*Syntheses of Peptides Related to the N-Terminal Structure of Corticotropin. III. The Synthesis of L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophan, the Smallest Peptide Exhibiting the Melanocyte-stimulating and the Lipolytic Activities**

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This investigation was undertaken to find out the minimum structure required for the two extra-adrenal activities of corticotropin as a melanocyte-stimulating hormone (MSH) and a lipolytic agent.

There have been reports on the relationship between these hormonal activities and the chemical structure. Hofmann and Yajima,[1] for instance, concluded from their synthetic studies that two things were essential for the MSH activity, the sequence His-Phe-Arg-Try-Gly (positions 6 to 10) in the corticotropin and in the α-MSH molecules and the presence of the histidine and the tryptophan residues at their respective positions. Pickering and Li[2] reached the same conclusion from their studies of synthetic peptides.

Concerning the activity as lipolytic or adipokinetic agents, Raben et al.[3] first reported that a peptide, H-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-OH, which corresponded to the γ-amide derivative of the *N*-terminal octapeptide sequence of the corticotropin and α-MSH molecules, was inactive, when it was lengthened at the carboxyl end to a decapeptide, H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-OH, the activity was encountered. Tanaka, Pickering and Li[4] also reported that the smallest peptide which was active in rat adipose tissue was the *N*-terminal decapeptide, whereas a pentapeptide, H-His-Phe-Arg-Try-Gly-OH, was still active in the rabbit.

It has not yet been examined whether or not the glycine next to the tryptophan (position 9) is essential to these activities. Therefore, we synthesized a tetrapeptide, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan, and assayed it for MSH activity by the in vitro frog skin method.[5] It was found that the peptide exhibited the same level of potency

* A part of this work has been briefly communicated: H. Otsuka and K. Inouye, This Bulletin, **37**, 289 (1964).

1) K. Hofmann and H. Yajima, "Recent Progress in Hormone Research," Vol. 18, Academic Press Inc., New York (1962), p. 41.

2) B. T. Pickering and C. H. Li, *Biochim. Biophys. Acta*, **62**, 475 (1962).

3) M. S. Raben, Ruth Landolt, F. A. Smith, K. Hofmann and H. Yajima, *Nature*, **189**, 681 (1961).

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

5) K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954); A. B. Lerner and M. R. Wright, "Methods of Biochemical Analysis," Vol. 8, Interscience Publishers, New York (1960), p. 295.

(3.6×10^4 units per gram) as pentapeptides, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine[6,7] and its D-phenylalanine analog.[8] This may show that the glycine is not only not essential for the activity, but that it is not so stimulating, as is the case with a hexapeptide, H-Gly-His-Phe-Arg-Try-Gly-OH,[9] in which the *N*-terminal glycine shows a tenfold increase in potency as compared with the pentapeptide without the *N*-terminal glycine. The tetrapeptide was also active as a lipolytic agent at a dose of 1000 μg. in rabbit perirenal adipose tissue. This potency is comparable to that reported for the pentapeptide.[4] From this finding, it may be concluded that the glycine is not essential also for the lipolytic activity.

One of the difficulties in the synthesis of the tetrapeptide is that the tryptophan resides at the carboxyl end of the molecule. The saponification of the tryptophan ester was accompanied by the fission of the peptide bonds. We have failed also in an attempt to prepare the *t*-butyl ester of tryptophan which may be removed by a mild acid solvolysis. Therefore, it appeared most promising to employ the benzyl ester, which could be removed by means of catalytic or chemical reduction. In order to minimize the racemization which occurs during peptide synthesis, a step-by-step procedure, by which a peptide chain is extended from the carboxyl end, may be recommended. However, all the intermediate products that we have tried so far, namely, the various derivatives of N^G-tosyl(or nitro)-L-arginyl-L-tryptophan and L-phenylalanyl-N^G-tosyl(or nitro)-L-arginyl-L-tryptophan, failed to crystallize. Because of this disadvantage, it was very difficult to remove the impurities[10] which were produced during the repeated treatment of the tryptophan peptide in order to remove its amino-protecting group with such an acid reagent as hydrogen bromide-acetic acid or trifluoroacetic acid. Fortunately, we could obtain the hydrazide of *t*-butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginine in a crystalline state. As is well known, the azide coupling procedure is free from racemization.[11] Therefore, the procedure for the synthesis of the tetrapeptide was designed as follows:

