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Synthesis of Corticotropin Peptides. XII. The Effect of Amino-terminal Substitution in the Corticotropin 1-18 Peptide

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The synthesis and biological properties are described of an octadecapeptide amide, α-aminoisobutyryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide, corresponding to the first eighteen amino acid residues of corticotropin (ACTH) except for the amino terminal residue. The amino-terminal decapeptide as intermediate is built up from a tripeptide (positions 1—3) and a heptapeptide (4—10), where a benzyloxycarbonyl and a benzyl ester groups are used for protection of the terminal α-amino group and the γ-carboxyl group of 5-glutamic acid, respectively. These protecting groups are removed in the final step with hydrogen fluoride. The new method is successfully applied to the corresponding 1-β-alanine octadecapeptide amide, which has been synthesized previously by a different procedure. Adrenal-stimulating properties of the 1-α-aminoisobutyric acid peptide are compared with those of the 1-β-alanine peptide to show that the former peptide is more active than the latter when assayed by the adrenal-ascorbic acid depletion and the *in vivo* steroidogenesis methods. The lipotropic activity of the new peptide is also shown to be more prolonged than that of the 1-β-alanine peptide.

In 1965 we reported the synthesis of octadecapeptides, ACTH(1-18)-OH(Ia) and ACTH(1-18)-NH_2 (Ib), corresponding to the first eighteen amino acid residues of corticotropin (ACTH).[1] The amide analog (Ib) was also described by Ramachandran *et al.*[2] and Bajusz and Medzihradszky,[3] independently. Later the synthesis of the 1-glycine analog of Ib, [1-glycine]-ACTH(1-18)-NH_2 (Ic) was reported from this laboratory.[4] From synthetic and biological data on corticotropin peptides, including those of our own work, which have been compiled over the past decade[5] we have learned that the aminoterminal eighteen residues fulfill the minimal requirements for eliciting hig hadrenal-stimulating activity. The corresponding peptides Ia, Ib and Ic were, however, still found to be much less active than the native hormone, especially when they were administered for assays by subcutaneous or intramuscular routes.[1,6] This is probably associated with the fact that these peptides are inactivated very rapidly in the tissues, compared with the native hormone. In an effort to find out the mechanism of inactivation we have tried to improve the biological properties of octadecapeptide Ib by partially modifying its structure.

We synthesized [1-β-alanine]-ACTH(1-18)-NH_2 (Id) and found that the amino-terminal substitution by β-alanine caused not only remarkable enhancement but also prolonged duration of action of the adrenal-stimulating and the lipotropic activities over the corresponding 1-serine and 1-glycine peptides.[6,7] This finding offered evidence to support the view that the marked increase in biological

1) H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, *J. Biochem.* (Tokyo), **58**, 512 (1965).

2) J. Ramachandran, D. Chung and C. H. Li, *J. Amer. Chem. Soc.*, **87**, 2696 (1965).

3) S. Bajusz and K. Medzihradszky, "Peptides," Proceedings of the 8th European Peptide Symposium, ed. by H. C. Beyerman, A. van de Linde and W. Maassen van den Brink, North-Holland Publishing Co., Amsterdam (1967), p. 209.

4) H. Otsuka, M. Shin, Y. Kinomura and K. Inouye, This Bulletin, **43**, 196 (1970).

5) a) E. Schröder and K. Lübke, "The Peptides," Vol. 2, Academic Press, New York and London (1966), p. 194; b) J. Ramachandran and C. H. Li, *Advan. Enzymol.*, **29**, 391 (1967).

6) K. Inouye, A. Tanaka and H. Otsuka, This Bulletin, **43**, 1163 (1970).

7) [Gly^1]-ACTH(1-18)-NH_2, (Ic) and [β-Ala^1]-ACTH(1-18)-NH_2 (Id) were found to possess 6.7×10^8 MSH units/g and 9.6×10^9 MSH units/g, respectively, as assayed by the *in vitro* frog skin method;[8] ACTH-(1-24)-OH (Synacthen, Ciba) possessed 2.1×10^8 MSH units/g in a narallel determination. H. Otsuka, K. Kondo and K. Inouye, unpublished.

8) K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinol.*, **54**, 553 (1954); A. B. Lerner and M. R. Wright, Method of Biochemical Analysis, **8**, 295 (1960).

activities, which was reported by Boissonnas *et al.*[9] and by Kappeler *et al.*[10] to occur when the amino-terminal L-serine of corticotropin peptides was replaced by D-amino acids, is a consequence of the decreased susceptibility of peptides toward the action of intracellular aminopeptidase. Geiger *et al.*[11] have also reported that [1-β-alanine]-ACTH(1-23)-NH_2 possesses a high potency over the 1-L-serine analog. The present communication will describe the synthesis of an octadecapeptide amide, α-aminoisobutyryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide, [Ibu[1]]-ACTH(1-18)-NH_2 (Ie),[12] corresponding to the first eighteen amino acid residues of corticotropin except that the amino terminal serine in the native hormone has been replaced by α-aminoisobutyric acid.

R_1-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-R_2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

ACTH(1—18)-OH (Ia)[1]:

$R_1 = NH_2\text{-}C(CH_2OH)H\text{-}CO$, $R_2 = OH$

ACTH(1—18)-NH_2 (Ib)[1]:

$R_1 = NH_2\text{-}C(CH_2OH)H\text{-}CO$, $R_2 = NH_2$

[Gly[1]]-ACTH(1—18)-NH_2 (Ic)[4]:

$R_1 = NH_2\text{-}CH_2\text{-}CO$, $R_2 = NH_2$

[β-Ala[1]]-ACTH(1—18)-NH_2 (Id)[6]:

$R_1 = NH_2\text{-}CH_2\text{-}CH_2\text{-}CO$, $R_2 = NH_2$

[Ibu[1]]-ACTH(1—18)-NH_2 (Ie):

$R_1 = NH_2\text{-}C(CH_3)_2\text{-}CO$, $R_2 = NH_2$

α-Aminoisobutyric acid (α-methylalanine) has no hydrogen atom on the α-carbon and therefore belongs to the type of amino acids which forms peptide bonds resisting the action of aminopeptidase. If the current view is right, the amino-terminal substitution by α-aminoisobutyric acid in a corticotropin peptide should result in a remarkable improvement in its biological activities, to the same extent as that produced by D-amino acids[9,10] or β-alanine.[6,11] The present synthesis was undertaken to examine this point and to provide more evidence for the view.

In the present work a new procedure was applied to the synthesis of the decapeptide intermediate corresponding to positions 1—10 of Ie. The procedure is outlined in Fig. 1. A benzyloxycarbonyl group and a benzyl ester group were used to protect the amino group of position 1 and the γ-carboxyl group of glutamic acid residue at position 5, respectively. Formation of the decapeptide was performed by coupling of the tripeptide azide (positions 1—3) with the heptapeptide (4—10) which was synthesized stepwise from the glycine at position 10 *via* a free pentapeptide H-His-Phe-Arg-Trp-Gly-OH (VI)[13] as an intermediate. This procedure eliminates the possibility of partial oxidation by nitrous acid of methionine that may occur when the hydrazide of a methionine containing peptide is subjected to azide formation prior to coupling reaction. Because of the presence of benzyloxycarbonyl and benzyl ester groups the final step to generate the free octadecapeptide amide (Ie) was hydrogen fluoride treatment,[14] by which all the protecting groups were removed very smoothly. The procedure was also applied to the synthesis of 1-β-alanine peptide (Id), which has been prepared before by a different procedure,[6] in order to provide a proof of its utility.

