Asn-Leu-Gln-Ser-Leu-OMe——Under cooling with ice, an etheral solution of diazomethane was added to a solution of Z(OMe)-Asn-Leu-Gln-Ser-Leu-OH (0.74 g) in DMF (25 ml) and the yellow color persisted for 60 min. The excess diazomethane was destroyed by addition of a few drops of AcOH. The solvent was evaporated and the residue was treated with MeOH. The resulting gelatinous mass was collected by filtration and washed with MeOH; yield 0.63 g (84%), mp 225—230°, $[\alpha]_D^{18}$ −23.1° (c=0.8, DMSO), Rf_1 0.48. *Anal.* Calcd. for $C_{34}H_{53}O_{12}N_7$: C, 54.32; H, 7.11; N, 13.04. Found: C, 54.05; H, 7.20; N, 12.82.

Z(OMe)-Asn-Leu-Gln-Ser-Leu-NHNH$_2$ (C)——To a solution of Z(OMe)-Asn-Leu-Gln-Ser-Leu-OMe[1] (0.58 g) in DMF (25 ml), 90% hydrazine hydrate (1 ml) was added and the solution was kept on standing at room temperature for 48 hr. The resulting gelatinous mass was collected by filtration and washed with MeOH; yield 0.48 g (83%), mp 262° dec. $[\alpha]_D^{18}$ = +19.0° (c=0.5, DMSO), Rf_1 0.35. *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. Z(OMe)-Asn-Leu-Gln-Ser-Leu-OBzl was similarly converted to the hydrazide; yield 81%.

Z(OMe)-Val-Ser-Met-Ile-Lys(Z)-OMe——Z(OMe)-Met-Ile-Lys(Z)-OMe[1] (7.03 g) was treated with TFA (14 ml) in the presence of anisole (3.5 ml) containing 2% ethanedithiol in an ice-bath for 45 min. Petroleum ether was added and the resulting oily precipitate was treated with ether to form a fine powder, which was dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml) containing Et_3N (1.4 ml). To this ice-cold solution, the azide (prepared from 4.59 g of Z(OMe)-Val-Ser-NHNH$_2$ with 12 ml of 2.03N HCl–DMF, 1.6 ml of isoamylnitrite and 4.98 ml of Et_3N) in DMF (20 ml) was added and the mixture, after stirring at 4° for 48 hr, was condensed *in vacuo*. Treatment of the residue with AcOEt afforded a fine powder, which was washed with 5% citric acid and H_2O and then recrystallized from MeOH; yield 6.00 g (67%), mp 230—232°, $[\alpha]_D^{18}$ −10.8° (c=1.0, DMSO), Rf_2 0.66. Amino acid ratios in an acid hydrolysate: Ser 1.04, Val 1.08, Met 0.77, Ile 1.00, Lys 0.81, (average recovery 99%). *Anal.* Calcd. for $C_{43}H_{64}O_{12}N_6S$: C, 58.09; H, 7.26; N, 9.45. Found: C, 58.16; H, 7.37; N, 9.63.

Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-OMe——Z(OMe)-Val-Ser-Met-Ile-Lys(Z)-OMe (4.45 g) was treated as stated above with TFA (8.9 ml) in the presence of anisole (2.2 ml) containing 2% ethanedithiol in an ice-bath for 60 min and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml) containing Et_3N (0.7 ml). DCC (1.24 g) was added and to a solution of Z(OMe)-Arg(Tos)-OH (2.46 g) and 2,4-dinitrophenol (1.11 g) in THF (20 ml) and the mixture, after stirring for 3 hr, when the spot of the starting material on thin layer chromatography disappeared, was combined with the above solution containing pentapeptide ester. The mixture was stirred at room temperature for 24 hr and then condensed *in vacuo*. Treatment of the residue with AcOEt afforded a fine powder, which was washed batchwisely with 5% citric acid, 5% sodium bicarbonate and H_2O and then precipitated from DMF with MeOH; yield 4.33 g (72%), mp 225—227°, $[\alpha]_D^{18}$ −12.4° (c=1.0, DMSO), Rf_2 0.64. Amino acid ratios in an acid hydrolysate: Arg 1.13, Val 0.93, Ser 1.26, Met 0.92, Ile 1.00, Lys 0.81 (average recovery 90%). *Anal.* Calcd. for $C_{56}H_{82}O_{15}N_{10}S_2$: C, 56.07; H, 6.89; N, 11.68. Found: C, 56.12; H, 7.07; N, 11.59.

Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-NHNH$_2$ (B)——To a solution of Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-OMe (2.55 g) in DMF (15 ml), 90% hydrazine hydrate (3 ml) was added and the solution was kept on standing overnight to form a gelatinous mass. After addition of MeOH (30 ml), the product was collected by filtration, washed with MeOH and precipitated from DMF with MeOH; yield 2.10 g (82%), mp 242—244°, $[\alpha]_D^{18}$ +1.0° (c=0.3, DMSO), Rf_1 0.57. *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-Pro-Ser-Gly-OH——In the usual manner, the azide was prepared from Z-Pro-Ser-NHNH$_2$ (16.0 g) dissolved in DMF (80 ml) with 4.21N HCl-dioxane (28.5 ml), isoamylnitrite (8 ml) and Et$_3$N (16.6 ml). This solution was combined with a solution of H-Gly-OH (7.5 g) in H$_2$O (40 ml) containing Et$_3$N (20.7 ml). The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was dissolved in 3% NH$_4$OH, which after washing with AcOEt, was acidified with 6N HCl. The solvent was evaporated *in vacuo* and MeOH was added. NH$_4$Cl thereby formed was removed by filtration and the filtrate was condensed. The residue was dissolved in a small amount of the solvent consisting of CHCl$_3$–MeOH–H$_2$O (8: 3: 1) and the solution was applied to a column of silica (80×3 cm), which was eluted with the same solvent system. Fractions containing the substance of Rf_1 0.18 were combined and the solvent was evaporated. The residue was triturated with ether. The resulting powder was washed with a small amount of ice-chilled H$_2$O and recrystallized from MeOH and ether; yield 10.0 g (51%), mp 99—101°, $[\alpha]_D^{18}$ −33.2° (c=0.5, DMF), Rf_1 0.18. *Anal.* Calcd. for $C_{18}H_{23}O_7N_3$: C, 54.95; H, 5.89; N, 10.68. Found: C, 54.51; H, 6.08; N, 10.22.

H-Pro-Ser-Gly-OH——Z-Pro-Ser-Gly-OH (8.73 g) dissolved in MeOH (100 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst in the usual manner for 5 hr. The catalyst was removed by filtration, the filtrate was condensed *in vacuo* and the residue was recrystallized from H$_2$O and MeOH; yield 5.31 g (92%), mp 250—253°, $[\alpha]_D^{18}$ −63.8° (c=0.5, H$_2$O), Rf_2 0.15. *Anal.* Calcd. for $C_{10}H_{17}O_5N_3$: C, 46.32; H, 6.61; N, 16.21. Found: C, 46.23; H, 6.51; N, 15.95.

Z-Lys(Z)-Ala-Pro-Ser-Gly-OH——The azide (prepared from 11.21 g of Z-Lys(Z)-Ala-NHNH$_2$ with 18.1 ml of 2.98N HCl–DMF, 3.6 ml of isoamylnitrite and 7.44 ml of Et$_3$N) in DMF (30 ml) was added to a solution of H-Pro-Gly-Ser-OH (3.89 g) and Et$_3$N (4.16 ml) in H$_2$O (20 ml). The mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH$_4$OH, which was washed with AcOEt and then acidified with 1N HCl. The oily precipitate turned to the solid under cooling with ice, which was recrystallized from MeOH and AcOEt; yield 7.80 g (72%), mp 96—99°, $[\alpha]_D^{18}$ −16.5° (c=0.6, DMF), Rf 0.42, Rf_2 0.35. Amino acid ratios in an acid hydrolysate: Lys 0.96, Ala 1.05, Pro 1.13, Ser 0.89, Gly 1.00 (average recovery 96%). *Anal.* Calcd. for $C_{35}H_{46}O_{11}N_6 \cdot H_2O$: C, 56.44; H, 6.50; N, 11.28. Found: C, 56.76; H, 6.54; N, 11.53.

Z-Lys(Z)-Ala-Pro-Ser-Gly-OMe——An etheral diazomethane was added to a solution of Z-Lys(Z)-Ala-Pro-Ser-Gly-OH (5.45 g) in MeOH (80 ml) and the yellow color was persisted for 40 min. A few drops of AcOH was added and the solvent was evaporated. Treatment of the residue afforded a fine powder, which was recrystallized from MeOH and ether; yield 5.0 g (90%), mp 110—112°, $[\alpha]_D^{18}$ 54.5° (c=0.9, DMF), Rf_1 0.67, Rf_2 0.53. *Anal.* Calcd. for $C_{36}H_{48}O_{11}N_6$: C, 58.36; H, 6.53; N, 11.35. Found: C, 58.15; H, 6.58; N, 11.11.

Z-Lys(Z)-Ala-Pro-Ser-Gly-NHNH$_2$ (A)——To a solution of Z-Lys(Z)-Ala-Pro-Ser-Gly-OMe (4.45 g) in MeOH (50 ml), 90% hydrazine hydrate (3.6 ml) was added. The solution was kept on standing overnight and then condensed. The residue was treated with ether and then recrystallized from MeOH and ether; yield 3.91 g (89%), mp 89—91°, $[\alpha]_D^{18}$ −13.3° (c=0.3, DMSO), Rf_1 0.47. *Anal.* Calcd. for $C_{35}H_{48}O_{10}N_8 \cdot 2H_2O$: C, 54.11; H, 6.75; N, 14.42. Found: C, 54.40; H, 6.70; N, 14.17.