The crystalline N^G-tosyl-L-arginine methyl ester (III, free base) was coupled with *t*-butyloxycarbonyl-L-phenylalanine by the *N*, *N'*-dicyclohexylcarbodiimide (DCCI) method.[12] The resultant protected dipeptide (VII) was converted into the crystalline hydrazide (IX). The coupling of the azide (derived from compound IX) with L-tryptophan benzyl ester gave the protected tripeptide, *t*-butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophan benzyl ester (XI). The amino-protecting group of compound XI was removed by treatment with trifluoroacetic acid,[13] affording the tripeptide ester, L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophan benzyl ester. This tripeptide ester was next condensed with N^α, N^{Im}-dicarbobenzoxy-L-histidine[14] by the DCCI procedure to obtain the protected tetrapeptide, N^α, N^{Im}-dicarbobenzoxy-L-histidyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophan benzyl ester (XII). Then, the reduction of compound XII in sodium in liquid ammonia was performed in order to remove all the protecting groups, namely, the two carbobenzoxy, one tosyl and one benzyl ester, groups. The resultant free tetrapeptide (XIII) was purified on a carboxymethyl cellulose column. The purified product was then submitted to paper chromatography in a solvent system consisting of *n*-butanol - acetic acid - water (4 : 1 : 2), and to electrophoresis on paper at pH 3.8, 6.6 and 11.1; these tests indicated that it behaves as a homogeneous substance. Moreover, the tetrapeptide obtained was found to be completely digestible by leucine aminopeptidase to give an amino acid composition in molar ratios which are consistent with the theoretical values.

The azide-coupling procedure has never before been employed when the nitroarginine residue has been included in the carboxyl moiety to be activated, because the nitroguanidino function may be decomposed by the action of hydrazine.[15] As is shown above, it is a valuable advantage that the N^G-tosyl-arginine is entirely free from this trouble. In the step of coupling Phe-Arg with Try for the preparation of compound XI, the DCCI method was also tried in place of the azide procedure, but the product had a low optical rotation ($[\alpha]_D^{25}$ $-4.4°$ (methanol)). This means that some racemization occurred in the arginine residue.

6) K. Hofmann, M. E. Woolner, H. Yajima, G. Spühler, T. A. Thompson and E. T. Schwartz, *J. Am. Chem. Soc.*, **80**, 6458 (1958).
7) R. Schwyzer and C. H. Li, *Nature*, **182**, 1669 (1958).
8) E. Schnabel and C. H. Li, *J. Am. Chem. Soc.*, **82**, 4576 (1960).
9) E. Schnabel and C. H. Li, *J. Biol. Chem.*, **235**, 2010 (1960).
10) D. M. Theodoropoulos and J. S. Fruton, *Biochemistry*, **1**, 933 (1962).
11) M. B. North and G. T. Young, *Chem. & Ind.*, **1955**, 1597; N. A. Smart, G. T. Young and M. W. Williams, *J. Chem. Soc.*, **1960**, 3902.
12) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
13) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1453 (1960).
14) K. Inouye and H. Otsuka, *J. Org. Chem.*, **27**, 4236 (1962).
15) J. S. Fruton, "Advances in Protein Chemistry," Vol. 5, Academic Press Inc., New York (1949), p. 1.

In connection with these studies, we have next achieved an improved synthesis of the tetrapeptide; this synthesis consists of the catalytic hydrogenation of a derivative with low solubility, N^{α}, N^{Im}-dicarbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophan benzyl ester (m. p. 167~168°C decomp., $[\alpha]_D^{24.5}$ −12.3° (dimethylformamide)). This compound was obtained from crystalline N^{α}, N^{Im}-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester (m. p. 112~113°C, $[\alpha]_D^{24.5}$ −15.0° (ethyl acetate))[16] and L-phenylalanyl-nitro-L-arginyl-L-tryptophan benzyl ester, which was synthesized in a step-by-step manner from the carboxyl end by the use of the *t*-butyloxycarbonyl group[19,20] as an amino-protecting element in conjugation with the DCCI procedure. This improved procedure for the synthesis of the protected tetrapeptide gave an over-all yield, based on L-tryptophan benzyl ester, of approximately 50%. The details of these experiments will be published elsewhere.

Experimental

All melting points are uncorrected.