Synthesis of [Ibu[1]]-Octadecapeptide Amide (Ie). The acylation of α-aminoisobutyric acid with benzyl chloroformate proceeded smoothly under the ordinary conditions to give Z-Ibu-OH (II) in moderate yield, whereas acylation with *t*-butyl azidoformate gave the desired Boc-Ibu-OH in a yield of only 9%, even when the reaction was continued for over a week in the presence of excess azidoformate. This is probably due to the presence of the additional α-methyl group, which will reduce the reactivity of α-amino group sterically. Attempts to prepare the Ibu-Tyr-Ser sequence were made by two different procedures. There was no difficulty in coupling compound II with tyrosine methyl ester by the DCC method. The resulting dipeptide ester was readily converted into the hydrazide Z-Ibu-Tyr-$NHNH_2$. In the subsequent steps, however, the products could not be characterized as the

9) a) R. A. Boissonnas, St. Guttmann and J. Pless, *Experientia*, **22**, 526 (1966); b) W. Doepfner, *ibid.*, **22**, 527 (1966); c) M. Jenny, A. F. Muller and R. S. Mach, *ibid.*, **22**, 528 (1966).

10) H. Kappeler, B. Riniker, W. Rittel, P. Desaulles, R. Maier, B. Schär and M. Staehelin, "Peptides," Proceedings of the 8th European Peptide Symposium, ed. by H. C. Beyerman, A. van de Linde and W. Maassen van den Brink, North-Holland Publishing Co., Amsterdam (1967), p. 214.

11) R. Geiger, H.-G. Schröder and W. Siedel, *Ann. Chem.*, **726**, 177 (1969).

12) All amino acid residues are of the L-configuration, unless otherwise indicated. The abbreviated designation of amino acids, peptides and their derivatives accords with the proposal of the IUPAC-IUB Commission of Biochemical Nomenclature, which appeared in *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967). Ibu: α-aminoisobutyric acid.

13) See Ref. 6. An alternative synthesis of VI with the use of N^G-tosyl-arginine will be described elsewhere.

14) S. Sakakibara and Y. Shimonishi, This Bulletin, **38**, 1412 (1965); S. Sakakibara, Y. Shimonishi, M. Okada and Y. Kishida, "Peptides," Proceedings of the 8th European Peptide Symposium, ed. by H. C. Beyerman, A. van de Linde and W. Maassen van den Brink, North-Holland Publishing Co., Amsterdam (1967), p. 44.

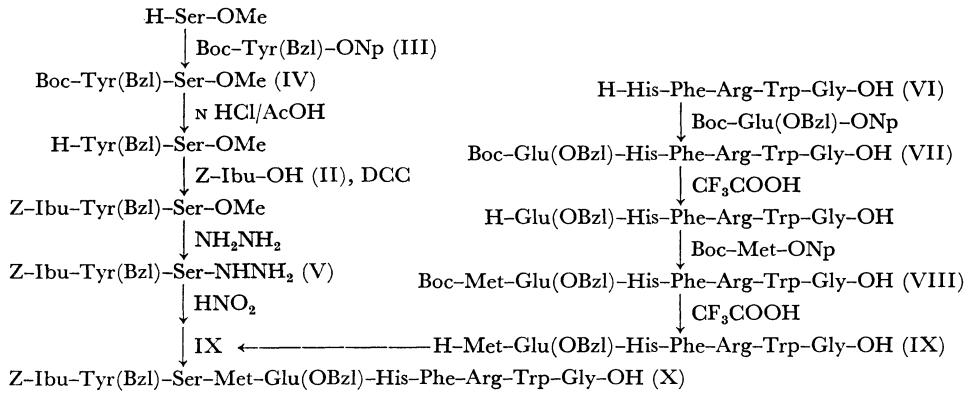

Fig. 1. Synthetic procedure of decapeptide intermediate X[12]

desired compounds, Z-Ibu-Tyr-Ser-OMe and its hydrazide. There must have been some complication in the azide coupling reaction. On the other hand, Boc-Tyr(Bzl)-ONp (III) was allowed to react with serine methyl ester to yield Boc-Tyr(Bzl)-Ser-OMe (IV). Compound II was then coupled by the DCC method with a crystalline dipeptide ester, derived from IV with N hydrogen chloride in acetic acid, to give a tripeptide Z-Ibu-Tyr(Bzl)-Ser-OMe, which was converted into the corresponding hydrazide (V).

A pentapeptide H-His-Phe-Arg-Trp-Gly-OH (VI), which was derived from Z-His-Phe-Arg-(NO_2)-Trp-Gly-OH[6] or from Z-His-Phe-Arg(Tos)-Trp-Gly-OH[13] by hydrogen fluoride treatment,[14] was acylated with Boc-Glu(OBzl)-ONp in the absence of added amine. The reaction was fairly complete in 24 hr. The product VII, isolated in 80% yield, was treated with trifluoroacetic acid and the resulting amino-free hexapeptide trifluoroacetate was then coupled with Boc-Met-ONp in the presence of triethylamine to obtain a heptapeptide Boc-Met-Glu(OBzl)-His-Phe-Arg-Trp-Gly-OH (VIII). Removal of the Boc group from VIII yielded the N^{α}-free heptapeptide (IX) as the key intermediate in the present synthesis of both Ie and Id. Coupling of IX with the azide derived from V gave the decapeptide derivative Z-Ibu-Tyr(Bzl)-Ser-Met-Glu(OBzl)-His-Phe-Arg-Trp-Gly-OH (X). Compound X was then converted into the dihydrochloride by lyophilization from acetic acid containing a small excess of hydrogen chloride, followed by esterification with *N*-hydroxysuccinimide by the carbodiimide method.[15] The active ester of X, which was isolated, was allowed to react with a partially protected octapeptide, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2,[16] in the presence of triethylamine to yield a protected octadecapeptide Z-Ibu-Tyr(Bzl)-Ser-Met-Glu(OBzl)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 (XI). The crude XI was, without purification, subjected to treatment with hydrogen fluoride[14] in the presence of anisole and methionine as radical scavengers. The crude deblocked octadecapeptide (Ie), obtained in the form of hydrofluoride, was treated with an anion exchange resin (acetate) to be converted into the acetate form, which was then chromatographed, for purification, on a column of carboxymethyl cellulose using an ammonium acetate buffer with a linear concentration gradient. After repeated chromatography, peptide Ie was obtained in pure form. The over-all yield of Ie for the final coupling, deblocking and purification steps was 50%. The product behaved as a single component in thin-layer chromatography and in paper electrophoresis. Quantitative amino acid analysis[17] showed that an acid hydrolysate of the product contained the individual amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio of intact Ie was determined spectrophotometrically to be unity.

