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Studies on Peptides. LIX.[1,2] Synthesis of the Nonatriacontapeptide Corresponding to the Entire Amino Acid Sequence of Porcine Adrenocorticotropic Hormone

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The nonatriacontapeptide corresponding to the newly revised amino acid sequence of porcine adrenocorticotropic hormone (ACTH) was synthesized by successive condensations of 4 peptide fragments; Z(OMe)-(15—19)-OH, Z(OMe)-(11—14)-OH, Z(OMe)-(5—10)-OH and Z-(1—4)-$NHNH_2$, with H-(20—39)-OBzl, a synthetic intermediate of porcine corticotropin-like intermediate lobe peptide. The synthetic peptide exhibited the identical *Rf* value with that of natural porcine ACTH in two different solvent systems and its *in vivo* steroidogenetic activity was 148.2 IU/mg.

In 1971, Graf, *et al.*[4] revised the amino acid sequence of porcine adrenocorticotropic hormone (ACTH). The new sequence (I) differs from the one previously published by Shepherd, *et al.*[5] in 1956 only in the location of one amide group; Asn in position 25 instead of Asp and Glu in position 30 instead of Gln. Shortly after this publication, the revised structure of porcine ACTH and that of human ACTH were published by Riniker, *et al.*[6] They have arrived at the same conclusion with regard to the position of amide group. This amide group has the tendency to undergo preferential deamination under alkaline conditions. The synthesis of the nonatriacontapeptide corresponding to the 1956 formula of porcine ACTH was reported by Schwyzer and Sieber[7] in 1963. They utilized protecting groups removable by trifluoroacetic acid for this synthesis.

Our synthetic route to the nonatriacontapeptide corresponding to the newly revised amino acid sequence of porcine ACTH is illustrated in Fig. 1. An available synthetic intermediate of porcine ACTH-like intermediate lobe peptide (CLIP),[8] served as a starting amino component to the present synthesis. To the C-terminal eicosapeptide (II),[8b] three peptide subunits (III, IV, V) possessing Gly or Pro at the C-terminus were successively condensed. To the resulting pentatriacontapeptide, the N-terminal tetrapeptide unit (VI) was condensed by the

1) Part LVIII: H. Yajima, K. Kitagawa, T. Segawa, and T. Nakata, *Chem. Pharm. Bull.* (Tokyo), **24**, 544 (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.
3) Location: *a) Sakyo-ku, Kyoto; b) Fukushima, Osaka.*
4) L. Graf, S. Bajusz, A. Patthy, E. Barat, and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **6**, 415 (1971).
5) R.G. Shepherd, S.D. Willson, K.S. Howard, P.H. Bell, D.S. Davis, S.B. Davis, E.A. Eiger, and N.E. Shekespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1956).
6) B. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature New Biol.*, **235**, 114 (1972).
7) R. Schwyzer and P. Sieber, *Nature*, **199**, 172 (1963); *idem*, *Helv. Chim. Acta*, **49**, 134 (1966).
8) *a)* A.P. Scott, J.G. Ratcliffe, L.H. Rees, J. Landon, H.P.J. Bennett, P.J. Lowry, and C. McMartin, *Nature New Biol.*, **244**, 65 (1973); A.P. Scott, P.J. Lowry, H.P.J. Bennett, and C. McMartin, *J. Endocr.*, **61**, 369 (1974); *b)* K. Koyama, Y. Mori, Y. Kiso, S. Hirabayashi, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2301 (1975).