N^{G}-Tosyl-L-arginine Monohydrate (I).—To 15 g. of crystalline N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginine (m. p. 102~106°C),[21] 130 g. of 25% (w/w) hydrogen bromide in acetic acid was added; the mixture then stood at room temperature for 30 min., after which time the solvent was removed at 40~45°C in vacuo. The oily residue was dissolved in 60 ml. of water and shaken with 60 ml. of ethyl acetate. The aqueous layer was washed further with ethyl acetate, cooled in an ice-bath, and neutralized to pH 8.6~8.8 by the addition of 28% aqueous ammonia to separate colorless crystals. After refrigeration overnight, the crystals were collected, washed with cold water, and dried; yield, 10.33 g. (92.1%); m. p. 142~147°C. Recrystallization from water gave lustrous crystals; recovery 94%, m. p. 145~149°C, $[\alpha]_D^{23.5}$ −3.9±1° (*c* 2.435, methanol), $[\alpha]_D^{24}$ +18.1±0.8° (*c* 2.518, N HCl); lit.[21]: m. p. 146~150°C, $[\alpha]_D^{25}$ −5.5° (*c* 1.3, methanol).

Found: C, 45.36; H, 6.41; N, 16.46; S. 9.11; H_2O, 5.18. Calcd. for $C_{13}H_{20}O_4N_4S \cdot H_2O$: C, 45.07; H, 6.40; N, 16.17; S. 9.26; H_2O, 5.20%.

N^{α}-Carbobenzoxy-N^{G}-tosyl-L-arginine Methyl Ester (II).—To 50 ml. of anhydrous methanol which had previously been cooled below −10°C, 1.20 ml. (0.0165 mol.) of thionyl chloride and 6.93 g. (0.015 mol.) of N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginine were added. The solution was stirred for 4 hr. and then let stand overnight at 0°C. After the removal of the solvent in vacuo the resulting sirup was dissolved in 50 ml. of ethyl acetate, washed with water, 5% sodium bicarbonate and with water, and dried over sodium sulfate. The evaporation of the solvent gave a foamy residue, which was then dried over phosphorus pentoxide in vacuo; yield, 6.59 g. (92.2%), colorless amorphous powder, $[\alpha]_D^{25.5}$ −8.7±0.4° (*c* 4.972, methanol). A single spot was visible in thin-layer chromatography on silica gel in ethyl acetate (R_f =0.43).

Found: C, 55.39; H, 6.36; N, 11.15; S, 6.28. Calcd. for $C_{22}H_{28}O_6N_4S$: C, 55.45; H, 5.92; N, 11.76; S. 6.73%.

N^{G}-Tosyl-L-arginine Methyl Ester (Free Base) (III).—Compound II (15.00 g.) was dissolved in 97 g. of 38% (w/w) hydrogen bromide in acetic acid, and the reaction mixture was allowed to stand at room temperature for 60 min. and evaporated in vacuo at 40~45°C. The residue was dissolved in 40 ml. of water and washed three times with 50 ml. portions of ethyl acetate to afford an aqueous solution of the ester hydrobromide, which gave a single ninhydrin reactive spot (R_f=0.63) on paper in the *n*-butanol-acetic acid-water (4:1:2) system.

To the aqueous solution of the hydrobromide obtained above, 60 ml. of methylene chloride was added, the mixture was then shaken with 30 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The aqueous phase was extracted again with methylene chloride, and the combined extract was dried over anhydrous sodium sulfate and evaporated in vacuo at 30°C to afford a gummy residue, which crystallized upon being dissolved in a small volume of methylene chloride. After the addition of ether, the mixture was refrigerated overnight and the crystals were filtered off, washed with ether and dried; yield, 9.71 g. (94.6% based on compound II used); m.p. 97°C (soften over 93°C). Recrystallization from the same solvent gave a sample for analyses; m. p. 98.5°C (soften over 95°C), $[\alpha]_D^{25}$ +14.8±1° (*c* 2.424, methanol).

Found: C, 48.78; H, 6.48; N, 15.92; S, 9.66. Calcd. for $C_{14}H_{22}O_4N_4S$: C, 49.11; H, 6.48; N, 16.36; S, 9.36%.

