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Synthesis of a Biologically Active Nonadecapeptide Corresponding to the First Nineteen Amino Acid Residues of Adrenocorticotropins[1a]

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The synthesis of a nonadecapeptide, L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline, which has an amino acid sequence identical with the first 19 residues from the NH_2-terminus of adrenocorticotropin (ACTH), is described. The synthetic nonadecapeptide has been shown by bioassay to possess high ACTH as well as melanotropic (MSH) activities.

Adrenocorticotropic hormone (ACTH) in highly purified form has been isolated from sheep,[1b] pig[2] and beef[3] pituitaries. The complete structures of the porcine,[4] ovine[5] and bovine[6] hormones, which are straight-chain polypeptides composed of 39 amino acids, have been established. In Table I, which summarizes the known structural data on the hormones isolated from these three species, differences in composition and in the arrangement of amino acids at positions 25–33 are evident among the three. From these data, it can be inferred that the removal of that portion of the COOH-terminal sequence comprising positions 25–39 would not impair the adrenal-stimulating activity. Indeed, in the late 1940's it already was suspected that the active portion of ACTH was a relatively small polypeptide, after it had been demonstrated that digestion of the ACTH protein with pepsin did not destroy its activity,[7] and now there is some experimental evidence[4] to indicate that the biological potency of ACTH probably resides in the core consisting of the first 24 amino acid residues in the whole peptide molecule. It is obvious, however, that the minimal structural requirement for activity in the ACTH polypeptide molecule can be established with certainty only by the chemical synthesis of a peptide possessing adrenocorticotropic activity. In this paper is presented an account of the synthesis of a biologically active nonadecapeptide, L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-arginyl-L-arginyl-L-proline (V), which has an amino acid sequence identical with the first 19 residues from the NH_2-terminus of ACTH. A short account of the synthesis has already appeared.[8]

The nonapeptide N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-N^{ϵ}-tosyl-L-lysyl-N^{ϵ}-tosyl-L-lysyl-N^{G}-tosyl-L-arginyl-N^{G}-tosyl-L-arginyl-L-proline methyl ester (Id) was prepared by coupling the *p*-nitrophenyl ester Ib of the protected tetrapeptide N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl-L-prolyl-L-valyl-glycine with the product which was obtained by catalytic hydrogenation of the protected pentapeptide N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl-N^{ϵ}-tosyl-L-lysyl-N^{G}-tosyl-L-arginyl-N^{G}-tosyl-L-arginyl-L-proline methyl ester (Ic).

Peptide Ic was synthesized by the stepwise lengthening of the chain one amino acid at a time starting from the COOH-terminal proline methyl ester. A similar stepwise procedure performed by the nitrophenyl ester method[9] has been employed recently for the syntheses of oxytocin[10] and lysine-vasopressin[11] and their 2-phenylalanine analogs.[12] In our synthesis we employed dicyclohexylcarbodiimide coupling[13] during the first two steps, since we did not succeed in preparing a stable nitrophenyl ester of N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginine. It was thought that this nitrophenyl ester might possibly be formed when $N\alpha$-carbobenzoxy-N^{G}-tosyl-L-arginine was treated with *p*-nitrophenol in the presence of dicyclohexylcarbodiimide[13,14]; however, only the corresponding lactam could be isolated.[15] This lactam formation was also reported when peptide synthesis was performed with dicarbobenzoxy-L-arginine[16] by the dicyclohexylcarbodiimide method.

For the last two steps in the preparation of the protected pentapeptide Ic, N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysine *p*-nitrophenyl ester[11] was employed. The dipeptide intermediate (N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginyl-L-proline methyl ester) was obtained in crystalline form. Attempts at crystal-

(1) (a) This Communication is Paper XXIV of the adrenocorticotropins (ACTH) series; (b) C. H. Li, I. I. Geschwind, A. L. Levy, J. I. Harris, J. S. Dixon, N. G. Pon and J. O. Porath, *Nature*, **173**, 251 (1954); C. H. Li, I. I. Geschwind, J. S. Dixon, A. L. Levy and J. I. Harris, *J. Biol. Chem.*, **213**, 171 (1955).

(2) P. H. Bell, *J. Am. Chem. Soc.*, **76**, 5565 (1954); R. G. Shepherd, K. S. Howard, P. H. Bell, A. R. Cacciola, R. G. Child, M. C. Davies, J. P. English, B. M. Finn, J. M. Meisenhelder, A. W. Moyer and J. van der Scheer, *ibid.*, **78**, 5051 (1956).

(3) C. H. Li and J. S. Dixon, *Science*, **124**, 934 (1956).

(4) K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies and P. H. Bell, *J. Am. Chem. Soc.*, **77**, 3419 (1955); R. G. Shepherd, S. D. Wilson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner and N. E. Shakespeare, *ibid.*, **78**, 5067 (1956).

(5) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955); J. Leonis, C. H. Li and D. Chung, *J. Am. Chem. Soc.*, **81**, 419 (1959).

(6) C. H. Li, J. S. Dixon and D. Chung, *ibid.*, **80**, 2587 (1958); *Biochem. Biophys. Acta*, **46**, 324 (1961).

(7) C. H. Li, Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, N. Y., 17th Meeting, 1948, p. 114.

(8) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. B. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960).

(9) M. Bodanszky, *Nature*, **175**, 685 (1955); *Acta Chim. Hung.*, **10**, 335 (1957); *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).

(10) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(11) M. Bodanszky, J. Meienhofer and V. du Vigneaud, *ibid.*, **82**, 3195 (1960).

(12) M. Bondanszky and V. du Vigneaud, *ibid.*, **81**, 1258 (1959).