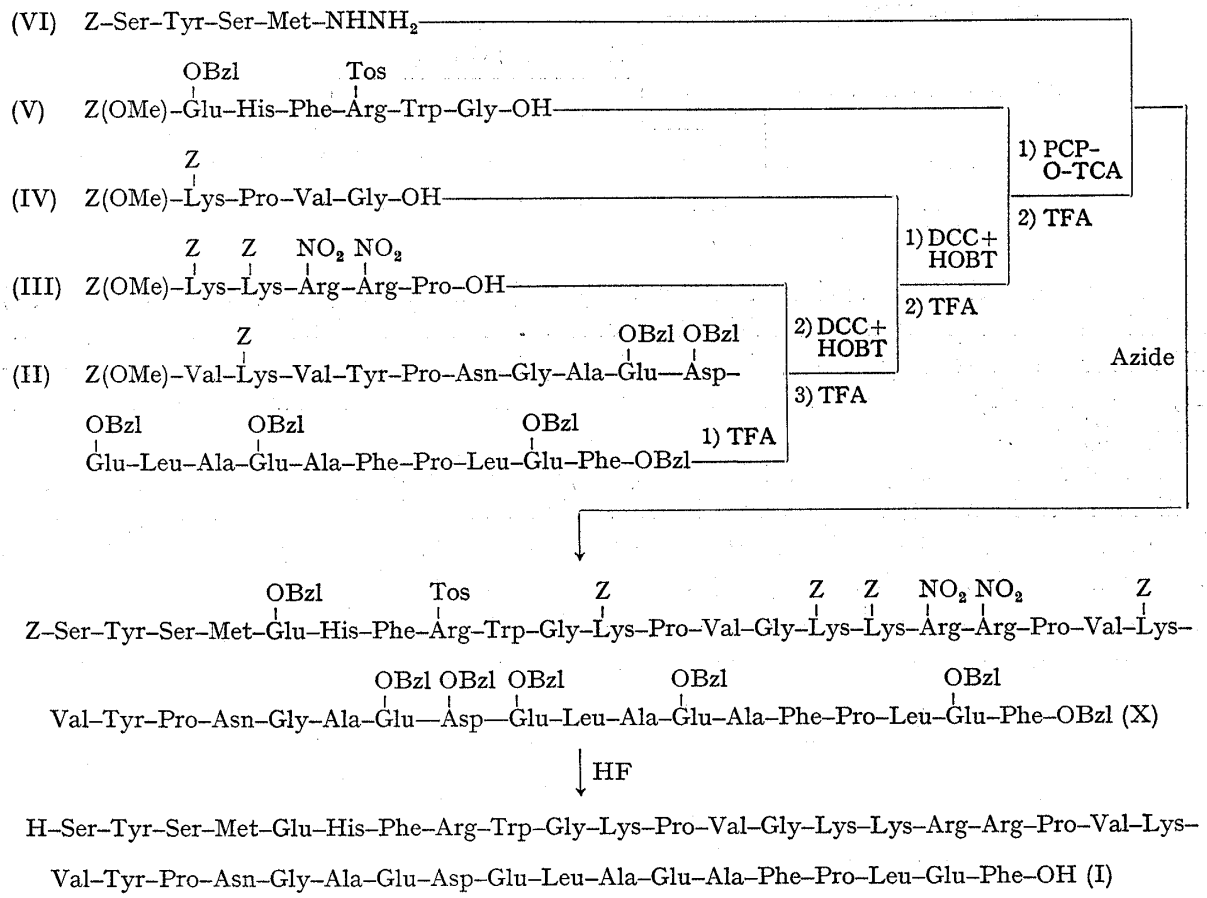

Fig. 1. Synthetic Route to Porcine ACTH

azide procedure. With this procedure, the risk of racemization during the fragment condensation was avoided.

For the present synthesis, amino acid derivatives bearing protecting groups removable by hydrogen fluoride[9] were employed; *i.e.*, Arg(NO_2), Arg(Tos), Lys(Z), Glu(OBzl) and Asp(OBzl). These side chain protecting groups survive mostly intact under careful trifluoroacetic acid (TFA) treatment for the removal of the Z(OMe) group[10] employed as a temporary α-amino protecting group.

For the synthesis of the N-terminal tetrapeptide hydrazide, Z–Ser–Tyr–Ser–Met–$NHNH_2$ (VI),[11] the previous route was slightly modified as shown in Fig. 2. Z(OMe)–Ser–$NHNH_2$ was condensed with H–Met–OH by the modified azide procedure[12] to give Z(OMe)–Ser–Met–OH, which after treatment with TFA, was condensed with Z–Ser–Tyr–$NHNH_2$[13] *via* the same azide procedure to give the known protected tetrapeptide, Z–Ser–Tyr–Ser–Met–OH.[14] This was converted to the corresponding hydrazide (VI) in the usual manner through its methyl ester. Hofmann, *et al.*[13] and Li, *et al.*[11] prepared this protected tetrapeptide ester by the direct coupling of Z–Ser–Tyr–$NHNH_2$ and H–Ser–Met–OMe.

9) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
10) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962); H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970); H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *ibid.*, **21**, 1380 (1973).
11) C.H. Li, J. Meienhofer, E. Schnabel, D. Chung, T.B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **83**, 4449 (1961).
12) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
13) K. Hofmann, A. Johl, A.E. Furlenmeier, and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957).
14) H. Yajima, K. Kawasaki, Y. Okada, H. Minami, K. Kubo, and I. Yamashita, *Chem. Pharm. Bull.* (Tokyo) **16**, 919 (1968).

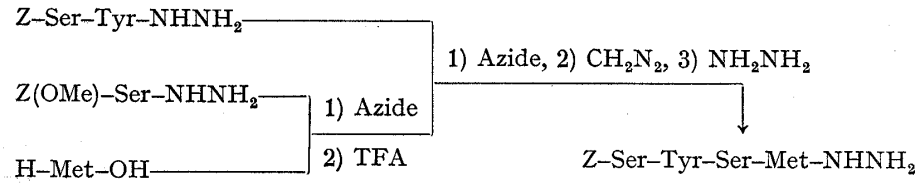

Fig. 2. Synthetic Route to the protected Tetrapeptide Hydrazide, Z–(1—4)–$NHNH_2$ (VI)

The protected hexapeptide, Z(OMe)–Glu(OBzl)–His–Phe–Arg(Tos)–Trp–Gly–OH (V), available from our previous synthesis of porcine β-melanocyte-stimulating hormone,[15] was utilized for the present synthesis, since the starting tetrapeptide, Z–Phe–Arg(Tos)–Trp–Gly–OH, can be easily prepared by the azide condensation of Z–Phe–Arg(Tos)–$NHNH_2$ with H–Trp–Gly–OH.