***t*-Butyloxycarbonyl-L-phenylalanine Dicyclohexylamine Salt (VI).**—*By t-Butyl p-Nitrophenyl Carbonate.*[19]—The free acid was synthesized by almost the same procedure as that described by Anderson and McGregor.[19] The product was ultimately crystallized from ethyl acetate and a large volume of petroleum ether as colorless fine needles. Since the isolation of the crystals was rather troublesome, the product was isolated and estimated as dicyclohexylamine salt.[22]

To an ethereal solution of the sirupy product (4.72 g.), which was obtained from 3.30 g. (0.02 mol.) of L-phenylalanine and 7.18 g. (0.03 mol.) of *t*-butyl *p*-nitrophenyl carbonate, a solution of 3.23 g. (0.018 mol.) of dicyclohexylamine (DCHA) in ether was added at 0°C. The DCHA salt which

16) Lit.: m. p. 86~90°C,[17] 109~110°C;[18] the optical rotation was not given.
17) F. Sakiyama, This Bulletin, **35**, 1943 (1962).
18) J. Meienhofer, *Chimia*, **16**, 385 (1962).
19) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).
20) L. A. Carpino, ibid., **79**, 98 (1957); L. A. Carpino, C. A. Giza and B. A. Carpino, ibid., **81**, 955 (1959).
21) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).
22) E. Klieger, E. Schröder and H. Gibian, *Liebigs Ann. Chem.*, **640**, 157 (1961); F. Weygand and K. Hunger, *Z. Naturforsch.*, **13b**, 50 (1958); *Chem. Abstr.*, **52**, 10880 (1958).

separated soon was filtered off, washed with ether, and dried; yield, 7.86 g. (88.0% based on L-phenylalanine used); m. p. 209~214°C decomp., $[\alpha]_D^{27}$ +29.2±2° (*c* 1.025, methanol).

Found: C, 70.13; H, 9.67; N, 6.36. Calcd. for $C_{26}H_{42}O_4N_2$: C, 69.92; H, 9.48; N, 6.27%.

By t-Butyl Azidoformate.[20]—The amorphous free acid, which was obtained by the method of Schwyzer et al.[23] from 8.3 g. (0.05 mol.) of L-phenylalanine and 14.3 g. (0.10 mol.) of *t*-butyl azidoformate,[24] was converted into the DCHA salt as described above; yield, 17.43 g. (78.2% based on L-phenylalanine used); m. p. 210~212°C decomp., $[\alpha]_D^{25.5}$ +28.9±0.5° (*c* 1.048, methanol).

Found: C, 71.09; H, 9.70; N, 6.37. Calcd. for $C_{26}H_{42}O_4N_2$: C, 69.92; H, 9.48; N, 6.27%.

L-Tryptophan Methyl Ester (V).—*L-Tryptophan Methyl Ester Acetate.*—A crude sample of L-tryptophan methyl ester hydrochloride[25] was converted into the acetate for purification. A mixture of the hydrochloride dissolved in a small volume of water and ether was shaken with 50% (w/v) potassium carbonate at 0°C. The ether phase was dried over sodium sulfate, to which an appropriate amount of glacial acetic acid was added to separate L-tryptophan methyl ester acetate as colorless thin needles; m. p. 106~108°C, $[\alpha]_D^{24.5}$ +15.3±1° (*c* 2.615, water).

Found: C, 60.50; H, 6.67; N, 10.09: Calcd. for $C_{12}H_{14}O_2N_2 \cdot CH_3COOH$: C, 60.42; H, 6.52; N, 10.07%.

Free Ester (V).—A suspension of 1.67 g. (6.0 mmol.) of the ester acetate obtained above in 20 ml. of methylene chloride was shaken with 5 ml. of ice-cold 50% (w/v) potassium carbonate in an ice-salt bath. The aqueous phase was then extracted again with methylene chloride. The combined organic extract was, after having been dried over sodium sulfate, evaporated at 25°C in vacuo to afford a sirupy residue, which crystallized easily from ether-petroleum ether; yield, 1.28 g. (97.8%); m. p. 90~91°C, $[\alpha]_D^{23.5}$ +36.3±1° (*c* 2.577, methanol).

Found: C, 66.12; H, 6.54; N, 12.92. Calcd. for $C_{12}H_{14}O_2N_2$: C, 66.04; H, 6.47; N, 12.84%.

L-Tryptophan Benzyl Ester (VI).—*L-Tryptophan Benzyl Ester p-Toluenesulfonate.*—A mixture of 20.42 g. (0.1 mol.) of L-tryptophan and 20.9 g. (0.11 mol.) of *p*-toluenesulfonic acid monohydrate in 150 ml. of benzyl alcohol was stirred at 90~100°C under a slow stream of nitrogen. After 6 hr. anhydrous ether (750 ml.) was introduced, and the precipitates (the salt of unesterified tryptophan) was filtered off (14.94 g.). From the filtrate the crystalline *p*-toluenesulfonate of the benzyl ester separated slowly in the form of needles; yield, 25.15 g.; m. p. 171~173°C.