(13) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(14) M. Rothe and F. W. Kunitz, *Ann. Chem. Liebigs*, **609**, 88 (1957).

(15) E. Schnabel and C. H. Li, *J. Am. Chem. Soc.*, **82**, 4576 (1960).

(16) L. Zervas, T. T. Otani, M. Winitz and J. P. Greenstein, *ibid.*, **81**, 2878 (1959).

TABLE I

STRUCTURAL DIFFERENCES AMONG ADRENOCORTICOTROPINS ISOLATED FROM PIG, SHEEP AND BEEF PITUITARY GLANDS

Structure of bovine ACTH

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

NH_2

Lys-Val-Tyr-Pro-Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

Species	Laboratory	Amino acid residue in position 25 26 27 28 29 30 31 32 33
		NH_2
Pig	American Cyanamid Co.[4]	Asp-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu
		NH_2
Sheep	University of Calif.[5]	Ala-Gly-Glu-Asp-Asp-Glu-Ala-Ser-Glu
		NH_2
Beef	University of Calif.[6]	Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-Glu

lization of the other intermediates have so far proved unsuccessful; consequently, countercurrent distribution has been used at various stages of the synthesis for the purification of the intermediates.

The protected tetrapeptide N^{α}-carbobenzoxy-N^{ϵ} - tosyl - L - lysyl - L - prolyl - L - valyl - glycine (Ia) was synthesized by coupling crystalline L-prolyl-L-valyl-glycine methyl ester with carbobenzoxy-N^{ϵ}-tosyl-L-lysine by means of the *p*-nitrophenyl ester[9] method. Carbobenzoxy-L-prolyl-L-valine was allowed to react with glycine methyl ester, with dicyclohexylcarbodiimide as the condensing agent; the resulting protected tripeptide, carbobenzoxy-L-prolyl-L-valyl-glycine methyl ester,[17] was obtained in crystalline form and in high yield. The protected tripeptide ester was hydrogenated to give a free crystalline tripeptide ester. This material was allowed to react with N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysine *p*-nitrophenyl ester in ethyl acetate to give the crystalline protected tetrapeptide ester. Saponification of this tetrapeptide derivative yielded crystalline N^{α} - carbobenzoxy - N^{ϵ} - tosyl - L - lysyl - L-prolyl-L-valyl-glycine (Ia) in 93% yield. The *p*-nitrophenyl ester of Ia was prepared with the use of dicyclohexylcarbodiimide[13,14]; the product was obtained in crystalline form in good yield.

Preparation of N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl - L - prolyl - L - valyl - glycyl - N^{ϵ} - tosyl - L-lysyl - N^{ϵ} - tosyl - L - lysyl - N^{G} - tosyl - L - arginyl-N^{G}-tosyl-L-arginyl-L-proline (I) by saponification of the protected nonapeptide ester Id presented some difficulties. If the alkali was not allowed to react long enough, the saponification was incomplete. On the other hand, prolonged reaction with the alkali caused considerable damage to the material, so that increasing amounts of decomposition products were obtained. We found that the most satisfactory procedure to follow was to allow the alkali to react for one or two hours and subsequently to separate the ester and the acid either by extraction or countercurrent distribution; the unreacted ester was then re-saponified in the same manner. The protected nonapeptide acid I also was prepared in good yield by coupling the saponified protected pentapeptide Ic after hydrogenation with the tetrapeptide nitrophenyl ester Ib.

The nonapeptide I was hydrogenated catalytically and then allowed to react with the hexapeptide N^{α}-carbobenzoxy-γ-benzyl-L-glutamyl-Im - benzyl - L - histidyl - L - phenylalanyl - N^{G}-tosyl-L-arginyl-L-tryptophyl-glycine (II) *via* the mixed anhydride procedure with isobutyl chloroformate.[18] The resulting pentadecapeptide III was then purified by countercurrent distribution in the system consisting of toluene–chloroform–methanol–water (5:5:8:2 by volume) for 242 transfers. The protected pentadecapeptide III was isolated from those tubes falling within the theoretical distribution curve corresponding to the main peak which had a distribution coefficient (*K*) of 0.34.

Two slightly different routes of synthesis were used in the preparation of peptide II. For the first, N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginyl-L-tryptophylglycine methyl ester[15] was subjected to catalytic hydrogenation, and the product was then allowed to react with N^{α}-carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanine *p*-nitrophenyl ester. The resulting protected pentapeptide ester was saponified and the resulting acid then was subjected to catalytic hydrogenation; the hydrogenated product next was coupled with the *p*-nitrophenyl ester of carbobenzoxy-γ-benzyl-L-glutamic acid. The second route of the synthesis of peptide II differs from the first only in the saponification step. For this step, the N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginyl-L-tryptophyl-glycine methyl ester was saponified, hydrogenated and then condensed with carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanine *p*-nitrophenyl ester. This method was used in order to avoid degradation of the protected pentapeptide ester during saponification; moreover, the tripeptide acid is easier to purify than the pentapeptide acid. The remainder of the synthesis was carried out in the same manner as in the first procedure.

The protected pentadecapeptide III was submitted to catalytic hydrogenation and the product was allowed to react with peptide IV, N^{α}-carbobenzoxy - L - seryl - L - tyrosyl - L - seryl - L - methioninhydrazide, by the azide procedure. Peptide IV was obtained by the condensation of carbobenzoxy-

(17) K. Hofmann, E. Sturz, G. Spuhler, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3727 (1960).

(18) J. R. Vaughan, Jr. and R. L. Osato, *ibid.*, **74**, 676 (1952).