Z(OMe)-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$_2$, Z(OMe)-(CCK-PZ 12—33)-NH$_2$——The protected heptadecapeptide amide (1.00 g) was treated with TFA (1.5 ml) in the presence of anisole (1.1 ml) containing 2% ethanedithiol in an ice-bath for 60 min and dry ether was added. The resulting TFA salt, collected by filtration, was dried over KOH pellets *in vacuo* and then dissolved in DMF (8 ml) containing 10% Et$_3$N–DMF (1.4 ml). To this ice-cold solution, the azide (prepared from 0.51 g of Z(OMe)-Asn-Leu-Gln-Ser-Leu-NHNH$_2$, 0.8 ml of 2.03N HCl–DMF, 1.18 ml of 10% isoamylnitrite-DMF, and 2.27 ml of 10% Et$_3$N–DMF) in DMSO–DMF (2—1 ml) was added. The mixture was stirred at 4° for 48 hr and the solvent was evaporated *in vacuo* and the residue was treated with AcOEt. The resulting powder was washed with MeOH and dissolved in a small amount of DMSO. The solution was applied to a column of Sephadex LH-20 (3×130 cm), which was eluted with DMSO–DMF (50: 10). Individual fractions (4 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the front main peak (tube No. 71—76) were combined and the solvent was evaporated *in vacuo*. The residue was triturated with AcOEt and precipitated from DMF with MeOH; yield 0.65 g (55%), mp 212—214°, $[\alpha]_D^{18}$ −16.5° (c=0.6, DMSO), Rf_1 0.53. Amino acid ratios in an acid hydrolysate: Asp 4.66, Leu 1.80, Glu 0.95, Ser 3.03, Pro 1.13, His 1.03, Arg 2.75, Ile 1.34, Tyr 0.79, Met 1.88, Gly 1.00, Phe 1.06 (average recovery 87%). *Anal.* Calcd. for $C_{167}H_{216}O_{43}N_{34}S_4 \cdot 9H_2O$: C, 54.53; H, 6.41; N, 12.94. Found: C, 54.90; H, 6.45; N, 12.49.

Z(OMe)-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$_2$, Z(OMe)-(CCK-PZ 6—33)-NH$_2$——The above protected docosapeptide amide, Z(OMe)-(CCK-PZ 12—33)-NH$_2$ (0.80 g) was treated with TFA (1.7 ml) in the presence of anisole (1.2 ml) containing 2% ethanedithiol as stated above. The resulting TFA salt, collected as fine powders, was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF

(5 ml) containing 10% Et_3N–DMF (0.96 ml). To this ice-cold solution, the azide (prepared from 0.55 g of Z(OMe)-Arg(Tos)-Val-Ser-Met-Ile-Lys(Z)-$NHNH_2$ with 0.37 ml of 2.98N HCl–DMF, 0.79 ml of 10% isoamylnitrite–DMF and 1.52 ml of 10% Et_3N-DMF) in DMSO–DMF (2 ml—1 ml) was combined and the solution was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed with H_2O and a half of this product was dissolved in a small amount of DMSO. The solution was applied to a column of Sephadex LH-20 (3×126 cm), which was eluted with DMSO–DMF (50: 10). Individual fractions (4 ml each) were collected and the desired fractions were collected as stated above (tube No. 58—82). The last half of the crude product was similarly purified. The combined product was precipitated from DMF with MeOH; yield 0.94 g (91%), mp 230° dec., $[\alpha]_D^{18}$ −12.4° (c=0.4, DMSO), Rf_1 0.60. Amino acid ratios in an acid hydrolysate: Arg 2.96, Val 1.11, Ser 4.30, Met 2.99, Ile 2.25, Lys 0.91, Asp 4.65, Leu 1.69, Glu 0.89, Pro 1.18, His 0.96, Tyr 0.72, Gly 1.00, Phe 1.10 (average recovery 85%). *Anal.* Calcd. for $C_{213}H_{286}O_{54}N_{44}S_6 \cdot 6H_2O$: C, 55.28; H, 6.49; N, 13.32. Found: C, 55.09; H, 6.46; N, 13.08.