The protected tetrapeptide, Z(OMe)–Lys(Z)–Pro–Val–Gly–OH (IV), corresponding to positions 11 to 14 of (I), was prepared in the similar manner as described in the preparation of the corresponding Boc-derivative[16,17] as shown in Fig. 3. The dicyclohexylcarbodiimide (DCC) condensation of Z(OMe)–Lys(Z)–OH with H–Pro–Val–Gly–OMe[18] gave Z(OMe)–Lys(Z)–Pro–Val–Gly–OMe, which was then saponified by alkali to give (IV). Its homogeneity was assessed by elemental analysis and acid hydrolysis.

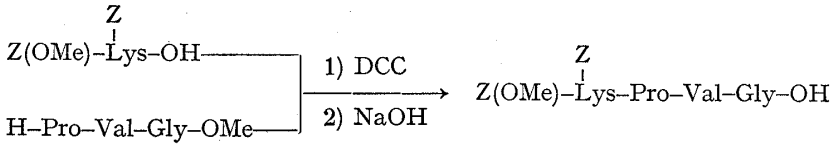

Fig. 3. Synthetic Route to the protected Tetrapeptide, Z(OMe)–(11—14)–OH (IV)

The next pentapeptide unit, Lys–Lys–Arg–Arg–Pro, corresponding to positions 15 to 19, is also a unit used for the synthesis of ACTH active peptide and analogues.[7,11,16,19] We have prepared this pentapeptide in the form of Z(OMe)–Lys(Z)–Lys(Z)–Arg(NO_2)–Arg(NO_2)–Pro–OH (III) by the azide coupling of Z(OMe)–Lys(Z)–Lys(Z)–$NHNH_2$ and H–Arg(NO_2)–Arg(NO_2)–Pro–OH as shown in Fig. 4. The former hydrazide was prepared by the DCC coupling of Z(OMe)–Lys(Z)–OH and H–Lys(Z)–OMe followed by treatment of the resulting protected dipeptide ester with hydrazine hydrate in the usual manner. The latter tripeptide was prepared by saponification followed by the hydrogen bromide treatment of the known protected tripeptide ester, Z–Arg(NO_2)–Arg(NO_2)–Pro–OMe.[7,16,20] Homogeneity of (III) was confirmed by elemental analysis and thin-layer chromatography.

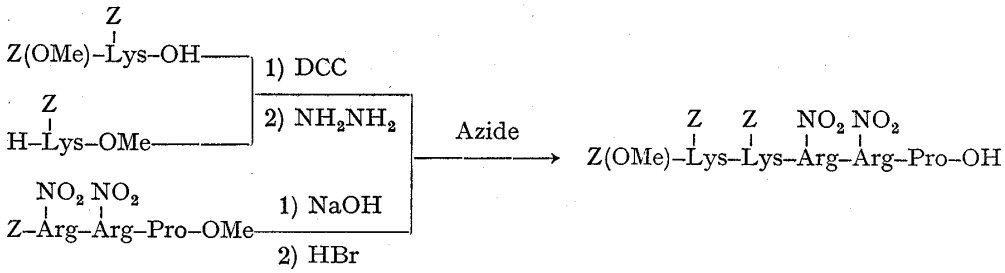

Fig. 4. Synthetic Route to the protected Pentapeptide, Z(OMe)–(15—19)–OH (III)

15) H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).
16) M. Fujino, O. Nishimura, and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **17**, 2135 (1969).
17) H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).
18) K. Hofmann, E.Stutz, G. Spuler, H. Yajima, and E.T. Schwart, *J. Am. Chem. Soc.*, **82**, 3727 (1960).
19) J. Meienhofer and C.H. Li, *J. Am. Chem. Soc.*, **84**, 2434 (1962); S. Bajusz, K. Mediharadszky, Z. Paulay, and Z. Lang, *Acta, Chim. Acad. Sci. Hung.*, **52**, 335 (1967).
20) K. Sturm, R. Geiger, and W. Siedel, *Chem. Ber.*, **96**, 609 (1963).

Assembling of five peptide subunits obtained as outlined above were then performed according to the scheme illustrated in Fig. 1. The protected eicosapeptide ester, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu-(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, (II, abbreviated as Z(OMe)-(20—39)-OBzl),[8b] was treated with TFA in the presence of anisole and the resulting trifluoroacetate was converted to the corresponding hydrochloride, which after neutralization, was condensed with Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OH (III) (1.5 equiv.) by DCC in the presence of N-hydroxybenzotriazole (HOBT).[21] The latter additive suppressed the formation of an acylurea, known as the side product of DCC. The resulting protected pentacosapeptide ester, Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (VII, abbreviated as Z(OMe)-(15—39)-OBzl), was purified by column chromatography on silica, using the solvent system of $CHCl_3$-MeOH-H_2O (8:3:1). This solvent system was useful in detecting the purity of every intermediate in the latter synthesis also.