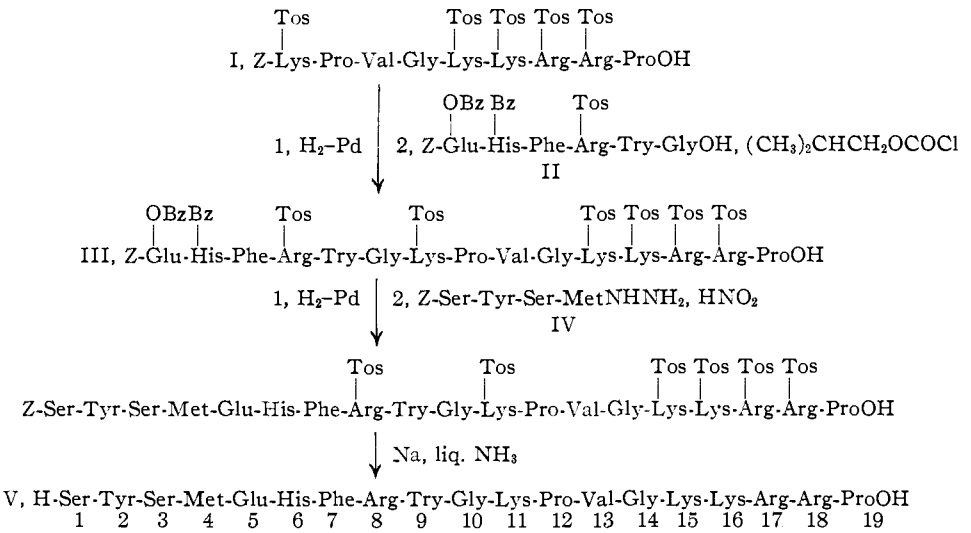

Fig. 1.—Outline of the synthesis of L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline; Z, carbobenzoxy; Bz, benzyl; Tos, *p*-toluenesulfonyl.

L-seryl-L-tyrosine azide with L-seryl-L-methionine methyl ester similar to the procedure employed by previous workers.[19] The protected nonadecapeptide was treated with sodium–liquid ammonia[20]; the crude nonadecapeptide was desalted and then purified by countercurrent distribution in two solvent systems. Figure 1 presents an outline of the synthetic steps involved in the preparation of the nonadecapeptide.

Even though countercurrent distribution was used extensively for purification of the intermediates in the course of our synthesis, the final products were still difficult to obtain in crystalline form. In only one instance, peptide II, was crystallization achieved after purification by countercurrent distribution. In Table II are given the distribution coefficients of the large peptide fragments obtained during the synthesis.

TABLE II

DISTRIBUTION COEFFICIENTS (K) OF VARIOUS SYNTHETIC PEPTIDES

Peptide[a]	Solvent system[b]	K
I	Toluene	0.26
II	Carbon tetrachloride	.16
III	Toluene	.34
IV	Pyridine	.08
V	2-Butanol	.58

[a] See Fig. 1. [b] Toluene, $CHCl_3$–$C_6H_5CH_3$–CH_3OH–H_2O (5:5:8:2, by volume); carbon tetrachloride; $CHCl_3$–CCl_4–CH_3OH–H_2O (3:1:3:1, by volume); pyridine, 0.1% acetic acid–1-butanol–pyridine (11:5:3, by volume); 2-butanol, 2-butanol–0.5% trichloroacetic acid.

In order to avoid the danger of racemization during the synthesis, we planned the coupling reactions to involve optically inactive carboxyl terminal glycine (as has been done in the synthesis of melanocyte-stimulating hormone derivatives[21]), since it is known that any carboxyl terminal amino acid is quite likely to be racemized in the course of the reactions that are carried out by means of the mixed anhydride procedure[18] and the *p*-nitrophenyl ester method.[9] In the preparation of the nonapeptide I, the *p*-nitrophenyl ester of the N^{α}-carbobenzoxy - N^{ϵ} - tosyl - L - lysyl - L - prolyl-L-valyl-glycine was allowed to react with the pentapeptide N^{ϵ}-tosyl-L-lysyl-N^{ϵ}-tosyl-L-lysyl-N^{G}-tosyl - L - arginyl - N^{G} - tosyl - L - arginyl - L -proline. For the synthesis of the pentadecapeptide III, the mixed anhydride of the hexapeptide II, which has glycine as carboxyl terminal residue, was used for reaction with the hydrogenated peptide I. Finally, since there is no evidence that azide coupling involves any racemization,[22] this procedure was used to link the final tetrapeptide IV to the hydrogenated pentadecapeptide III to form the protected nonadecapeptide V.

During the preparation of the various peptide fragments, much effort was directed toward obtaining crystalline intermediates in the hope that it will reduce the amount of racemized product. In those peptides where arginine was present, however, attempts at crystallization were unsuccessful except for the dipeptide N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginyl-L-proline methyl ester, and the hexapeptide II.

In the solvent system consisting of 2-butanol–0.5% trichloroacetic acid, peptide V distributed in accordance with the theoretical curve for a partition coefficient of 0.58. Peptide V also was subjected to amino acid analyses by both the paper–fluorodinitrobenzene method[23,24] and the chromatographic procedure[25] as well as by a spectrophotometric method[26] for the quantitative determination of tyrosine and tryptophan; these analyses gave

(19) K. Hofmann, A. Jöhl, A. E. Furlenmeier and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957).

(20) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(21) K. Hofmann, *Ann. N. Y. Acad. Sci.*, **88**, 689 (1960).

(22) M. Goodman and G. W. Kenner, *Advances in Protein Chem.*, **12**, 465 (1957).

(23) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(24) A. L. Levy, *Nature*, **174**, 126 (1954).