Found: C, 64.59; H, 5.73; N, 6.22. Calcd. for $C_{25}H_{26}O_5N_2S$: C, 64.36; H, 5.62; N, 6.00%.

Free Ester (VI).—To 3.50 g. (7.5 mmol.) of the *p*-toluenesulfonate obtained above, 10 ml. of water and 30 ml. of ether were added. The mixture was then shaken with 10 ml. of 50% (w/v) potassium carbonate. The aqueous phase was extracted again with ether. The combined extract was dried over sodium sulfate and evaporated in vacuo to give a solid residue, which was then recrystallized from ether-petroleum ether; yield, 1.97 g. (89.4%); m. p. 71°C, $[\alpha]_D^{26.5}$ +12.8±1° (*c* 2.067, methanol); lit.[26] m. p. 71~72°C.

Found: C, 73.47; H, 6.22; N, 9.63. Calcd. for $C_{18}H_{18}O_2N_2$: C, 73.45; H, 6.16; N, 9.52%.

***t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginine Methyl Ester (VII).**—To a suspension of 3.125 g. (7.0 mmol.) of compound IV in 70 ml. of 50% ethanol, 7 ml. (wet volume) of Dowex-50W×8 (H^+-form) was added; the mixture was then shaken at room temperature for 30 min. After the removal of the resin, the filtrate was evaporated in vacuo at 40~45°C. The resulting sirup was dissolved in ether, and the solution was, after having been dried over anhydrous sodium sulfate, evaporated again to obtain *t*-butyloxycarbonyl-L-phenylalanine in an essentially quantitative yield.

The acyl amino acid obtained above was dissolved in methylene chloride; to the mixture 2.40 g. (7.0 mmol.) of compound V and a methylene chloride solution of 1.45 g. (7.0 mmol.) of *N*,*N'*-dicyclohexylcarbodiimide (DCCI) were then added at 0°. The reaction mixture (total volume 25 ml.) was let stand at 0°C overnight and then filtered to remove the separated urea (1.49 g., 95.2%). The filtrate was evaporated to afford a sirupy residue, which was redissolved in 40 ml. of ethyl acetate, washed successively with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate, water and finally with saturated sodium chloride, and dried over anhydrous sodium sulfate. After the removal of the solvent, the residue was dissolved in 7 to 8 ml. of acetonitrile, cooled at 0°C for 30 min., and then filtered to remove a slight amount of the urea. The evaporation of the solvent gave a foamy residue which was dried over phosphorous pentoxide; yield, 4.13 g. (100%); m. p. 93~98°C, $[\alpha]_D^{25}$ −5.9±0.5° (*c* 4.647, methanol). Thin-layer chromatography on silica gel in ethyl acetate gave a single spot reactive to ninhydrin when previously treated with concentrated hydrochloric acid.

Found: C, 56.78; H, 7.01; N, 11.82; S. 5.55. Calcd. for $C_{28}H_{39}O_7N_5S$: C, 57.03; H, 6.67; N, 11.88; S, 5.44%.

***t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginine (VIII).**—To a solution of 3.245 g. (5.5 mmol.) of compound VII in 16 ml. of methanol, 5.5 ml. of 2 N sodium hydroxide was added. The mixture was shaken at room temperature for 30 min. and then neutralized at 0°C by the addition of 11.0 ml. of ice-cold N hydrochloric acid and 20 ml. of water. The product thus precipitated was extracted three times with ethyl acetate. The combined organic phase was washed twice with

23) R. Schwyzer, P. Sieber and H. Kappeler, *Helv. Chim. Acta*, **42**, 2662 (1959).

24) *t*-Butyl carbazate, the key intermediate to the azide, was also easily obtained from *t*-butyl *p*-nitrophenyl carbonate[19] in a 90% yield.

25) R. A. Boissonnas, St. Guttmann, R. L. Huguenin, P.-A. Jaquenoud and Ed. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).