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, Z-(CCK-PZ 1—33)-NH_2——The above protected octacosapeptide amide, Z(OMe)-(CCK-PZ 6—33)-NH_2 (0.89 g) was treated with TFA (1.5 ml) in the presence of anisole (1.1 ml) containing 2% ethanedithiol as stated above. The resulting TFA salt was dissolved in DMF (5 ml) containing 10% Et_3N–DMF (0.83 ml). To this ice-cold solution, the azide (prepared from 0.29 g of Z-Lys(Z)-Ala-Pro-Ser-Gly-$NHNH_2$ with 0.32 ml of 2.98N HCl–DMF, 0.69 ml of 10% isoamylnitrite–DMF and 1.33 ml of 10% Et_3N–DMF) in DMF (2 ml) was combined and the mixture was stirred at 4° for 72 hr. The solvent was evaporated and the residue was treated with AcOEt to give a fine powder; yield 1.35 g. A half of this product was dissolved in a small amount of DMSO–DMF (50: 10) and the solution was applied to a column of Sephadex LH-20 (3×130 cm), which was eluted with the same solvent. Ultraviolet spectrum (UV) absorbancy at 280 mμ was determined in each fraction (4 ml). Fractions corresponding to the front main peak (tube No. 74—94) were collected and the solvent was evaporated. The residue was treated with AcOEt to afford a fine powder. The last half of the crude sample was similarly purified and the combined product was precipitated from DMF with AcOEt; yield 0.88 g (89%), mp 220—223° $[\alpha]_D^{18}$ −38.4° (c=0.4, DMSO).[27] Rf_1 0.65. 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.

H-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2, [27-Tyr]-CCK-PZ (I)——The above protected tritriacontapeptide amide, Z-(CCK-PZ 1-33)-NH_2 (200 mg) was treated with HF (approximately 5 ml) in the presence of anisole (1.8 ml) containing 2% ethanedithiol and skatol (150 mg) in an ice-bath for 40 min. The excess HF was evaporated *in vacuo* at 0° and dry ether was added. The resulting powder was collected by filtration and dissolved in H_2O (5 ml), which was treated with Amberlite IR-4B (acetate form, approximately 2 g) for 30 min and then filtered. The filtrate was lyophilized to give a fluffy powder. The product was then dissolved in a small amount of 5% AcOH and the solution was applied to a column of Sephadex G-25 (1.6×150 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and absorbancy at 280 mμ was determined. The fractions corresponding to the front main peak (tube No. 46—71, Fig. 5-a) were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 119 mg (deblocking step 78%). This powder (42 mg) was dissolved in H_2O (1 ml) and the solution was applied to a column of CM-cellulose (2.0×5.2 cm) which was eluted with 0.1M NH_4HCO_3 (pH 7.8) through a mixing flask containing H_2O (80 ml) and finally with 5% AcOH. Individual fractions (4 ml each) were collected and UV absorbancy at 280 mμ was similarly determined. Three peaks were detected (Fig. 5-b) F-1 (main, tube No. 16—23), F-2 (minor, tube No. 24—47) and F-3 (minor, tube No. 66—72 in 5% AcOH eluates). Fractions corresponding

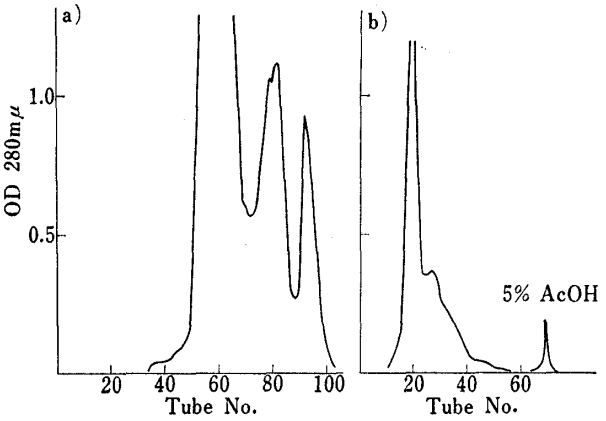


Fig. 5. Column Chromatografic Purification of [27-Tyr]-CCK-PZ

a) Sephadex G-25 purification
column 1.6×150 cm, solvent 5% AcOH, flow rate 12 ml/hr, fraction 4 ml
b) CM-cellulose purification
column 2.0×5.2 cm, fraction 4 ml, gradient elution 0.1M NH_4HCO_3 (pH 7.8), mixing flask H_2O (80 ml)

27) Previously recorded erroneously by disorder of the polarimeter, *Chem. Pharm. Bull.* (Tokyo), **24**, 1110 (1976).

to these tubes were collected and the solvent was removed by lyophilization respectively; F-2 (5 mg) and F-3 (2 mg) were not further examined. F-1: yield 20 mg (purification step 49%). Rf_3 0.44, Rf_4 0.50, Rf_5 0.05. $[\alpha]_D^{24}$ −35.8° (c=0.2, 3% AcOH). Amino acid ratios in 3N Tos-OH hydrolysates and AP-M digest (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, 7.13, N, 15.67. Found: C, 48.06, H, 7.05, N, 15.37.

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