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

(26) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

the composition in molar ratios which are consistent with the theoretically calculated values (mol. wt. 2346.7)

$Ser_1Tyr_1Met_1Glu_1His_1Phe_1Arg_3Try_1Gly_2Lys_3Pro_2Val_1$

Successive hydrolysis of peptide V by trypsin, chymotrypsin and leucine aminopeptidase caused complete degradation of the peptide V to its constituent amino acids with the exception of the arginyl–proline bond at the C-terminus. Apparently, the arginylproline linkage is not only resistant to tryptic digestion as is the case with the adrenocorticotropins[4-6] but also is unaffected by leucine aminopeptidase.

It is well established[4,27] that adrenocorticotropins possess an intrinsic melanocyte-stimulating activity. Indeed, one of the melanocyte-stimulating hormones, α-MSH, from pig[28] and horse[29] pituitaries, consists of 13 amino acids and contains a sequence identical with ACTH except that the α-amino group is blocked with an acetyl residue and the C-terminus with an amide. The synthesis of α-MSH in its natural form has been achieved recently by Guttman and Boissonnas,[30] and its glutaminyl and formyl-lysyl analog has been synthesized by Hofmann, *et al.*[31] Moreover, a heptapeptide sequence, L-methionyl-L-glutamyl-L - histidyl - L - phenylalanyl - L - arginyl - L-tryptophyl-glycine, which occurs in preparations of ACTH as well as in the synthetic nonadecapeptide, forms a part of the structure of β-MSH from pig,[32,33] beef[34] and human[35] pituitary glands. It was therefore to be expected that peptide V should exhibit melanocyte-stimulating activity. By the *in vitro* frog skin assay procedure[36] peptide V was shown to possess an MSH activity of 1.4×10^7 units per gram. Moreover, a single dose of 0.1 microgram of the peptide caused a change in melanophore index[37] in hypophysectomized *Rana pipiens* of from 1+ to 3+ within one hour. It is thus clearly evident that peptide V has an MSH potency comparable to that of the native adrenocorticotropins. As far as we are aware, this is the first synthetic peptide which possesses both adrenocorticotropic and melanotropic activities in *high* potency; hence, it represents the most convincing evidence yet obtained in favor of intrinsic melanocyte-stimulating activity in adrenocorticotropins.

According to the results of bioassay by the *in vitro* adrenal method,[38] peptide V was found to have an ACTH activity of 40 U.S.P. units per mg. with a 95% confidence limit for the assay in the range of 33–49. When the synthetic nonadecapeptide was tested for adrenocorticotropic activity by the adrenal ascorbic acid depletion method,[39] it was found to have a potency of 74 U.S.P. units per mg. if injections were given subcutaneously, and a potency of 35 units per mg. by the intravenous route. It may be recalled[1-3,27,40] that native adrenocorticotropins from various species possess an ACTH activity of 80–150 U.S.P. units per mg. as estimated by both *in vivo* and *in vitro* methods. Thus, peptide V, which has an amino-acid sequence corresponding to the first 19 amino acid residues of the adrenocorticotropins, has approximately 50% the ACTH potency of the native hormone. It will be of interest to ascertain the minimal structural requirement for full adrenocorticotropic potency and to obtain synthetic peptides which possess either *minimal* ACTH activity, or none at all, but which exercise other physiological functions including lipolysis, adipokinesis, erythropoiesis, etc.

Experimental[41]

N$^\alpha$-Carbobenzoxy-N^G-tosyl-L-arginyl-L-proline Methyl Ester.—L-Proline methyl ester (7.0 g.), prepared from a solution of L-proline methyl ester hydrochloride,[14] was dissolved in acetonitrile (50 ml.) along with N$^\alpha$-carbobenzoxy-N^G-tosyl-L-arginine[15] (23.3 g.). The solution was cooled to 0° before the addition of dicyclohexylcarbodiimide (12.5 g.). After being allowed to stand for 6 hours at 0° and 40 hours at room temperature, the heavy precipitate (dicyclohexylurea) was filtered off and washed once with hot acetonitrile and twice with boiling acetone. When the solution was concentrated *in vacuo*, the product crystallized in the form of prisms. From the mother liquor, more of the protected dipeptide was isolated by evaporating to dryness, dissolving the material in ethyl acetate, and washing in the usual manner with acid and base; yield 20.6 g. (72%), m.p. 151–152°. A sample was recrystallized from much ethyl acetate; m.p. 152–153°, $[\alpha]^{25}D$ −39.0° (*c* 1, methanol).

Anal. Calcd. for $C_{27}H_{35}O_7N_5S$ (573.7): C, 56.5; H, 6.15; N, 12.2; S, 5.59. Found: C, 56.8; H, 6.01; N, 12.0; S, 5.42.

N$^\alpha$-Carbobenzoxy-N^G-tosyl-L-arginyl-N^G-tosyl-L-arginyl-L-proline Methyl Ester.—N$^\alpha$-Carbobenzoxy-N^G-tosyl-L-arginyl-L-proline methyl ester (19.2 g.) was suspended in methanol (150 ml.) and concentrated hydrochloric acid (3 ml.) containing palladium black (freshly prepared from 4 g. of $PdCl_2$). Hydrogen was passed through the solution which was stirred vigorously with a Vibro-Mixer.[43] After 4 hours the catalyst was filtered from the clear solution and the solvent was removed by evaporation *in vacuo*. The residual colorless oil was dissolved in acetonitrile (100 ml.), and N$^\alpha$-carbobenzoxy-N^G-tosyl-L-arginine[15] (15.5 g.) was added along with triethylamine (5 ml.). The solution was cooled and dicyclohexylcarbodiimide (8.25 g.) was added. After being allowed to stand for 6 hours at 0° and for 34 hours at room temperature, the precipitated dicyclohexylurea was removed by filtration and the solvent was removed by evaporation *in vacuo*, leaving a yellow oil, which was dis-

(27) C. H. Li, P. Fønss-Bech, I. I. Geschwind, T. Hayashida, G. Hungerford, A. J. Lostroh, W. R. Lyons, H. D. Moon, W. O. Reinhardt and M. Sideman, *J. Exp. Med.*, **105**, 335 (1957).