Synthesis of [β-Ala[1]]-Octadecapeptide Amide (Id). A synthesis of this octadecapeptide amide has been reported already in the preceding paper,[6] in which the terminal amino group was protected with a *t*-butyloxycarbonyl group and the γ-carboxyl group of the glutamic acid residue at position 5 by the formation of the *t*-butyl ester and the first decapeptide sequence was made up of tetrapeptide (positions 1—4) and hexapeptide (5—10). An alternative synthesis developed in the present work is summarized in Fig. 2. The amino-terminal tripeptide sequence was synthesized in the form of Z-β-Ala-Tyr-Ser-OMe (XIV) *via* Z-β-Ala-Tyr-$NHNH_2$ (XIII) as an intermediate. The tripeptide azide, derived from XV, was allowed to react with heptapeptide (IX) to give a decapeptide Z-

15) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964); *ibid.*, **85**, 3039 (1963).

16) H. Otsuka, K. Inouye, M. Kanayama and F. Shinozaki, This Bulletin, **39**, 882 (1966).

17) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

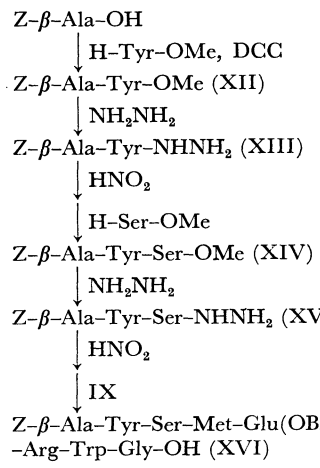

Fig. 2. Synthetic procedure of decapeptide intermediate XVI[12]

β-Ala-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg-Trp-Gly-OH (XVI) in pure form in 62% yield. Compound XVI was then esterified with *N*-hydroxysuccinimide in the usual manner and the active ester was made react with the octapeptide H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2.[16] The resulting crude preparation of protected octadecapeptide Z-β-Ala-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 (XVII) was, without purification, deblocked with hydrogen fluoride to liberate peptide Id. The crude Id was then purified by chromatography on a column of carboxymethyl cellulose. These procedures, the final coupling, deblocking and purification steps, were carried out in a manner almost the same as that used in the synthesis of Ie. The over-all yield of Id thus obtained was 45%. The product was found to be homogeneous in thin-layer chromatography and in paper electrophoresis. The quantitative amino acid analysis[17] showed that an acid hydrolysate of the product contained the individual amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio of intact Id was determined spectrophotometrically to be unity.[18] Optical rotation and chromatographic behaviors of the present preparation of Id were identical with those of a previous preparation obtained by a different synthetic procedure.[6]

Biological Properties of the Octadecapeptide Amides. Adrenal-stimulating activities of Ibu[1]-octadecapeptide amide (Ie) were compared with those of the present and previous preparations of β-Ala[1]-analog (Id) by six different assay procedures: 1) adrenal-ascorbic acid depletion,[19] 2) *in vitro* steroidogenesis,[20] 3) *in vivo* steroidogenesis in adrenal cannulation,[21] 4) *in vivo* steroidogenesis in dexamethasone-pentobarbital-blocked mouse,[22] 5) *in vivo* steroidogenesis in peripheral blood (intravenous administration), and 6) *in vivo* steroidogenesis in peripheral blood (intramuscular administration). The results are summarized in Table 1, in which the data on Gly[1]-analog (Ic)[4,6] are included for comparison. As is seen in Table 1, there is no significant difference in potency between the present preparation and the previous preparation of Id, proving their identity. This also proves the usefulness and safety of the new synthetic procedure, by which both peptides Id and Ie were prepared. The data in Table 1 clearly demonstrate that Ibu[1]-peptide (Ie) is endowed with a very high adrenal-stimulating activity and its potency appears to be more than twice that of β-Ala[1]-peptide (Id), when assayed by the adrenal-ascorbic acid depletion method and by the *in vivo* steroidogenesis methods. In addition, it is to be noted that the activity of Ie, estimated by the 11-hydroxycorticosteroid (11-OHCS) level in rat peripheral blood, is unchanged regardless of whether the peptide is administered intravenously or intramuscularly. In Ic and Id, however, the potency ratio of intramuscular *vs.* intravenous administration is 1/5 and 1/2, respectively. Tanaka has estimated the half-lives of a native corticotropin and the synthetic peptides Ic, Id, and Ie with the *in vitro* lipotropic activity[23] as a parameter. The results are shown in Table 2.[24] When the synthetic peptides are incubated in plasma, their half-lives are all the same, indicating that the inactivation of peptides in plasma is little associated with the action of aminopeptidase. On the other hand, when incubated with muscle fragments, the inactivation rate largely depends on the nature of the amino-terminal residue of peptides; Ibu[1]-peptide (Ie) can remain active rather longer than β-Ala[1]-peptide (Id). This may be correlated with the above observation that the intramuscular *vs.* intravenous potency ratio of Ie is larger than that of Id. In the preceding paper we described that β-Ala[1]-peptide (Id) was remarkably more potent, with a longer action in adrenal-stimulating activity than the corresponding Ser[1]-peptide (Ib) or Gly[1]-

18) G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.*, **7**, 319 (1952); T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

19) The United States Pharmacopeia, XVII, 147 (1965).

20) M. Saffran and A. V. Schally, *Endocrinol.*, **56**, 523 (1955).

21) H. S. Lipscomb and D. H. Nelson, *ibid.*, **71**, 13 (1962).

22) A. Tanaka and M. Nakamura, "Integrative Mechanism of Neuroendocrine System," Hokkaido University Medical Library Series, Vol. 1 (1968), p. 49.

23) A. Tanaka, B. T. Pickering and C. H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962).

24) We thank Dr. A. Tanaka for of this Laboratory permission to include these data in the present communication.

Table 1. Adrenal-stimulating activities of synthetic octadecapeptide amides[a]

Method	Administration route	Peptide[b]		
		Ic	Id	Ie
Adrenal-ascorbic acid depletion[19]	subcutaneous	25.6	— (135)	140—286
In vitro steroidogenesis[20]		27.2	— (237)	82.7
In vivo steroidogenesis in:				
adrenal cannulation[21]	intravenous	151	220 (285)	480
dexamethasone-Nembutal-blocked mouse[22]	intravenous	170	250 (259)	757
peripheral blood	intravenous	177	286 (—)	590
peripheral blood	intramuscular	50.5	115 (124)	495

a) The activities are expressed in USP units/mg, relative to the Third USP Corticotropin Reference Standard. The figures in parentheses are the data on the previous preparation.[6]
b) Ic: [Gly^1]-ACTH(1-18)-NH_2, Id: [β-Ala^1]-ACTH(1-18)-NH_2, and Ie: [Ibu^1]-ACTH(1-18)-NH_2.