Similar technique used above in the deprotection of the Z(OMe) group, coupling reaction and subsequent purification were extended to the next chain elongation reaction. Condensation reaction of Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (IV) and the α-deprotected pentacosapeptide ester (VII) by the DCC plus HOBT procedure went smoothly. It should be recalled that condensation of Z-Lys(For)-Pro-Val-Gly-OH by DCC alone resulted in the predominant formation of the acylurea.[22] The resulting protected nonacosapeptide ester, Z(OMe)-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (VIII, abbreviated as Z(OMe)-(11—39)-OBzl), after deprotection with TFA, was submitted to the coupling reaction with Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-OH (V). Rink and Riniker[23] pointed out that DCC has a tendency to mask the N^{im}-function of the His residue and this side reaction is accelerated in the presence of HOBT. Therefore the pentachlorophenyl trichloroacetate (PCP-O-TCA) procedure[24] was selected for this coupling reaction. It seems worthwhile to note that our synthetic deblocked nonatriacontapeptide initially prepared by the DCC plus HOBT procedure at this step exhibited essentially no activity. By usual analytical tools, it was not easy to identify the presence of one mole of DCC attached at a site of such a relatively large peptide.

The resulting protected pentatriacontapeptide ester, Z(OMe)-Glu(OBzl)-His-Phe-Arg-(Tos)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (IX, abbreviated as Z(OMe)-(5—39)-OBzl) was treated with TFA as stated above and then submitted to the final coupling reaction with Z-Ser-Tyr-Ser-Met-$NHNH_2$ by the modified azide procedure. Purification of the resulting fully protected nonatriacontapeptide ester, Z-Ser-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Lys-(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (X, abbreviated as Z-(1—39)-OBzl), was achieved by column chromatography on silica as described above. The purified peptide exhibited a single spot on thin-layer chromatography. Its homogeneity was further assessed by elemental analysis and hydrolysis with 3N *p*-toluenesulfonic acid.[25]

According to Sakakibara, *et al.*,[9] protecting groups employed, Z, Tos, NO_2 and Bzl groups, were deblocked from Z-(1—39)-OBzl by hydrogen fluoride at 0°. Anisole, Trp and Met

21) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
22) K. Hofmann, T.Y. Liu, H. Yajima, N. Yanaihara, and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961).
23) H. Rink and B. Riniker, *Helv. Chim. Acta*, **57**, 831 (1974).
24) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
25) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **216**, 2842 (1971).

were added as scavengers. The excess hydrogen fluoride was evaporated *in vacuo*. The product was soon converted to the corresponding acetate by Amberlite IR-4B and incubated with dithiothreitol[26] at 50° for 6 hr to reduce Met–sulfoxide formed possibly during the above treatment. The deblocked peptide was passed through a column of Sephadex G-25 to remove scavengers and then purified by column chromatography on CM-Sephadex. After the column was eluted with water, gradient elution was established using 0.3M ammonium acetate buffer at pH 6.9. A single peak was detected in the gradient eluates, which were examined by measurement of the absorbancy at 280 mμ. Ammonium acetate was mostly removed from the desired fractions by Sephadex G-25 and finally by repeated lyophilization. The fluffy powder thus obtained exhibited a single spot on thin-layer chromatography and behaved as a single component in the field of disc electrophoresis on polyacrylamide gel at two different pH values. The hydrolysate with 3N *p*-toluenesulfonic acid contained the constituent amino acids in ratios predicted by theory. The presence of Trp was confirmed by the above hydrolysis and further by enzymatic digestion. By increasing the amount of aminopeptidase (AP-M),[27] it was possible to digest this Pro-containing nonatriacontapeptide completely. In this amino acid analysis, the Asn peak was overlapped with that of Ser. From the difference of the Asp recovery between acid and enzymatic hydrolysates and that of Ser recovery, the presence of the Asn residue in our synthetic peptide could be confirmed.

Experimental evidences cited above may justify the conclusion that our synthetic peptide which embodies the entire amino acid sequence of porcine ACTH is homogeneous and possesses the L-configuration of the constituent amino acids. When our synthetic peptide was compared by thin-layer chromatography with natural porcine ACTH (supplied by Dr. S. Lande, Yale university), both peptides exhibited the identical *Rf* values in two different solvent systems.

The *in vivo* steroidogenetic activity of our synthetic peptide, after comparison to that of natural porcine ACTH (99.4 IU/mg) and that of [1-Gly]-ACTH (1—18)-NH_2 (161 IU/mg),[28] was judged as 148.2 IU/mg. The peptide initially synthesized by applying the DCC+HOBT condensation of (V) as stated above exhibited only the *in vivo* activity of 1.3—4.4 IU/mg. Treatment of this peptide with methanol–2N acetic acid (4: 1) at 60° for 4 hr, brought the activity up to 20 IU/mg. The result suggests that the N,N′-dicyclohexylamidino moiety presumably attached at the His residue was partially removed during the above treatment. Since removal of this residue from a relatively large peptide seems to proceed in somewhat slow rate, it is evident as pointed out by Rink and Riniker[23] that the DCC condensation reaction of peptides containing the N^{im}-unprotected His residue should be performed with great care, especially when this residue participates as an essential part of biologically active peptides.

Experimental

Thin-layer chromatography was performed on silica gel (Kieselgel 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–AcOH (9: 1: 0.5), Rf_3 $CHCl_3$–MeOH–H_2O (45: 10: 1), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24). Rf_5 *n*-BuOH–pyridine–AcOH–H_2O (4: 1: 1: 2).