26) M. Brenner and W. Huber, ibid., **36**, 1109 (1953).

water and shaken with 15 ml. of M sodium bicarbonate and twice with water. The aqueous extracts were combined, washed three times with ethyl acetate, and then acidified at 0°C with ice-cold 4 N hydrochloric acid in the presence of 30 ml. of ethyl acetate. The aqueous phase was extracted twice with ethyl acetate. The combined ethyl acetate solution was washed once with ice-cold N hydrochloric acid and once with water until the washing became almost neutral; it was then dried over sodium sulfate and evaporated in vacuo at 30°C to afford a colorless, foamy residue which was dried over phosphorus pentoxide; yield, 3.05 g. (96.4%); m. p. 100~110°C, $[\alpha]_D^{24}$ +1.0±0.5° (*c* 4.226, methanol). A sample was treated with an-anhydrous trifluoroacetic acid at room temperature for 60 min. and then chromatographed on paper in the *n*-butanol - acetic acid - water (4 : 1 : 2) system to give a single ninhydrin reactive spot with R_f =0.80.

Found: C, 56.04; H, 6.78; N, 11.43; S. 5.81. Calcd. for $C_{27}H_{37}O_7N_5S$: C, 56.33; H, 6.48; N, 12.17; S, 5.57%.

***t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginine Hydrazide (IX).**—Compound VII (4.86 g., 8.25 mmol.) was dissolved in 48 ml. of anhydrous ethanol, and then 4.05 ml. of hydrazine hydrate was added at 0°C. The mixture was allowed to stand at 0°C for 3 days and then, after the addition of 50 ml. of water, concentrated to about 40 ml. in vacuo at 40°C. The sirupy precipitate was extracted three times with ethyl acetate, and the organic extracts were combined, washed twice, with water and with saturated sodium chloride, and dried over sodium sulfate. The crystalline hydrazide, which separated during the concentration of the ethyl acetate solution, was collected, washed twice with ice-cold ethyl acetate, and dried; yield, 4.58 g. (94.1%); m. p. 110~114°C. Recrystallization from methanol - ethyl acetate did not alter the melting point; $[\alpha]_D^{24}$ −6.3±1° (*c* 2.440, methanol).

Found: C, 55.22; H, 7.19; N, 16.61; S, 5.50. Calcd. for $C_{27}H_{39}O_6N_7S$: C, 54.99; H, 6.67; N, 16.63; S, 5.44%.

***t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophan Methyl Ester (X).**—*A) By the Carbodiimide Method.*—Compound VIII (2.88 g., 5.0 mmol.) and 1.09 g. (5.0 mmol.) of L-tryptophan methyl ester (V) were dissolved in 15 ml. of acetonitrile; to this solution was added under ice-cooling 1.03 g. (5.0 mmol.) of DCCI dissolved in a small volume of acetonitrile. The reaction mixture was then allowed to stand at 0°C overnight. After the removal of the separated urea (1.04 g., 92.5%), the filtrate was evaporated in vacuo at 35°C. The resulting sirupy residue was dissolved in 30 ml. of ethyl acetate, and the solution was washed with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. The evaporation of the solvent gave a foamy residue which was dried over phosphorus pentoxide in vacuo; yield, 3.68 g. (94.8%). The crude acyltripeptide ester (1.20 g.) obtained above was dissolved in 10 ml. of ethyl acetate, and the solution was passed through a column of dry silica gel (about 200 g.) with more ethyl acetate. The 3 ml.-fractions were collected, and those of tube Nos. 41—146, which contained the desired compound, were pooled and evaporated to dryness in vacuo to give an amorphous solid; wt. 0.86 g. (recovery 71.5%), m. p. 110~120°C, $[\alpha]_D^{25}$ −3.4±0.5° (*c* 4.546, methanol). Thin-layer chromatography on silica gel in ethyl acetate gave a single Ehrlich reactive spot. The single spot was also visible when the plate was heated after having been treated with sulfuric acid.

Found: C, 60.13; H, 6.66; N, 12.03; S, 4.33. Calcd. for $C_{39}H_{49}O_8N_7S$: C, 60.37; H, 6.37; N, 12.64; S, 4.13%.