Table 2. Half-lives of corticotropin and related synthetic peptides as *in vitro* lipotropic agents[24]

Peptide[a]	*In vivo*[b] (Intravenous injection)	*In vitro*,[c] incubation with	
		Plasma	Muscle
	min	min	min
Ic	2.0	30.0	42.6
Id	3.1	35.4	88.2
Ie	3.5	32.4	122.9
ACTH	4.4	59.2	250.9

a) Ic: [Gly^1]-ACTH (1-18)-NH_2, Id: [β-Ala^1]-ACTH (1-18)-NH_2, Ie: [Ibu^1]-ACTH (1-18)-NH_2, ACTH: native sheep corticotropin.
b) A peptide sample was injected intravenously into vena cava inferior and blood samples, collected from abdominal aorta in appropriate time intervals and acidified, were assayed for the *in vitro* lipotropic activity.[23]
c) A sample was dissolved in the fresh plasma from anesthetized rat or in the Krebs-Ringer bicarbonate buffer containing thigh muscle slices of rat, bovine serum albumin and glucose. These mixtures were then incubated at 37°C and the aliquots taken from the mixtures in appropriate time intervals were assayed for the *in vitro* lipotropic activity.[23]

peptide (Ic).[6] The present results show that Ibu^1-peptide (Ie) also exceeds Ic greatly in the biological activity, exhibiting a similarity to Id. Since α-aminoisobutyric acid amide and β-alanine amide are equally known not to be cleaved by hog kidney leucine aminopeptidase,[25] this similarity is not surprising, and it provides additional support for the view that the marked increase in biological activities as a main effect of the amino-terminal substitution is a consequence of the decreased susceptibility of peptide toward aminopeptidase action. However, peptide Ie still differs from peptide Id as seen in Tables 1 and 2, indicating that α-aminoisobutyric acid is preferred to β-alanine as the amino-terminal residue of corticotropin peptide, probably because of some favorable effects of the α-amino group in the former amino acid. Geiger *et al.*[26] reported that removal of 1-glycine residue from [Gly^1]-ACTH(1-23)-tricosapeptide amide caused a 50% loss of activity. This finding would suggest a significant importance for activity of the distance separating the terminal amino group and the center of active site of the peptide. In Ibu^1-peptide (Ie) the amino-terminal amino group occupies the place where it sits in a native corticotropin, whereas the corresponding group in β-Ala^1-peptide (Id) is located one methylene unit away from the regular position. In addition, it is to be noted that the amino group of α-aminoisobutyric acid will have a pK value close to those of ordinary α-amino acids (*e. g.* serine 9.15) rather than that of β-alanine (10.19).[27] These properties would affect a specific binding of the peptide to its receptor site and the tighter binding may, in turn, serve to further stabilize the peptide; peptide Ie thus could be more potent and have a longer action than Id. One of other possibilities which explain the difference between Id and Ie is the participation of an enzyme which can act on β-alanine peptides specifically. Because of the occurrence of β-alanine peptides, such as carnosine and anserine, in animal tissues, it is not unreasonable to assume the presence of such enzyme.

It has been shown that the amino-terminal substitution by unusual amino acids, such as β-alanine and α-aminoisobutyric acid, in a corticotropin pep-

25) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).

26) R. Geiger, K. Sturm, G. Vogel and W. Siedel, *Z. Naturforsch.*, **19b**, 858 (1964).

27) J. P. Greenstein and M. Winitz, "Chemistry of Amino Acids," Vol. 1, John Wiley & Sons, Inc., New York (1961), p. 486.

tide improves its biological properties greatly in both enhancement of potency and prolongation of action. As is apparent in Table 2, however, the amino-terminal substitution improves the stability of peptide in tissues, but not the stability in blood, indicating that the inactivation of peptide in blood may be associated with the action of some endopeptidase, but not with an aminopeptidase action. The presence of such an endopeptidase is known.[28] A further improvement in allowing the short-chain peptide to come closer to a native corticotropin should be achieved by reducing the susceptibility of peptide toward the endopeptidase action without a considerable loss of activity. The synthesis of [β-Ala^1, Orn^{15}]-ACTH(1-18)-NH_2 may be one solution.[29] This new peptide possesses a moderate adrenal-stimulating activity and shows an excellent prolongation of action; the half-life in plasma is longer than that of the native hormone (76 min, see Table 2 for comparison).

Experimental

All melting points were uncorrected.

Benzyloxycarbonyl-α-aminoisobutyric Acid (II). To a solution of α-aminoisobutyric acid (5.16 g, 0.05 mol) and anhydrous sodium carbonate (5.30 g, 0.05 mol) in N sodium hydroxide (50 m*l*) was added benzyl chloroformate (9.39 g, 0.055 mol) at 0°C and the mixture was stirred for 3 hr. The reaction mixture was washed with ether to remove the excess reagent and acidified with 6N hydrochloric acid, followed by extraction with ethyl acetate. The organic extracts were combined, dried over sodium sulfate and evaporated *in vacuo* to give a crystalline residue, which was recrystallized from ethyl acetate - petroleum ether; yield 8.75 g (73%), mp 69—70°C. Lit,[30] mp 78°C.

Found: C, 61.04; H, 6.36; N, 6.07%. Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90%.

***t*-Butyloxycarbonyl-α-aminoisobutyric Acid.** To a solution of α-aminoisobutyric acid (4.75 g, 0.046 mol) and sodium bicarbonate (4.25 g, 0.05 mol) in N sodium hydroxide (46 m*l*) and dioxane (30 m*l*) was added dropwise a dioxane solution of *t*-butyl azidoformate (7.25 g, 0.05 mol) and the mixture was stirred at 45—50°C for 5 days. An additional quantity of *t*-butyl azidoformate (6.6 g, 0.046 mol) and N sodium hydroxide (46 m*l*) was introduced and the mixture was stirred for 2 days at the same temperature and then concentrated *in vacuo* to remove most of the organic solvent. The concentrate was chilled and acidified with ice-cold 4N hydrochloric acid to pH 3, followed by extraction with ethyl acetate. The organic solution was dried over sodium sulfate and evaporated. The resulting crystals were recrystallized from ether; yield 0.81 g (8.7%), mp 119—120°C.

Found: C, 53.64; H, 8.39; N, 7.19%. Calcd for $C_9H_{17}NO_4$: C, 53.19; H, 8.43; N, 6.89%.

Benzyloxycarbonyl-α-aminoisobutyryl-tyrosine Hydrazide. Compound II (1.54 g, 6.5 mmol) and tyrosine methyl ester (free base, 1.27 g, 6.5 mmol) were dissolved in acetonitrile and to this solution was added *N,N'*-dicyclohexylcarbodiimide (DCC, 1.34 g, 6.5 mmol) at 0°C with acetonitrile as solvent. The mixture was kept at 4°C overnight. *N,N'*-dicyclohexylurea (DCU) which formed was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with N hydrochloric acid and 5% sodium bicarbonate, dried over sodium sulfate and evaporated *in vacuo* to give the dipeptide ester as a sirupy residue. The residue was dissolved in ethanol (15 m*l*) and hydrazine hydrate (0.8 m*l*) was added. After the reaction mixture had been kept at room temperature for 2 days, water was added to separate the desired dipeptide hydrazide as crystals; yield 2.35 g (87%), mp 160—162°C. Recrystallization from aqueous ethanol yielded the pure material in a recovery of 92%; mp 164—165°C, $[\alpha]_D^{27}$ $-31.4\pm0.7°$ (*c* 1.078, methanol).

Found: C, 60.95; H, 6.41; N, 13.31%. Calcd for $C_{21}H_{26}N_4O_5$: C, 60.86; H, 6.32; N, 13.52%.