Z(OMe)-Ser-Met-OH——To a solution of Z(OMe)-Ser-$NHNH_2$ (7.08 g) in DMF (70 ml), 2.5N HCl–DMF (22 ml) and isoamyl nitrite (3.5 ml) were added under cooling with ice–NaCl and the mixture was stirred for 5 min, when the hydrazine test became negative. The solution, after neutralization with Et_3N (7.0 ml), was combined with a solution of H-Met-OH (7.46 g) in H_2O (70 ml) containing Et_3N (10.5 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in 5% citric acid. The resulting precipitate was extracted with AcOEt. The organic phase was washed with H_2O–NaCl,

26) D. Yamashiro and C.H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
27) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963). Complete digestion of the synthetic peptide (0.1 μmole) was achieved by AP-M (2U) purchased from Rohm & Haas (Lot. No. 191226).
28) H. Otsuka, M. Shin, Y. Kinomura and K. Inouye, *Bull. Chem. Soc. Japan*, 43, 196 (1970)

dried over Na_2SO_4 and then evaporated. The solid residue was recrystallized from MeOH; yield 7.20 g (72%), mp 77—78°, $[\alpha]_D^{26}$ −10.5° (c=0.7, MeOH), Rf_1 0.27. *Anal.* Calcd. for $C_{17}H_{24}O_7N_2S$: C, 50.99; H, 6.04; N, 7.00. Found: C, 51.19; H, 6.15; N, 6.77.

Z-Ser-Tyr-Ser-Met-OH——Z(OMe)-Ser-Met-OH (12.0 g) was treated with TFA (20 ml) in the presence of anisole (5 ml) in an ice-bath for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in a mixture of H_2O and DMF (50 ml). To this ice-cold solution, Et_3N (8.4 ml) and the azide (prepared from 12.50 g of Z-Ser-Tyr-$NHNH_2$[13] with 15 ml of 4N HCl-DMF, 4.42 ml of isoamyl nitrite and 8.4 ml of Et_3N as described above) were combined and the mixture was stirred at 4° for 48 hr. The product was purified as described previously; yield 11.33 g (61%), mp 193—196°, $[\alpha]_D^{25}$ −5.5° (c=0.7, DMF) (lit.[14] mp 194—196°, $[\alpha]_D$ −7.7° in DMF). Rf_1 0.12. *Anal.* Calcd. for $C_{28}H_{36}O_{10}N_4S\cdot H_2O$: C, 52.74; H, 6.01; N, 8.79. Found: C, 52.84; H, 5.96; N, 8.56.

Z-Ser-Tyr-Ser-Met-OMe——An ethereal solution of diazomethane was added to a solution of Z-Ser-Tyr-Ser-Met-OH (2.40 g) in MeOH (40 ml). The yellow color was persisted for 5 min, when a few drop of AcOH was added. The solvent was evaporated and the solid residue was recrystallized from MeOH and ether; yield 2.33 g (89%), mp 182—184°, $[\alpha]_D^{26}$ −38.8° (c=1.0, MeOH). (lit.[13] mp 190°, $[\alpha]_D$ −39.0° in MeOH. The title compound was prepared by the azide condensation of Z-Ser-Tyr-$NHNH_2$ and H-Ser-Met-OMe). *Anal.* Calcd. for $C_{29}H_{38}O_{10}N_4S\cdot 1/2H_2O$: C, 54.11; H, 6.11; N, 8.70. Found: C, 53.95; H, 6.03; N, 8.80.

Z-Ser-Tyr-Ser-Met-$NHNH_2$ (VI)——The title compound was prepared according to Li, *et al.*[11] mp 222—223° (lit.[11] mp 245—247°). *Anal.* Calcd. for $C_{28}H_{38}O_9N_6S\cdot H_2O$: C, 51.52; H, 6.17; N, 12.87. Found: C, 51.40; H, 5.83; N, 12.80.

Z(OMe)-Lys(Z)-Pro-Val-Gly-OMe——Z-Pro-Val-Gly-OMe[17] (4.19 g) in tetrahydrofuran (THF) (50 ml) containing 1N HCl (10 ml) was hydrogenated over a Pd catalyst in the usual manner until the evolution of CO_2 ceased. The solution was filtered and the filtrate was condensed to about one-third of the original volume *in vacuo*. The residue was dissolved in DMF (50 ml) and Et_3N (1.4 ml) was added. This solution was combined with a solution of Z(OMe)-Lys(Z)-OH (4.44 g) and DCC (2.3 g) in THF (40 ml). After the mixture was stirred at room temperature for 24 hr, the solution was filtered, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% Na_2CO_3, 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue turned to the solid by treatment with ether; yield 5.20 g (73%), mp 114—117°, $[\alpha]_D^{26}$ −37.6° (c=0.8, DMF). *Anal.* Calcd. for $C_{34}H_{47}O_8N_5\cdot H_2O$: C, 60.78; H, 7.35; N, 10.42. Found: C, 60.76; H, 7.15; N, 10.56.

Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (IV)——Z(OMe)-Lys(Z)-Pro-Val-Gly-OMe (5.40 g) in MeOH (25 ml) was treated with 1N NaOH (10 ml) at room temperature for 60 min. The solvent was evaporated *in vacuo* at 28° and the residue was dissolved in H_2O. The aqueous phase was washed with ether and then acidified with citric acid. The resulting precipitate was extracted with AcOEt, which after washing with H_2O–NaCl, was evaporated. The residue was triturated with petroleum ether; yield 4.96 g (94%), mp 77—82°, $[\alpha]_D^{25}$ −29.7° (c=0.8, DMF). Amino acid ratios in an acid hydrolysate Lys 1.04, Pro 1.05, Val 1.00, Gly 1.00 (average recovery 91%). *Anal.* Calcd. for $C_{33}H_{45}O_8N_5\cdot 1.5H_2O$: C, 59.45; H, 7.22; N, 10.50. Found: C, 59.38; H, 7.23; N, 10.01.

Z-Arg(NO_2)-Arg(NO_2)-Pro-OH——The title compound was prepared according to Geiger, *et al.*[20] mp 134—136°, $[\alpha]_D^{26}$ −34.4° (c=0.8, DMF). (lit.[20] mp 105°, $[\alpha]_D$ −37.7° in MeOH). *Anal.* Calcd. for $C_{25}H_{37}O_{10}N_{11}\cdot 1/2C_2H_5OH$: C, 46.27; H, 6.00; N, 22.81. Found: C, 46.54; H, 6.13; N, 22.89.

Z(OMe)-Lys(Z)-Lys(Z)-OMe——To a solution of H-Lys(Z)-OMe (prepared from 9.92 g of the hydrochlo- stirring. ride and 4.2 ml of Et_3N) in DMF (160 ml), Z(OMe)-Lys(Z)-OH (13.34g) and DCC (7.42 g) were added with The mixture was stirred at room temperature for 24 hr and then filtered. The filtrate was condensed and the residue was dissolved in AcOEt, which after washing successively with 10% citric acid, 5% Na_2CO_3 and H_2O- NaCl, was dried over Na_2SO_4 and then evaporated. The resulting gelatinous mass was precipitated from MeOH with ether; yield 13.50 g (63%), mp 103—105°, $[\alpha]_D$ +4.0° (c=0.5, DMF). Rf_2 0.87. *Anal.* Calcd for $C_{38}H_{48}O_{10}N_4$: C, 63.32; H, 6.71; N, 7.77. Found: C, 63.42; H, 6.97; N, 7.96.

Z(OMe)-Lys(Z)-Lys(Z)-$NHNH_2$——To a solution of Z(OMe)-Lys(Z)-Lys(Z)-OMe (12.0 g) in MeOH (100 ml), 80% hydrazine hydrate (5.1 ml) was added. The gelatinous mass formed on standing at room temperature overnight, was collected by filtration and recrystallized from MeOH; yield 9.50 g (80%), mp 179—180°, $[\alpha]_D^{26}$ −1.8° (c=1.1, DMF). Rf_2 0.43. *Anal.* Calcd. for $C_{37}H_{48}O_9N_6$: C, 61.65; H, 6.71; N, 11.66. Found: C, 61.65; H, 6.78; N, 11.49.

Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OH (III)——Z-Arg(NO_2)-Arg(NO_2)-Pro-OH (1.50 g) was treated with 25% HBr in AcOH (5 ml) at room temperature for 1.5 hr and dry ether was added. The solid precipitate was collected by filtration, dried over KOH pellets *in vacuo* overnight and then dissolved in DMF (10 ml). The pH of the solution was adjusted to 8 with Et_3N (0.6 ml). To this solution, was added Z(OMe)-Lys(Z)-Lys(Z)-azide (prepared from 1.65 g of the hydrazide with 4.6 ml of 1N HCl–DMF, 0.3 ml of isoamyl nitrite and 0.6 ml of Et_3N) in DMF (15 ml). After the mixture was stirred at 4° for 48 hr, a few drop of AcOH was added and the solvent was evaporated *in vacuo*. Addition of AcOEt to the residue gave fine powder, which after washing with AcOEt, 10% citric acid and H_2O, was precipitated from MeOH with ether; yield 1.98 g (72%), mp 96—98°, $[\alpha]_D^{26}$ −23.7° (c=0.7, DMF). Rf_2 0.49. Amino acid ratios in an acid hydrolysate

Lys 1.88, Pro 1.00 (average recovery 94%). *Anal.* Calcd. for $C_{54}H_{75}O_{17}N_{15}$: C, 53.77; H, 6.27; N, 17.42. Found: C, 53.96; H, 6.41; N, 16.89.

Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(15—39)-OBzl (VII)——Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl[8-6] (3.08 g) was treated with TFA (10 ml) in the presence of anisole (2 ml) in an ice-bath for 60 min. Dry ether was added and the resulting powder was collected by filtration and then dissolved in a small amount of DMF. After addition of 3N HCl–dioxane (0.5 ml), dry ether was added and the resulting powder was collected. This hydrochloride was again dissolved in a small amount of dioxane and Et_3N (0.14 ml) was added. Addition of dry ether afforded the free base as fine powder, which was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml). To this solution, Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OH (2.41 g), HOBT (0.54 g) and DCC (0.62 g) were successively combined. The mixture was stirred at room temperature for 72 hr. The solution, after filtration, was condensed *in vacuo*. The residue was treated with AcOEt and the resulting powder was washed batchwisely with 3% $NaHCO_3$, 5% citric acid and H_2O and then dissolved in a small amount of the solvent of $CHCl_3$–MeOH–H_2O (8:3:1). The solution was applied to a column of silica (3×15 cm), which was eluted with the same solvent system. Fractions containing the substance of Rf_1 0.52 were combined and the solvent was evaporated. The residue was treated with H_2O and the resulting fine powder was precipitated from THF with MeOH; yield 2.53 g (61%), mp 234—237°, $[\alpha]_D$ −15.6° (c=0.3, DMF). Amino acid ratios in an acid hydrolysate: Lys 2.94, Arg 1.52, Pro 3.05, Val 2.01, Tyr 0.52, Asp 2.00, Glu 4.38, Gly 1.00, Ala 3.25, Phe 2.22, Leu 2.24 (average recovery 85%). *Anal.* Calcd. for $C_{208}H_{267}O_{51}N_{37}\cdot 3H_2O$: C, 60.12; H, 6.62; N, 12.47. Found: C, 60.44; H, 6.38; N, 12.09.