(28) J. I. Harris and A. B. Lerner, *Nature*, **179**, 1346 (1957).

(29) J. S. Dixon and C. H. Li, *J. Am. Chem. Soc.*, **82**, 4568 (1960).

(30) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **42**, 1257 (1959).

(31) K. Hofmann, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3732 (1960).

(32) J. I. Harris and P. Roos, *Nature*, **178**, 90 (1956); *Biochem. J.*, **71**, 434 (1959).

(33) I. I. Geschwind, C. H. Li and L. Barnafi, *J. Am. Chem. Soc.*, **78**, 4494 (1956); **79**, 620 (1957).

(34) I. I. Geschwind, C. H. Li and L. Barnafi, *ibid.*, **79**, 6394 (1957).

(35) J. I. Harris, *Nature*, **184**, 167 (1959).

(36) K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(37) L. T. Hogben and D. Slome, *Proc. Roy. Soc.* (*London*), **B108**, 10 (1931).

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

(39) M. A. Sayers, G. Sayers and L. A. Woodbury, *ibid.*, **42**, 379 (1949).

(40) R. Guillemin, *ibid.*, **66**, 819 (1960).

(41) Melting points were performed on a Fisher–Johns melting point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. All samples for microanalyses were dried in an Abderhalden drying pistol with P_2O_5 at 77° for 16 hours at 0.3 mm. pressure. Paper chromatography was carried out on Whatman No. 1 filter paper at room temperature; the solvents used were 1-butanol–acetic acid–water (BAW) in a ratio of 4:1:1 and *sec*-butyl alcohol–10% NH_3 (SBA) in a ratio of 85:15; the location of the peptide spot was either revealed by the ninhydrin reagent or the chlorine method.[42]

(42) H. Zahn and E. Rexroth, *Z. anal. Chem.*, **148**, 181 (1955).

(43) Vibro-Mixer, A. G. Fuer chemie-Apparatebau, Zurich, Model E1.

solved in ethyl acetate. The solution was washed in the usual manner with acid and base and dried over $MgSO_4$. The product, which was obtained by evaporation of the solvent, was isolated by precipitation from acetone–ether. The product was obtained in the form of a white amorphous powder, yield 21.5 g. (73.5%), m.p. 112–120°, $[\alpha]^{23}D$ −31.0° (*c* 1, methanol).

The product was further purified by countercurrent distribution in the solvent system methanol–water–chloroform–carbon tetrachloride (4:1:5:5). After 240 transfers two peaks were well separated. The first peak with K = 0.17 represented less than 5% of an impurity, whereas the second peak with K = 0.41 followed closely the theoretical distribution curve and represented the tripeptide derivative which was recovered in 80% yield, m.p. 115–120°, $[\alpha]^{24}D$ −32.0° (*c* 1, methanol).

Anal. Calcd. for $C_{40}H_{53}O_{10}N_9S_2$ (884.0): C, 54.3; H, 6.04; N, 14.3; S, 7.25. Found: C, 54.3; H, 6.21; N, 14.4; S, 7.10.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline Methyl Ester.—The tripeptide base was prepared from Nα-carbobenzoxy-NG-tosyl-L-arginyl-NG-tosyl-arginyl-L-proline methyl ester (19.0 g.) by hydrogenation as described above and dissolved in acetonitrile (110 ml.); to this solution was added Nα-carbobenzoxy-Nε-tosyl-L-lysine *p*-nitrophenyl ester[11] (12.0 g.). After the mixture had been allowed to stand for 40 hours at room temperature, the solvent was evaporated *in vacuo*. The residual oil was dissolved in acetone (100 ml.) and the solution was slowly poured into ether (900 ml.) with vigorous stirring. This precipitation procedure was repeated twice, yielding 21.0 g. (84%) of a white amorphous powder, m.p. 110–115°, $[\alpha]^{24}D$ −30.5° (*c* 1, methanol).

Anal. Calcd. for $C_{58}H_{71}O_{13}N_{11}S_3$ (1166.4): C, 54.6; H, 6.14; N, 13.2; S, 8.26. Found: C, 54.8; H, 6.33; N, 13.1; S, 8.05.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline Methyl Ester.—The free tetrapeptide base was prepared from Nα-carbobenzoxy-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-arginyl-L-proline methyl ester (1.99 g.) by hydrogenation as described above and dissolved in acetonitrile (5 ml.); to this solution was added Nα-carbobenzoxy-Nε-tosyl-L-lysine *p*-nitrophenyl ester[11] (1.14 g.). After the mixture had stood for 45 hours at 35°, the solvent was evaporated *in vacuo* and the residual material was purified by three successive precipitations from acetone–ether. The product was obtained in the form of a white amorphous powder, yield 2.24 g. (91%), m.p. 108–113°, $[\alpha]^{25}D$ −29.0° (*c* 1, methanol).

Anal. Calcd. for $C_{66}H_{89}O_{16}N_{13}S_4$ (1448.7): C, 54.7; H, 6.19; N, 12.6; S, 8.85. Found: C, 54.5; H, 6.19; N, 12.7; S, 8.84.