***t*-Butyloxycarbonyl-*O*-benzyl-tyrosine *p*-Nitrophenyl Ester (III).** To a solution of *t*-butyloxycarbonyl-*O*-benzyl-tyrosine (2.97 g, 8 mmol)[31] and *p*-nitrophenol (1.12 g, 8 mmol) in ethyl acetate was added an ethyl acetate solution of DCC (1.65 g, 8 mmol) at 0°C and the mixture was kept at 4°C for 3 hr. The DCU which formed was removed by filtration and the filtrate was evaporated *in vacuo*. The resulting solid residue was suspended in hot ethanol and the crystals were filtered off after refrigeration, washed with cold ethanol and dried *in vacuo* to give the desired active ester; yield 3.39 g (86%), mp 140—141°C, $[\alpha]_D^{22}$ $-0.3\pm0.4°$ (*c* 0.983, ethyl acetate).

Found: C, 65.93; H, 5.73; N, 5.67%. Calcd for $C_{27}H_{28}N_2O_7$: C, 65.84; H, 5.73; N, 5.69%'

***t*-Butyloxycarbonyl-*O*-benzyl-tyrosyl-serine Methyl Ester (IV).** To an ice-cold solution of serine methyl ester hydrochloride (1.03 g, 6.6 mmol) in dimethylformamide (DMF, 8 m*l*) was added triethylamine (0.92 m*l*, 6.6 mmol) and the amine hydrochloride which formed was filtered off. ADMF solution of III (2.96 g, 6.0 mmol) was added to the filtrate and the mixture was kept at 4°C for 2 days. After the solvent had been removed by evaporation *in vacuo* at a bath temperature of 45—50°C, the residue was dissolved in ethyl acetate and the solution was washed successively with ice-cold N hydrochloric acid, water, 2N aqueous ammonia and water, dried over sodium sulfate and evaporated *in vacuo*. The resulting gelatinous mass was precipitated from ethyl acetate - petroleum ether. Reprecipitation from methanol-water afforded the pure dipeptide; yield 2.75 g (97%), mp 73—77°C, $[\alpha]_D^{22}$ $+7.7\pm0.5°$ (*c* 1.046, methanol).

Found: C, 63.36; H, 6.97; N, 5.71.% Calcd for $C_{25}H_{32}N_2O_7$: C, 63.54; H, 6.83; N, 5.93%.

Benzyloxycarbonyl-α-aminoisobutyryl-*O*-benzyl-tyrosyl-serine Hydrazide (V). Compound IV (2.08 g,

28) W. F. White and A. M. Gross, *J. Amer. Chem. Soc.*, **79**, 1141 (1957).

29) K. Inouye, K. Watanabe, K. Namba and H. Otsuka, in preparation.

30) M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 325 (1935).

31) R. Schwyzer, P. Sieber and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

4.4 mmol) was dissolved in N hydrogen chloride in acetic acid (20 ml) and the mixture was allowed to stand at room temperature for 30 min. Evaporation of the solvent left an oily residue which solidified upon treatment with ether. The solid which was filtered off was dissolved in a mixture of water (6 ml) and dichloromethane (20 ml), and to this was added ice-cold 50% potassium carbonate (6 ml). The mixture was shaken well and the organic phase was separated, dried over sodium sulfate and evaporated *in vacuo*. The crystalline *O*-benzyl-tyrosyl-serine methyl ester (free base) thus obtained was dissolved in acetonitrile along with compound II (1.04 g, 4.4 mmol) and to this solution was introduced an acetonitrile solution of DCC (0.91 g, 4.4 mmol) at 0°C. The mixture was kept at 4°C overnight. The DCU which formed was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed successively with N hydrochloric acid, water and 5% sodium bicarbonate, dried over sodium sulfate and evaporated *in vacuo*, yielding the crude tripeptide ester which showed on a thin-layer chromatogram (in ethyl acetate) the presence of a smaller amount of an additional component. The crude ester was then dissolved in ethanol (20 ml) and hydrazine hydrate (1.0 ml, 20 mmol) was added. The reaction mixture was allowed to stand at room temperature for 2 days, followed by evaporation *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with water and dried quickly over sodium sulfate. The precipitates which separated on standing were filtered off, washed with cold ethyl acetate and ether and dried *in vacuo* to give the desired hydrazide; yield 1.91 g (72%). Reprecipitation from ethanol gave the pure material in a recovery of 80%, mp 151—153°C, $[\alpha]_D^{22}$ $-2.2\pm0.4°$ (*c* 1.062, glacial acetic acid).

Found: C, 62.98; H, 6.31; N, 11.81%. Calcd for $C_{31}H_{37}N_5O_7$: C, 62.93; H, 6.30; N, 11.84%.

Benzyloxycarbonyl-β-alanyl-tyrosine Methyl Ester (XII). To a solution of benzyloxycarbonyl-β-alanine (8.9 g, 0.04 mol)[32] and tyrosine methyl ester (free base, 7.8 g, 0.04 mol) in acetonitrile was added a dichloromethane solution of DCC (8.3 g, 0.04 mol) at 0°C and the mixture was kept at 4°C overnight. After removal of DCU by filtration the filtrate was evaporated *in vacuo*. The resulting residue was dissolved in ethyl acetate and the solution was washed with N hydrochloric acid and 5% sodium bicarbonate, dried over sodium sulfate and evaporated *in vacuo* to yield a sirupy residue which crystallized from ethyl acetate - ether. After recrystallization from the same solvent the product amounted to 13.2 g (83%); mp 114—116°C, $[\alpha]_D^{27}$ $+6.2\pm0.4°$ (*c* 1.040, methanol).

Found: C, 62.94; H, 6.06; N, 6.85%. Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 6.93%.

Benzyloxycarbonyl-β-alanyl-tyrosine Hydrazide (XIII). Compound XII (4.81 g, 0.012 mol) was dissolved in ethanol (50 ml) and hydrazine hydrate (2.9 ml, 0.06 mol) was added. The mixture was allowed to stand at room temperature for a few hours and kept at 4°C overnight. The crystalline hydrazide which separated was filtered off, washed with cold ethanol and dried *in vacuo*; yield 4.72 g (98%). After treatment with hot ethanol the mp was 225—228°C; $[\alpha]_D^{26}$ $+3.9\pm0.4°$ (*c* 1.032, 50% acetic acid).

Found: C, 60.09; H, 6.16; N, 13.97%. Calcd for $C_{20}H_{24}N_4O_5$: C, 59.99; H, 6.04; N, 13.99%.

Benzyloxycarbonyl-β-alanyl-tyrosyl-serine Methyl Ester (XIV). Compound XIII (2.40 g, 6 mmol) was dissolved in DMF (7 ml) and N hydrochloric acid (15 ml) was added. The solution was chilled in ice and to this was added ice-cold 2M sodium nitrite (3. 6ml). After the mixture had been kept at 0°C for 4 min, the azide which separated was extracted with ice-cold ethyl acetate (25 ml×2). The organic extracts were combined, washed with ice-cold 5% sodium bicarbonate, dried over sodium sulfate. The resulting solution was added to a mixture of serine methyl ester hydrochloride (0.93 g, 6 mmol) and triethylamine (0.84 ml, 6 mmol) in 90% tetrahydrofuran (20 ml) and the mixture was stirred at 4°C for 48 hr. The gelationous precipitates which had separated were filtered off, washed with cold ethyl acetate and with water and dried *in vacuo*; yield 1.58 g (54%). After treatment with hot methanol the mp was 195—196°C; $[\alpha]_D^{26}$ $+2.1\pm0.4°$ (*c* 1.091, methanol).