B) By the Azide Method.—To a solution of 2.95 g. (5.0 mmol.) of compound IX in dimethylformamide (12 ml.), which had previously been cooled below −10°C, 25 ml. of ice-cold N hydrochloric acid and 2.75 ml. of ice-cold 2M sodium nitrite were added; the mixture was then stirred at −10°C for three min., after which time 20 ml. of ice-cold saturated sodium chloride was introduced. The separated azide was then extracted three times with 20 ml.-portions of cold ethyl acetate. The organic extract was washed with 20 ml. of ice-cold M sodium carbonate and 20 ml. of ice-cold saturated sodium chloride, and dried over anhydrous sodium sulfate. To this ethyl acetate solution 1.07 g. (4.85 mmol.) of L-tryptophan methyl ester (V) was then added, and the mixture was allowed to stand at 0°C. After 47 hr. the reaction mixture was washed with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate, water and, finally, saturated sodium chloride, and dried over sodium sulfate. The evaporation of the solvent gave a foamy residue which was dried over phosphorus pentoxide in vacuo; yield, 3.41 g. (90.8%). The crude product obtained above was purified on a silica gel column in almost the same way as has been described in A) to afford 2.80 g. (recovery 82.2%) of the pure tripeptide derivative, which was homogeneous in thin-layer chromatography; m. p. 110~120°C $[\alpha]_D^{26}$ −3.2±0.5° (*c* 4.417, methanol).

Found: C, 59.31; H, 6.60; N, 12.08; S, 4.10. Calcd. for $C_{39}H_{49}O_8N_7S$: C, 60.37; H, 6.37; N, 12.64; S, 4.13%.

***t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophan Benzyl Ester (XI).**—*t*-Butyloxycarbonyl-L-phenylalanyl-N^G-tosyl-L-arginine azide (prepared from 2.95 g. (5.0 mmol.) of the hydrazide (IX) as described above) and 1.47 g. (5.0 mmol.) of L-tryptophan benzyl ester (VI) were coupled in a ethyl acetate solution, almost as in case of the corresponding methyl ester (X), to give 3.24 g. (76.2%) of the crude peptide. A 3.14 g.-sample of the crude product was purified on silica gel (150 g.) as in the case of compound X; wt., 2.70 g. (recovery 86%), $[\alpha]_D^{24.5}$ −6.6±0.5° (*c* 4.095, methanol).

Found: C, 63.31; H, 6.42; N, 11.12; S, 3.68. Calcd. for $C_{45}H_{53}O_8N_7S$: C, 63.44; H, 6.27; N, 11.51; S, 3.76%.

The carbodiimide procedure was also employed in coupling compound VIII with L-tryptophan

benzyl ester (VI), as in the case of compound X, to obtain the same tripeptide derivative. The product was homogeneous in chromatography, but it had a low optical rotation; $[\alpha]_D^{25}$ $-4.4\pm0.6°$ (*c* 2.428, methanol).

N^{α}, N^{Im}-Dicarbobenzoxy-L-histidyl-L-phenylalanyl-N^{G}-tosyl-L-arginyl-L-tryptophan Benzyl Ester (XII).—Compound XI (2.45 g., 2.88 mmol.) was dissolved in 7.0 ml. of anhydrous trifluoroacetic acid at 0°C, and the solution was let stand at room temperature for 1 hr. under nitrogen. To this solution was then introduced about 100 ml. of anhydrous ether under ice-cooling. The resulting precipitates were collected, washed thoroughly with anhydrous ether, and dried to give 2.34 g. (94.0%) of L-phenylalanyl-N^{G}-tosyl-L-arginyl-L-tryptophan benzyl ester trifluoroacetate.

The ester trifluoroacetate (0.866 g., 1.0 mmol.), which had been dissolved in 15 ml. of ethyl acetate, was shaken with 10 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The organic phase was then washed with saturated sodium chloride, dried over sodium sulfate, and evaporated in vacuo at 30°C under nitrogen to afford a foamy residue (0.86 g.) The free tripeptide ester thus obtained was then dissolved in 10 ml. of acetonitrile, along with 0.423 g. (1.0 mmol.) of N^{α}, N^{Im}-dicarbobenzoxy-L-histidine.[14] To this solution was added 0.206 g. (1.0 mmol.) of DCCI dissolved in a small volume of acetonitrile; the mixture was allowed to stand at 0°C for 20 hr. After the separated urea had been removed by filtration, the filtrate was evaporated in vacuo at 35°C. The sirupy residue was redissolved in ethyl acetate, washed with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate and water, and dried over sodium sulfate. The evaporation of the solvent gave an amorphous solid residue; yield, 1.15 g. (99.3%). The crude product (1.15 g.), which was dissolved in 10 ml. of ethyl acetate, was applied onto a column of silica gel (60 g.) which had been previously stabilized with ethyl acetate; it was then eluted with the same solvent. The fractions containing the desired compound were pooled and evaporated to afford an amorphous solid residue; wt. 0.81 g. (recovery 70%); m. p. 97~105°C, $[\alpha]_D^{24.5}$ $-10.9\pm1°$ (*c* 1.825, methanol). Thin-layer chromatography on silica gel in ethyl acetate gave a single spot, which was reactive to the Ehrlich but not to the Pauly reagent.