The compound withstood all attempts at crystallization. In order to check its purity, a sample was subjected to countercurrent distribution in the solvent system chloroform–toluene–methanol–water (5:5:8:2). After 405 transfers, the distribution followed closely the theoretical curve of K = 0.45.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline.—The protected pentapeptide ester (3.15 g.) was dissolved in acetone (10 ml.) and the solution was cooled in ice; 2 *N* sodium hydroxide (2.1 ml.) was added dropwise over a period of 20 minutes. The solution was stirred for 50 minutes longer at room temperature. It then was acidified with 1 *N* hydrochloric acid (5 ml.) and the solid precipitate was filtered, washed thoroughly with water, and dried; yield 3.1 g. The material was subjected to countercurrent distribution in the system consisting of chloroform–toluene–methanol–water (5:5:8:2). After 348 transfers, two well-separated major peaks were obtained with values for K of 0.47 (unsaponified ester) and 0.93 (acid). Decomposition impurities traveled faster; recoveries: 50% acid, 25% ester, 10% other impurities. The acid (1.57 g.) was obtained in the form of an amorphous white powder, m.p. 124–126°, $[\alpha]^{25}D$ −28.4° (*c* 1, methanol).

Anal. Calcd. for $C_{65}H_{87}O_{16}N_{13}S_4$ (1434.7): C, 54.5; H, 6.11; N, 12.7; S, 8.94. Found: C, 54.3; H, 6.12; N, 12.6; S, 8.76.

Carbobenzoxy-L-prolyl-L-valyl-glycine Methyl Ester.—Glycine methyl ester hydrochloride (9.42 g.) was dissolved in ice-cold saturated potassium carbonate solution (50 ml.) and extracted with three 50-ml. portions of dichloromethane. The dichloromethane extract was dried over anhydrous sodium sulfate and the dried material was added to a solution of carbobenzoxy-L-prolyl-L-valine[44] in dichloromethane (17.4 g. in 150 ml.). The solution was cooled to 0° and dicyclohexylcarbodiimide (10.4 g.) was added. This solution was allowed to stand for 15 hours at 5° and the dicyclohexylurea that formed then was filtered off and washed with dichloromethane. The combined filtrate and washings were washed successively with 0.1 *N* HCl, water, 1 *M* NH_4OH and water and dried over anhydrous sodium sulfate. The solution was concentrated to a volume of 150 ml. and an equal volume of petroleum ether (b.p. 30–60°) was added to yield a product in crystalline form; yield 19 g. (90%), m.p. 109–110°. A sample was recrystallized for analysis from dichloromethane–petroleum ether; m.p. 111–112°, $[\alpha]^{25}D$ −90.4° (*c* 1.6, methanol); in paper chromatography, R_f BAW = 0.81 and R_f SBA = 0.79.

Anal. Calcd. for $C_{21}H_{29}O_6N_3$ (419.5): C, 60.1; H, 6.97; N, 10.0. Found: C, 60.0; H, 6.80; N, 10.1.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine Methyl Ester.—Carbobenzoxy-L-prolyl-L-valyl-glycine methyl ester (14.0 g.) was dissolved in 150 ml. of methanol, and to this solution, palladium-black (freshly prepared from 3 g. $PdCl_2$) was added. Hydrogen was passed through the solution while it was being rapidly stirred by a Vibro Mixer.[43] After 8 hours the catalyst was removed by filtration and the solvent removed *in vacuo*. The methyl ester of the tripeptide base crystallized in the form of needles, m.p. 140–142°. The needles were immediately dissolved in ethyl acetate (100 ml.) along with Nα-carbobenzoxy-Nε-tosyl-L-lysine *p*-nitrophenyl ester[11] (18.9 g.). After this mixture had been allowed to stand for 60 hours at room temperature, a gelatinous precipitate formed. This precipitate was dissolved by the addition of more ethyl acetate (400 ml.) with slight warming. The solution was washed successively with 0.75 *N* ammonia (5 times), water, 1 *N* hydrochloric acid, and water, and dried over magnesium sulfate. Concentration to a small volume (30 ml.) yielded a product which crystallized in the form of needles. Recrystallization from ethyl acetate gave a yield of 17.6 g. (76%) Nα-carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine methyl ester in the form of white needles, m.p. 139–140°, $[\alpha]^{25}D$ −79.0° (*c* 1, methanol); in paper chromatography, R_f BAW = 0.91 and R_f SBA = 0.73.

Anal. Calcd. for $C_{34}H_{47}O_9N_5S$ (701.8): C, 58.2; H, 6.75; N, 9.98; S, 4.56. Found: C, 58.3; H, 6.69; N, 9.91; S, 4.79.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine.—The protected tetrapeptide ester (4.9 g.) was dissolved in acetone (15 ml.). The solution was cooled in ice and 2 *N* sodium hydroxide (7 ml.) was added dropwise over a period of 20 minutes. After being stirred for 1 hour at room temperature the solution was acidified with dilute hydrochloric acid. The crystalline precipitate was recrystallized from ethyl acetate which had been saturated with water; yield 4.46 g. (93%) of white needles, m.p. 109–110°, $[\alpha]^{25}D$ −73.0° (*c* 1, methanol); in paper chromatography, R_f BAW = 0.78 and R_f SBA = 0.38.