Found: C, 59.29; H, 6.08; N, 8.63%. Calcd for $C_{24}H_{29}N_3O_8$: C, 59.13; H, 6.00; N, 8.62%.

Benzyloxycarbonyl-β-alanyl-tyrosyl-serine Hydrazide (XV). To a solution of compound XIV (1.46 g, 3 mmol) in DMF (5 ml) was added hydrazine hydrate (0.73 ml, 15 mmol) and the mixture was kept at 4°C for 24 hr. The hydrazide had separated as crystals. After addition of ethanol (10 ml) the crystals were filtered off, washed with ethanol and dried *in vacuo* (1.40 g). Treatment with hot ethanol gave the pure hydrazide; yield 1.32 g (90%), mp 220—224°C decomp, $[\alpha]_D^{25}$ $+15.7\pm0.5°$ (*c* 1.042, glacial acetic acid).

Found: C, 56.35; H, 6.04; N, 14.57%. Calcd for $C_{23}H_{29}N_5O_7$: C, 56.66; H, 6.00; N, 14.37%.

***t*-Butyloxycarbonyl-γ-benzyl-glutamic Acid Dicyclohexylamine Salt.** Glutamic acid γ-benzyl ester (9.5 g, 0.04 mol)[33] and sodium bicarbonate (12.5 g) were suspended in water (100 ml) and dioxane (80 ml) and to this was added *t*-butyl azidoformate (8.6 g, 0.06 mol) with dioxane (20 ml). The mixture was stirred at 45—50°C for 2 days, followed by concentration *in vacuo* to remove most of the dioxane. The concentrated solution was then acidified to pH 3—4 with ice-cold 4N hydrochloric acid at 0°C and the product was extracted with ice-cold ethyl acetate. The organic solution was washed with ice-cold N hydrochloric acid and water, dried over sodium sulfate and evaporated *in vacuo* to give an oily residue. The residue was dissolved in ether (50 ml) and dicyclohexylamine (7.3 g, 0.04 mol) was added. Crystalline precipitates which had separated upon standing at 4°C overnight were filtered off, washed with ether and dried *in vacuo*; yield 16.7 g (81%). After recrystallization from ethyl acetate the product showed mp 140—142°C, $[\alpha]_D^{27}$ $+13.3\pm0.5°$ (*c* 1.024, methanol). Lit,[34] mp 138—139°C, $[\alpha]_D^{25}$ $+11.9°$ (*c* 1, methanol).

Found: C, 67.03; H, 9.02; N, 5.68%. Calcd for $C_{17}H_{23}NO_6 \cdot C_{12}H_{23}N$: C, 67.15; H, 8.94; N, 5.40%.

32) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

33) a) H. Yuki, *Nippon Kagaku Zasshi*, **77**, 44 (1956); b) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, **1950**, 3239; c) E. R. Blout and R. H. Karlson, *J. Amer. Chem. Soc.*, **78**, 941 (1956).

34) E. Schröder and E. Klieger, *Ann. Chem.*, **673**, 196 (1964).

***t*-Butyloxycarbonyl-γ-benzyl-glutamic Acid *p*-Nitrophenyl Ester.** *t*-Butyloxycarbonyl-γ-benzyl-glutamic acid dicyclohexylamine salt (9.0 g, 17.4 mmol) was shaken with Dowex 50W×8 (H^+ form, wet vol 18 cc) in 60% ethanol for 30 min. After the resin had been removed by filtration the filtrate was evaporated *in vacuo*. The residue was dissolved in ether and the solution was dried over sodium sulfate and evaporated *in vacuo* to afford the free acid quantitatively. This was dissolved in ethyl acetate together with *p*-nitrophenol (2.4 g, 17.4 mmol) and to this solution was added DCC (3.6 g, 17.4 mmol) at 0°C. The reaction mixture was kept at 4°C overnight. After removal of DCU by filtration the filtrate was evaporated *in vacuo* to give a residue which crystallized from ethanol. Recrystallization from the same solvent gave the active ester in a pure form; yield 7.0 g (88%), mp 118—119°C, $[\alpha]_D^{28}$ −33.1±0.7° (*c* 1.082, methanol). Lit,[35] mp 120—121°C, $[\alpha]_D^{25}$ −32.7° (*c* 1, methanol).

Found: C, 60.38; H, 5.80; N, 6.29%. Calcd for $C_{23}H_{26}N_2O_8$: C, 60.26; H, 5.72; N, 6.11%.

***t*-Butyloxycarbonyl-methionine Dicyclohexylamine Salt.** To a mixture of methionine (3.0 g, 0.02 mol) and sodium bicarbonate (3.8 g) in water-dioxane (1 : 1 by vol, 60 m*l*) was added *t*-butyl azidoformate (4.3 g, 0.03 mol) and the mixture was stirred at 45°C for 48 hr. The reaction mixture was worked up in the usual manner. The resulting acyl-methionine (oil, 4.3 g) was dissolved in ether and dicyclohexylamine (3.1 g, 0.017 mol) was added. The crystals were collected by filtration, washed with ether and dried *in vacuo*; yield 6.7 g (78%). A sample was recrystallized from ethyl acetate in a recovery of 92%; mp 141°C, $[\alpha]_D^{20}$ −18.2±0.9° (*c* 0.648, methanol).

Found: C, 61.56; H, 9.99; N, 6.68%. Calcd for $C_{10}H_{19}NO_4S \cdot C_{12}H_{23}N$: C, 61.36; H, 9.83; N, 6.51%.

***t*-Butyloxycarbonyl-methionine *p*-Nitrophenyl Ester.** *t*-Butyloxycarbonyl-methionine dicyclohexylamine salt (10.8 g, 0.025 mol) was treated with Dowex 50W×8 (H^+ form) in the same manner as above. The resulting oily acid and *p*-nitrophenol (3.5 g, 0.025 mol) were dissolved in ethyl acetate and to this was added an ethyl acetate solution of DCC (5.2 g, 0.025 mol) at 0°C. The mixture was allowed to stand at 4°C overnight. The DCU was filtered off and the filtrate was evaporated *in vacuo* to give a residue, which crystallized from ethanol. After recrystallization from ethanol the active ester was obtained in 84% yield; wt 7.8 g, mp 96—97°C, $[\alpha]_D^{27}$ −48.3±0.9° (*c* 1.020, methanol).

Found: C, 52.05; H, 5.97; N, 7.68%. Calcd for $C_{16}H_{22}N_2O_6S$: C, 51.88; H, 5.99; N, 7.56%.

***t*-Butyloxycarbonyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine (VII).** To a solution of histidyl-phenylalanyl-arginyl-tryptophyl-glycine monoacetate (VI, 1.20 g, 1.5 mmol)[36] in 50% DMF (10 m*l*) were added *t*-butyloxycarbonyl-γ-benzyl-glutamic acid *p*-nitrophenyl ester (1.03 g, 2.25 mmol) and DMF (25 m*l*), and the mixture was kept at 4°C for 3 days. The resulting solution was added dropwise into a mixture of ethyl acetate - ether (1 : 1 by volume, 200 m*l*) and the precipitates which formed were filtered off, washed with ethyl acetate and ether and dried *in vacuo* (1.86 g). These precipitates were redissolved in 50% acetic acid (*ca.* 10 m*l*) and ethanol (*ca.* 100 m*l*) was added. The resulting precipitates were collected and lyophilized from acetic acid; yield 1.37 g (89%), $[\alpha]_D^{22}$ −23.9±0.6° (*c* 1.020, 50% acetic acid).