Z(OMe)-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(11—39)-OBzl (VIII)——The above Z(OMe)-(15—39)-OBzl (1.64 g) was treated with TFA (8 ml) in the presence of anisole (1.5 ml) in an ice-bath for 60 min. The resulting TFA salt was converted, as stated above, to the corresponding hydrochloride using 3N HCl–dioxane (0.5 ml) and subsequently neutralized with Et_3N (0.06 ml). The free base thus obtained was dissolved in DMF (10 ml). To this solution, Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (0.56 g), HOBT (0.16 g) and DCC (0.21 g) were combined and the mixture was stirred at room temperature for 72 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was purified as stated above first by batchwise washing and then column chromatography on silica using the solvent system of $CHCl_3$–MeOH–H_2O (8:3:1). The product was finally precipitated from THF with MeOH; yield 1.42 g (81%), mp 250° decomp. $[\alpha]_D$ −30.3° (c=0.4, DMF). Amino acid ratios in an acid hydrolysate: Lys 3.83, Pro 3.74, Val 2.95, Gly 1.89, Arg 1.54, Tyr 0.50, Asp 2.00, Glu 4.37, Ala 3.21, Leu 2.23, Phe 2.15 (average recovery 85%). Rf_1 0.59. *Anal.* Calcd. for $C_{234}H_{304}O_{57}N_{42}4H_2O$: C, 59.94; H, 6.71; N, 12.54. Found: C, 59.98; H, 6.50; N, 12.24.

Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(5—39)-OBzl (IX)——Z(OMe)-(11—39)-OBzl (VIII) (1.15 g) was treated with TFA (2.3 ml) in the presence of anisole (0.6 ml) in an ice-bath for 45 min. As stated above, the resulting TFA salt was converted to the corresponding hydrochloride with 4.12N HCl–DMF (0.3 ml) and this hydrochloride was subsequently neutralized with Et_3N (0.3 ml). The free base precipitated from DMF with ether, was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (10 ml). This solution was kept under cooling with ice, until the following active ester was ready. To a solution of Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-OH (0.45 g) in DMF (4.5 ml), Et_3N (0.04 ml) and PCP-O-TCA (0.15 g) were added and the mixture was stirred at room temperature for 30 min. Thin-layer chromatographic examination revealed the appearance of a new spot of Rf_3 0.65 and the spot corresponding to the starting material disappeared. This solution was combined with the above solution containing the nonacosapeptide ester and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was purified as stated above by batchwise washing and column chromatography on silica (2.5×20 cm) using $CHCl_3$–MeOH–H_2O (8:3:1). The product was finally precipitated from DMF with MeOH; yield 0.87 g (61%), mp 228—230°, $[\alpha]_D^{26}$ −22.9° (c=0.4, DMF), Rf_3 0.50. Amino acid ratios in a Tos-OH hydrolysate: Glu 5.26, His 0.88, Phe 3.28, Arg not determined, Trp 1.13, Gly 2.80, Lys 4.32, Pro 3.76, Val 2.83, Asp 2.00, Tyr 0.82, Ala 3.18. Leu 2.00 (average recovery 87%). *Anal.* Calcd. for $C_{237}H_{364}O_{67}N_{54}S\cdot 3H_2O$: C, 60.17; H, 6.51; N, 13.12. Found: C, 60.36; H, 6.51, N, 12.94.

Z-Ser-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(1—39)-OBzl (X)——Z(OMe)-(5—39)-OBzl (IX) (0.85 g) was treated as usual with TFA (1.7 ml) in the presence of anisole (0.5 ml) in an ice bath for 45 min. Dry ether was added and the resulting TFA salt was collected by filtration, dried over NaOH pellets *in vacuo* for 3 hr and then dissolved in DMF (3 ml) containing Et_3N (0.08 ml). To this ice cold solution, the azide (prepared according to Honzl and Rudinger from 0.29 g of Z-Ser-Tyr-Ser-Met-$NHNH_2$ with 0.28 ml of 3.78N HCl–DMF,