Found: C, 64.26; H, 5.85; N, 11.94; S, 2.97. Calcd. for $C_{62}H_{64}O_{11}N_{10}S$: C, 64.34; H, 5.57; N, 12.10; S, 2.77%.

L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophan Monoacetate Dihydrate (XIII).—The protected tetrapeptide XII (500 mg.) was dissolved in 170 ml. of liquid ammonia which had been distilled from sodium; small pieces of sodium were then added over an interval of 20~25 min. until a blue color persisted. After the addition of 0.18 ml. of acetic acid, most of the ammonia was allowed to evaporate spontaneously; the rest was removed in vacuo to dryness over an ice-bath. The residue was dissolved in 40 ml. of 0.1 N acetic acid, and the solution was filtered through a Celite pad to remove any insoluble precipitates. The filtrate was passed through an Amberlite CG-50 column (wet vol. 14 cc.) in order to desalt it.[27] The column was washed with 700 ml. of 0.25% acetic acid and 50 ml. of water, and the peptide was then eluted with a solution consisting of pyridine-acetic acid-water (30:4:66 by volume). The eluate was evaporated in vacuo at 45°C, and the residue was dissolved in about 10 ml. of 0.1 N acetic acid. The lyophilization of this solution gave the crude tetrapeptide; yield, 306 mg. (95.8%). The crude product (230 mg.) was dissolved in about 20 ml. of a 0.025 M ammonium acetate buffer (pH 6.45), and this solution was added to a column (1.33×43 cm.) of carboxymethyl cellulose (Serva, 0.6 meq./g.) which had been equilibrated with the same buffer. The column was then eluted successively with the following ammonium acetate buffers (pH 6.45): 0.025 M (250 ml.), 0.0375 M (700 ml.), 0.05 M (350 ml.), and 0.075 M (1050 ml.). Individual fractions (7.5 ml. each) were collected at a flow rate of approximately 2 ml. per min. Absorbancy measurements at 280 mμ revealed the presence of one major and at least four minor components in the chromatogram. The fractions corresponding to the major peak, which was eluted with the 0.075 M buffer at the end, were pooled, the bulk of water was removed in vacuo, and the rest was lyophilized repeatedly to a constant weight; wt., 122 mg. (50.8% based on the protected peptide). The purified sample behaved as a single ninhydrin, Pauly, Ehrlich and Sakaguchi reactive spot in paper chromatography in the *n*-butanol-acetic acid-water (4:1:2) system (R_f=0.52~0.55), and in paper electrophoresis at pH 3.8, 6.6 and 11.1. λ_{max}^{HCl} 280.5 mμ (ε 5100), 288 mμ (ε 4250); $[\alpha]_D^{24.5}$ $-5.4\pm2°$ (*c* 0.947, N HCl). Amino acid ratios[28] in acid hydrolysate: His 1.00, Phe 1.00, Arg 1.03, (Try 0.79, decomposed partially with acid) (recovery 92.2%); amino acid ratios[28] in leucine aminopeptidase digest[29]: His 1.00, Phe 1.03, Arg 0.97, Try 0.98 (recovery 92.5%).

Found: C, 55.32; H, 6.71; N, 18.02. Calcd. for $C_{32}H_{40}O_5N_{10}\cdot CH_3COOH\cdot 2H_2O$: C, 55.12; H, 6.53; N, 18.91%.

Summary

1) A tetrapeptide, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan, corresponding to the amino-acid sequence of positions 6 to 9 in the corticotropin and α-MSH molecules has been synthesized.

2) This synthetic tetrapeptide has been found to exhibit an MSH potency of 3.6×10^4 units per gram in the in vitro frog skin assay, and it is also active on lipolysis in rabbit perirenal adipose tissue. These results may confirm that the glycine at position 10

27) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T-b. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **83**, 4449 (1961).

28) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1191 (1958).

29) LAP Lot No. 5930, Worthington Biochemical Corporation, Freehold, New Jersey, U. S. A.

in the molecules of corticotropin and α-MSH is not essential for these biological activities.

3) It has been shown that the azide-couling procedure can be employed successfully for the synthesis of arginyl peptides provided it is used in combination with the N^G-tosyl-arginine.

4) The preparation of N^G-tosyl-L-arginine and some of the relevant compounds has been described.

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