Found: C, 56.40; H, 6.22; N, 15.40; CH_3CO; 3.25%. Calcd for $C_{51}H_{64}N_{12}O_{11} \cdot CH_3COOH \cdot 3H_2O$: C, 56.07; H, 6.57; N, 14.81; CH_3CO, 3.79%.

***t*-Butyloxycarbonyl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine (VIII).** Compound VII (1.26 g, 1.1 mmol) was dissolved in trifluoroacetic acid (6 m*l*) and the mixture was kept at room temperature for 30 min and then chilled in an ice bath. Addition of ether afforded the partially deblocked hexapeptide as precipitates (1.34 g). These precipitates were dissolved in DMF (10 m*l*) and to this solution were added triethylamine (0.46 m*l*, 3.3 mmol) and *t*-butyloxycarbonyl-methionine *p*-nitrophenyl ester (0.74 g, 2 mmol). The mixture was allowed to stand at 4°C for 24 hr and then introduced into an ice-cold mixture of ethyl acetate - ether (1 : 4 by volume, 250 m*l*). The resulting precipitates were filtered off, washed with ether and dried *in vacuo* (1.56 g). To this product was added ethanol (15 m*l*) and the suspension was heated to boiling point and then chilled. The precipitates were collected, washed with cold ethanol and ether and lyophilized from acetic acid; yield 1.16 g (88%), $[\alpha]_D^{22}$ −18.5±0.5° (*c* 1.072, DMF).

Found: C, 54.51; H, 6.16; N, 13.94%. Calcd for $C_{56}H_{73}N_{13}O_{12}S \cdot CH_3COOH \cdot 2H_2O$: C, 54.23; H, 6.67; N, 14.18%.

Benzyloxycarbonyl-α-aminoisobutyryl-*O*-benzyl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine (X). Compound VIII (0.50 g, 0.4 mmol) was dissolved in trifluoroacetic acid (3 m*l*) containing one drop of water and the mixture was allowed to stand at room temperature for 30 min. The resulting heptapeptide trifluoroacetate (IX) was precipitated by the addition of ether. The precipitates collected by filtration were washed thoroughly with ether and dried *in vacuo*.

A mixture of V (0.47 g, 0.8 mmol), N hydrochloric acid (2 m*l*) and DMF (2 m*l*) was chilled in an ice bath and to this was added dropwise ice-cold 2M sodium nitrite (0.44 m*l*). The mixture was stirred at 0°C for 4 min and then extracted with ice-cold ethyl acetate. The organic extracts were combined, washed with ice-cold sodium bicarbonate and dried over sodium sulfate. The resulting solution was mixed with a solution of IX, obtained above, and triethylamine (0.25 m*l*, 1.6 mmol) in DMF (10 m*l*). The mixture was concentrated *in vacuo* at a bath temperature of 20°C until it became almost clear, and then kept at 4°C for 24 hr. Most of the solvent was removed by evaporation *in vacuo* at a bath temperature of 45°C. The precipitates which formed upon addition of ether were reprecipitated from methanol - ethanol (1 : 1 by vol); yield 0.96 g (82%), $[\alpha]_D^{24}$ −30.1±1.4° (*c* 0.515, DMF). Thin-layer chromatography:[38] R_f=0.44 (BAW), 0.68 (BAPW) and

35) C. H. Li, B. Gorup, D. Chung and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

36) See Ref. 6. In the present work, this pentapeptide (VI) was purified before use in the manner described previously.[37]

37) K. Inouye, This Bulletin, **38**, 1148 (1965).

38) Solvent systems of chromatography are: BAW, *n*-butanol - acetic acid-water (4 : 1 : 2 by vol); BAPW, *n*-butanol - acetic acid - pyridine - water (30 : 6 : 20 : 24 by vol).

0.50 (ethyl acetate - acetic acid-water=8 : 3 : 2 by vol).

Found: C, 57.31; H, 5.82; N, 12.33%. Calcd for $C_{82}H_{98}N_{16}O_{17}S \cdot 2CH_3COOH \cdot 4H_2O$: C, 57.26; H, 6.37; N, 12.42%.

Benzyloxycarbonyl-β-alanyl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine (XVI). A mixture of XV (0.86 g, 1.75 mmol), N hydrochloric acid (4 ml) and DMF (5 ml) was chilled in an ice bath and to this was added dropwise ice-cold 2M sodium nitrite (0.96 ml). The mixture was stirred at 0°C for 4 min, after which ice-cold DMF (10 ml) was introduced to dissolve the azide, which had separated, followed by addition of triethylamine (0.56 ml, 4.0 mmol). The resulting solution was added to an ice-cold solution of IX (derived from 1.09 g, 0.88 mmol, of VIII by treatment with trifluoroacetic acid) and triethylamine (0.49 ml, 3.5 mmol) in DMF (20 ml) and the mixture was allowed to stand at 0°C for 24 hr. After addition of acetic acid (1 ml) the solvent was removed by evaporation *in vacuo* at a bath temperature of 45°C to afford a sirupy residue. To this was then added ethanol (20 ml) and gelatinous precipitates were filtered off, washed thoroughly with ethanol, ethyl acetate and with ether and dried *in vacuo* (0.88 g). The filtrate and washes were combined and evaporated *in vacuo*. The residue was dissolved in ethyl acetate and insoluble precipitates were filtered off. These precipitates were heated with ethanol to boiling point and filtered off after refrigeration (0.27 g). The total yield amounted to 1.15 g (78%). The precipitates combined were boiled in ethanol for several minutes and collected by filtration in 80% recovery; $[\alpha]_D^{23}$ $-18.5 \pm 0.5°$ (*c* 1.067, DMF). Thin-layer chromatography: $R_f=0.75$ (BAPW). Paper chromatography: $R_f=0.8$ (BAW).

Found: C, 55.71; H, 6.06; N, 13.34%. Calcd for $C_{74}H_{90}N_{16}O_{17}S \cdot 2CH_3COOH \cdot 3H_2O$: C, 55.70; H, 6.23; N, 13.33%.

α-Aminoisobutyryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine Amide, [Ibu¹]-ACTH(1-18)-NH₂ (Ie). To a solution of X (0.32 g, 0.18 mmol) in acetic acid was added N hydrogen chloride in acetic acid (0.4 ml) and the mixture was immediately lyophilized and dried over sodium hydroxide pellets *in vacuo*. The decapeptide hydrochloride thus obtained was dissolved in DMF (3ml) together with *N*-hydroxysuccinimide (0.083 g, 0.72 mmol) and to this was added a solution of DCC (0.15 g, 0.72 mmol) in DMF (2 ml). The mixture was allowed to stand at 4°C overnight. The DCU which had separated was filtered off and the filtrate was introduced into ice-cold ethyl acetate - ether (1 : 1 by vol, 100 ml) The resulting precipitates were filtered off, washed with ethyl acetate and ether and dried *in vacuo* to yield the decapeptide active ester (0.36 g).