0.07 ml of isoamylnitrite and 0.15 ml of Et_3N) in DMF (5 ml) was combined and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with H_2O. The resulting powder was purified first by batchwise washing and then column chromatography on silica (5.5×10 cm) using $CHCl_3$–MeOH–H_2O (8: 3: 1) as stated above. The product was precipitated from DMF with MeOH; yield 0.67 g (73%), mp 236—238°, $[\alpha]_D^{26}$ −30.3° (c=0.4, DMF), Rf_1 0.53, Amino acid ratios in a Tos-OH hydrolysate: Ser 1.71, Tyr 1.67, Met 0.70, Glu 5.34, His 0.78, Phe 3.00, Arg not determined, Trp 0.85, Gly 2.95, Lys 4.34, Pro 3.77, Val 2.77, Asp 2.03, Ala 3.24, Leu 2.00 (average recovery 85%). *Anal.* Calcd. for $C_{306}H_{390}O_{73}N_{58}S_2 \cdot 2H_2O$: C, 59.77; H, 6.46; N, 13.25. Found: C, 60.01; H, 6.48; N, 13.00.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (I)——The above protected nonatriacontapeptide ester (X) (160 mg) was treated with HF (approximately 7 ml) in the presence of anisole (1 ml), Trp (100 mg) and Met (100 mg) in an ice-bath for 60 min. The excess HF was removed by evaporation *in vacuo* at 0° and the residue was dissolved in H_2O (10 ml), which was treated with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration and the filtrate, after incubation with dithiothreitol (100 mg) at 50° for 6 hr, was lyophilized. The resulting powder was dissolved in 0.5N AcOH (5 ml) and the solution was applied to a column of Sephadex G-25 (2.6×55 cm), which was eluted with 0.5N AcOH. Individual fractions (5 ml each) were collected and absorbancy at 280 mμ was determined. The fractions corresponding to the front peak(tube 35—49) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 104 mg (deblocking step 87%). This powder (100 mg) was dissolved in H_2O (10 ml) and the solution was applied to a column of CM-Sephadex (2.3×1.5 cm), which was eluted with H_2O (100 ml) and then with 0.3M ammonium acetate through a mixing flask containing H_2O (100 ml). Individual fractions (4 ml each) were collected and the absorbancy at 280 mμ was determined. Fractions corresponding to the main peak (tube No 42—54 in Fig. 5) were combined and the solution was condensed at 40° to approximately 5 ml. This solution was then applied to a column of Sephadex G-25 (2.6×55 cm), which was eluted with 0.5N AcOH. The desired fractions were collected as described above and the product was finally lyophilized to give a fluffy white powder; yield 32 mg (over-all yield 27%), Rf_4 0.75, Rf_5 0.32 positive to ninhydrin, Ehrlich, methionine and Sakaguchi tests. Amino acid ratios in a 3N Tos-OH hydrolysate: Ser 1.74, Tyr 1.52, Met 0.98, Glu 5.33, His 0.86, Phe 3.12, Arg 3.06, Trp 0.85, Gly 2.99, Lys 3.74, Pro 4.16, Val 2.80, Asp 2.19, Ala 3.07, Leu 2.00 (average recovery 88%). Amino acid ratios in AP-M digest (theory is given in parenthesis): Ser+Asn 2.41 (3 calcd. as Ser), Tyr 1.81 (2), Met 0.73 (1), Glu 4.92 (5),His 0.85 (1), Phe 3.28 (3), Arg 2.82 (3), Trp 0.81 (1), Gly 3.00 (3), Lys 4.03 (4), Pro 4.02 (4), Val 3.54 (3), Asp 0.75 (1), Ala 3.32 (3), Leu 2.10 (2) (average recovery 74%). Disc electrophoretic mobility on 15% polyacrylamide gel (0.5×6.1 cm, 5mA/tube) at pH 8.3 (0.38M glycine–Tris buffer) was 1.5 cm after 55 min from the origin to the anode (indicator, bromophenol blue) and at pH 4.0 (0.3M glycine–AcOH buffer) was 2.9 cm after 130 min to the cathod (indicator, methyl green). $[\alpha]_D^{27}$ −80.2° (c=0.3, 1% AcOH). *Anal.* Calcd. for $C_{210}H_{314}N_{56}O_{57}S \cdot 7CH_3COOH \cdot 10H_2O$: C, 52.06; H, 7.06; N, 15.18. Found: C, 51.78; H, 7.19; N, 15.41.

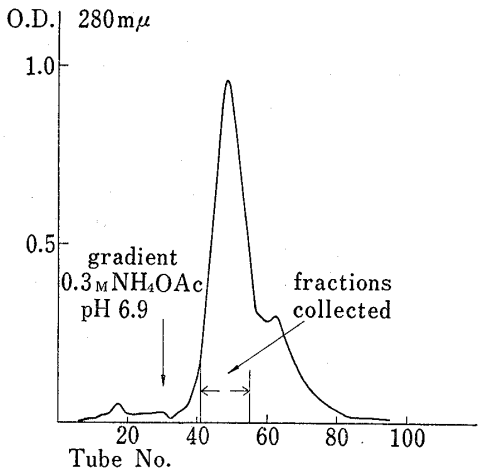

Fig. 5. Chromatographic Pattern of Synthetic Porcine ACTH on CM-Sephadex

Comparison of Rf values of synthetic peptide with natural porcine ACTH was performed on thin-layer chromatography in two different solvent systems at 26°. Both peptides exhibited Rf_4 0.75 and Rf_5 0.32 respectively.

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