The triacetate of N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide (derived from 0.12 mmol of the corresponding N^{α}-benzyloxycarbonyl derivative by catalytic hydrogenolysis)[16] was dissolved in DMF (2 ml) and triethylamine (0.18 ml) was added. This solution was mixed with a DMF solution of the active ester obtained above and the mixture (total volume, 4 ml) was kept at 4°C for 48 hr. The reaction mixture was then introduced into ice-cold ethyl acetate (100 ml) and the precipitates which separated were filtered off, washed with ethyl acetate and ether, lyophilized from acetic acid and dried over sodium hydroxide pellets *in vacuo*, affording the crude protected octadecapeptide (0.56 g).

The protected peptide obtained above was put into a reaction vessel (made of fluorinated polyethylene) with methionine (0.1 g) and anisole (0.5 ml), and hydrogen fluoride was introduced into the vessel placed in a dry ice-acetone bath. The mixture (*ca.* 10 ml) was stirred at 0°C for 90 min, followed by evaporation *in vacuo* to give a sirupy residue, which was dissolved in ice-cold water. The solution was washed with ethyl acetate and then passed through a column (1.7×12 cm) of Amberlite CG-400 (acetate form); the column was washed with portions of water. The aqueous solutions combined were concentrated to *ca.* 20 ml *in vacuo* and lyophilized. The crude deblocked peptide thus obtained (0.65 g) was subjected, for purification, to chromatography on a column (2.7×27 cm) of carboxymethyl (CM) celullose (Serva, 0.56 meq/g) using an ammonium acetate buffer (pH 6.8, 2000 ml) with a linear concentration gradient from 0.02 to 0.6M. Ten-ml fractions were collected and their absorptivity at 280 mμ was recorded. The fractions corresponding to a main peak (tubes 156—180) and its shoulder (tubes 181—200) were collected separately and the bulk of the solvent was removed by evaporation *in vacuo* at a bath temperature of 50—55°C. The residues were lyophilized to constant weights to give 189 mg (F-I) and 76 mg (F-II) of colorless powder from the former fractions and from the latter, respectively. F-I (189 mg) was rechromatographed on a CM cellulose column (2.2×25 cm) in the same manner as above and the pure peptide (137 mg, F-I-1) was obtained from a single peak. A small shoulder of the peak afforded F-I-2 (37 mg). F-II (76 mg) and F-I-2 (37 mg) were combined and the chromatography was repeated twice to obtain 29 mg of the pure peptide. Total yield of the octadecapeptide amide (Ie) thus obtained amounted to 166 mg (50%, as calculated on the basis of the amount of octapeptide used in the final coupling step); $[\alpha]_D^{23}$ $-58.4 \pm 1.9°$ (*c* 0.514, 0.1N acetic acid). $\lambda_{max}^{0.1N\ HCl}=279$ mμ ($E_{1cm}^{1\%}=25.1$), $\lambda_{shoulder}^{0.1N\ HCl}=288$ mμ ($E_{1cm}^{1\%}=19.7$); $\lambda_{max}^{0.1N\ NaOH}=281$ mμ ($E_{1cm}^{1\%}=25.2$), 288 mμ ($E_{1cm}^{1\%}=24.4$). The peptide behaved as a single component to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in thin-layer chromatography (BAPW as solvent) and in paper electrophoresis (600 V/36 cm, in 2N acetic acid). Amino acid ratios in acid hydrolysate (6N HCl, 105°C, 40 hr):[17] Ser 0.83, Glu 1.00, Pro 1.07, Gly 2.10, Ibu 0.99, Val 1.00, Met 1.02, Tyr 0.97, Phe 1.04, Lys 3.17, His 0.92, Arg 2.90. The Trp/Tyr ratio in intact Ie was determined spectrophotometrically to be 1.12.[18]

β-Alanyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine Amide, [β-Ala¹]-ACTH(1-18)-NH₂ (Id). Compound XVI (0.32 g, 0.18 mmol) was converted into the corresponding active ester hydrochloride by the same procedure as that described above in the preparation of Ibu¹-decapeptide active ester. A solution of the active ester in DMF (3 ml) was mixed with a solution of N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide triacetate (derived from 0.15 mmol of the corresponding N^{α}-benzyloxycarbonyl derivative)[16] and triethylamine (0.2 ml) in DMF (2 ml) and the mixture

was allowed to stand at 4°C for 4 days. The reaction mixture was then introduced into ice-cold ethyl acetate (100 m*l*) and the precipitates which formed were collected by filtration, washed with ethyl acetate and ether, lyophilized and dried over sodium hydroxide pellets *in vacuo*. The crude product (0.59 g) was dissolved in liquid hydrogen fluoride together with methionine (0.1 g) and anisole (0.5 m*l*) and the mixture (*ca.* 10 m*l*) was stirred at 0°C for 60 min, followed by evaporation *in vacuo*. A sirupy residue was dissolved in water and the solution was washed with ethyl acetate, treated with Amberlite CG-400 (acetate) and lyophilized, as described above, to yield the crude deblocked octadecapeptide amide (0.65 g). The crude product was subjected to chromatography on a column (2.6×23 cm) of CM cellulose (Serva, 0.56 meq/g) using an ammonium acetate buffer (pH 6.8, 2100 m*l*) with a linear concentration gradient of 0.02—0.6M. The fractions (10 m*l*/tube) corresponding to the main peak (tubes 151—190) and its shoulder (tubes 191—210) were collected separately. Removal of the bulk of the solvent, followed by lyophilization, yielded F-I (168 mg) and F-II (80 mg) from the peak and from its shoulder, respectively. F-I was rechromatographed on a CM cellulose column (2.2× 22 cm) in the same manner as above to give pure peptide (122 mg, F-I-1) and a less purified fraction (25 mg, F-I-2). F-II (80 mg) and F-I-2 (25 mg) were combined and rechromatographed to obtain an additional quantity of pure peptide (65 mg). Total yield of the octadecapeptide amide (Id) thus obtained amounted to 187 mg (45%, as calculated on the basis of the amount of octapeptide used in the final coupling step); $[\alpha]_D^{23}$ $-58.3\pm1.9°$ (*c* 0.527, 0.1N acetic acid). Lit,[6] $[\alpha]_D^{27}$ $-54.0\pm1.8°$ (*c* 0.515, 0.1N acetic acid). $\lambda_{max}^{0.1N\ HCl}$= 279 m$\mu$ ($E_{1\%}^{1cm}$=23.9), $\lambda_{shoulder}^{0.1N\ HCl}$=288 m$\mu$ ($E_{1cm}^{1\%}$=17.6), $\lambda_{max}^{0.1N\ NaOH}$=281 m$\mu$ ($E_{1cm}^{1\%}$=24.3), 288 mμ ($E_{1cm}^{1\%}$=23.8). The peptide behaved as a single component to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in thin-layer chromatography (BAPW as solvent) and in paper electrophoresis (600 V/36 cm, in 2N acetic acid). Amino acid ratios in acid hydrolysate (6N HCl, 105°C, 40 hr):[17] Ser 0.89, Glu 1.03, Pro 1.09, Gly 2.11, Val 1.00, Met 1.02, Tyr 1.09, Phe 1.07, β-Ala 1.08, Lys 2.91, His 0.92, Arg 2.71. The Trp/Tyr ratio in intact Id was 1.03 as determined spectrophotometrically.[18]

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