Anal. Calcd. for $C_{33}H_{45}O_9N_5S$ (687.8): C, 57.6; H, 6.60; N, 10.2; S, 4.66. Found: C, 57.5; H, 6.59; N, 10.4; S, 4.60.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine *p*-Nitrophenyl Ester.—Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine (4.82 g.) was dissolved in ethyl acetate (30 ml.) along with *p*-nitrophenol (1.07 g.). The solution was cooled to 0° and dicyclohexylcarbodiimide (1.59 g.) was added. After the mixture had been stirred for 1 hour at 0° and for 5 hours at room temperature, acetic acid (0.1 ml.) was added and the almost solidified mass was diluted with ethyl acetate (100 ml.). After the diluted mixture had been heated to the boiling point, the insoluble dicyclohexylurea was removed by filtration and was then washed repeatedly with boiling ethyl acetate. From the combined filtrate and washings, the solvent was removed by evaporation *in vacuo* and the residue was crystallized in the form of white prisms from absolute ethanol (70 ml.) to which a few drops of acetic acid had been added;

(44) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

yield 5.16 g. (91%), m.p. 152–153°, $[\alpha]^{24.5}D$ −40.5° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{89}H_{48}O_{11}N_8S$ (808.9): C, 57.9; H, 5.98; N, 10.4. Found: C, 58.0; H, 6.08; N, 10.4.

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline Methyl Ester.—The free base of the pentapeptide ester was prepared from Nα-carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline methyl ester (4.35 g.) by hydrogenation as described above and dissolved in acetonitrile (30 ml.); to this solution was added Nα-carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine *p*-nitrophenyl ester (2.67 g.). After the mixture had been allowed to stand for 60 hours at room temperature, the solvent was evaporated *in vacuo* and the residual product was precipitated twice from acetone–ether, yielding 5.76 g. (96%) of a white amorphous powder, m.p. 119–121°, $[\alpha]^{25}D$ −42.0° (*c* 1, methanol).

Anal. Calcd. for $C_{91}H_{126}O_{22}N_{18}S_5$ (1984.4): C, 55.1; H, 6.40; N, 12.7; S, 8.08. Found: C, 54.9; H, 6.24; N, 12.8; S, 8.05.

The product resisted all attempts at crystallization. A sample (1.25 g.) was subjected to countercurrent distribution in the solvent system consisting of chloroform–toluene–methanol–water (5:5:8:2). After 435 transfers, one main peak (K = 0.26), which followed the theoretical distribution curve, and two very small peaks on either side were obtained. From the main peak 850 mg. (68%) of material was recovered with m.p. 119–121°, $[\alpha]^{25}D$ −43.2° (*c* 1, methanol).

The protected nonapeptide ester also was prepared by the mixed anhydride procedure, with isobutyl chloroformate.[45] The free base of the pentapeptide ester was prepared from its carbobenzoxy derivative (1.45 g.) by hydrogenation as described above, and then dissolved in peroxide-free tetrahydrofuran (20 ml.). Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine (0.69 g.) was dissolved in peroxide-free tetrahydrofuran (25 ml.) and the solution was cooled to −15°. Triethylamine (0.14 ml.) and isobutyl chloroformate (136 mg.) were added and the mixture was stirred for 15 minutes at −10°. The cooled pentapeptide solution then was added. After this mixture had been allowed to stand at room temperature for 15 hours, the triethylammonium chloride was filtered off and the solvent removed by evaporation *in vacuo*. The residual oil was isolated by two successive precipitations from acetone–ether; yield 1.75 g. (88%) of a white amorphous powder, m.p. 120–124°, $[\alpha]^{25}D$ −42.3° (*c* 1, methanol).

Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-L-proline (I). a. By Saponification of the Corresponding Methyl Ester.—The protected nonapeptide ester (2.0 g.) was dissolved in acetone (20 ml.) and the solution was cooled in ice; 2 *N* sodium hydroxide (1.25 ml.) was added dropwise over a period of 20 minutes. After the solution had been stirred for 2 hours more at room temperature, water (25 ml.) was added until the solution became slightly turbid. It was washed with ethyl acetate with very gentle shaking to avoid formation of emulsions.[46] The water layer was acidified with dilute hydrochloric acid. The fine precipitate was collected, washed thoroughly with water, and dried over P_2O_5–KOH; yield 1.51 g. (76%), m.p. 134–136°, $[\alpha]^{25}D$ −37.4° (*c* 1, methanol).

Anal. Calcd. for $C_{90}H_{124}O_{22}N_{18}S_5$ (1970.3): C, 54.9; H, 6.34; N, 12.8. Found: C, 55.1; H, 6.41; N, 12.8.

To check the purity of this product, a sample was subjected to countercurrent distribution in the system consisting of chloroform–toluene–methanol–water (5:5:8:2) for 482 transfers. One single peak, which had a theoretical distribution curve with K = 0.75, was obtained.

b. By Nitrophenyl Ester Method.—Nα-Carbobenzoxy-Nε-tosyl-L-lysyl-Nε-tosyl-L-lysyl-NG-tosyl-L-arginyl-NG-tosyl-L-arginyl-proline (287 mg.) was submitted to catalytic hydrogenation as described above and the base formed was dissolved in acetonitrile (5 ml.) with 0.05 ml. of triethylamine; to this solution was added Nα-carbobenzoxy-Nε-tosyl-L-lysyl-L-prolyl-L-valyl-glycine nitrophenyl ester (178 mg.). After the mixture had been allowed to stand for 45 hours at room temperature, the solvent was removed *in vacuo* and the product purified by two successive precipitations from acetone–ether; yield 258 mg. (91%) of a white amorphous powder, m.p. 135–137°, $[\alpha]^{25.5}D$ −37.4° (*c* 1, methanol).

Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanine *p*-Nitrophenyl Ester.—Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanine[15] (3.71 g.), was suspended in 150 ml. of acetonitrile; *p*-nitrophenol (1.17 g.) was added and the mixture was cooled to 0°. Dicyclohexylcarbodiimide (1.44 g.) was added and the mixture was stirred for 2 hours at 0°, and then for 48 hours at room temperature. The precipitate, which contained both the *p*-nitrophenyl ester and dicyclohexylurea, was filtered and stirred with 250 ml. of hot acetone. The acetone suspension was cooled and the dicyclohexylurea was removed by filtration. After the acetone had been allowed to evaporate at room temperature, the product crystallized. The *p*-nitrophenyl ester was then filtered, washed with cold acetone, and dried (m.p. 152–155°, yield 3.08 g.); from the mother liquor, another 0.48 g. was recovered; total yield 3.60 g. (79%). After repeated crystallization from acetone, the melting point rose to 164–166°, $[\alpha]^{25}D$ +3.6° (*c* 1, methanol), $[\alpha]^{25}D$ −19.0° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{35}H_{33}O_7N_5$ (647.7). C, 66.8; H, 6.13; N, 10.8. Found: C, 66.9; H, 5.34; N, 10.7.

Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanyl-NG-tosyl-L-arginyl-L-tryptophyl-glycine Methyl Ester.—NG-Tosyl-L-arginyl-L-tryptophyl-glycine methyl ester[15] (3.25 g.) was dissolved in 25 ml. of dimethylformamide, and carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanine *p*-nitrophenyl ester (3.02 g.) was added, as well as a few drops of triethylamine to ensure basicity. After the mixture had been allowed to stand at room temperature for 24 hours, the dimethylformamide was evaporated *in vacuo* and the residue was dissolved in acetone and precipitated with anhydrous ether, giving a product, in a yield of 4.1 g., which was homogeneous in paper chromatography. By means of countercurrent distribution in the toluene system, some minor impurities were removed and the product with a partition coefficient (K) of 0.55 was obtained in the amount of 3.1 g. (63%). The material was dissolved in acetone, which was allowed to evaporate slowly at room temperature, and then in methanol, which was evaporated in the same manner; this yielded a material which melted at 144–146° with sintering from 142°; $[\alpha]^{25}D$ −22.5° (*c* 2, methanol), $[\alpha]^{25}D$ −25° (*c* 1, acetone).

Anal. Calcd. for $C_{57}H_{63}N_{11}O_{10}S$ (1094.23): C, 62.6; H, 5.81; N, 14.1. Found: C, 62.4; H, 5.61; N, 14.2.

Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanyl-NG-tosyl-L-arginyl-L-tryptophyl-glycine.—Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanyl-NG-tosyl-L-arginyl-L-tryptophyl-glycine methyl ester (2 g.) was dissolved in 20 ml. of 95% aqueous acetone at 0° and *N* NaOH (4.0 ml.) was added slowly at 0°. The reaction mixture was kept at 0° for 25 minutes with stirring and for an additional 30 minutes at room temperature, and then 4.0 ml. of 1 *N* HCl was added. Paper chromatography revealed that the saponification was complete and that some degradation had occurred. The acetone was evaporated *in vacuo* and the residue was washed with water and dried (yield 2 g.). The dried material was then submitted to countercurrent distribution in the carbon tetrachloride system for 100 transfers. The main peak with K = 0.26 was isolated by precipitation with anhydrous ether from a dimethylformamide solution; yield 1.5 g. (77%), m.p. 144–148°, $[\alpha]^{25}D$ −19.5° (*c* 1, methanol); in paper chromatography, $R_{f\,SBA}$ = 0.42.

Anal. Calcd. for $C_{56}H_{61}O_{10}N_{11}S_1 \cdot H_2O$(1080.2): C, 61.3; H, 5.74; N, 14.0. Found: C, 61.1; H, 5.62; N, 13.8.

Carbobenzoxy-γ-benzyl-L-glutamyl-Im-benzyl-L-histidyl-L-phenylalanyl-NG-tosyl-L-arginyl-L-tryptophyl-glycine (II).—Carbobenzoxy-Im-benzyl-L-histidyl-L-phenylalanyl-NG-tosyl-L-arginyl-L-tryptophyl-glycine (1.08 g.) was dissolved in 50 ml. of 90% methanol with heating and the solution was subjected to catalytic hydrogenation with Pd (from 1 g. of $PdCl_2$) for approximately 2 hours until there was no further evolution of carbon dioxide. As judged by the results of chromatography on paper, the decarbobenzoxylation was

(45) J. R. Vaughan, Jr., and J. A. Eichler, *J. Am. Chem. Soc.*, **75**, 5556 (1953).

(46) From the ethyl acetate layer 450 mg. (22.5%) of material was recovered after evaporation of the solvent and precipitation from acetone–ether; the product proved to be a mixture of unsaponified ester and acid, as indicated by paper chromatography.

complete (R_f BAW = 0.53; the material gave a positive reaction with both the Ehrlich reagent and with ninhydrin, but a negative reaction with the Pauly reagent).

In addition, about 30% of the material was also debenzylated (R_f BAW = 0.31; positive reaction with the Pauly reagent). The catalyst was removed by filtration and washed well with several small portions of dimethylformamide. Triethylamine, 0.05 ml., was added to the filtrate and the solvent was removed by evaporation *in vacuo*. The oily residue was dissolved in 5 ml. of dimethylformamide and 0.06 ml. of triethylamine, and, after the solution had been cooled to 0°, carbobenzoxy-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester[47a,b] (600 mg.) was added. The reaction was allowed to proceed at room temperature for 2 days. The debenzylated pentapeptide derivative L-histidyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophyl glycine condensed only very slowly, and most of it had not reacted in this interval. The dimethylformamide then was removed by distillation *in vacuo;* the glassy residue was washed thoroughly with 5% acetic acid and water, and then dried by washing with ethyl acetate and acetone. The yield of crude material was 950 mg. This product was submitted to countercurrent distribution in the carbon tetrachloride system for 608 transfers. Some impurities[48] were obtained, in addition to the main peak with K = 0.16, which was isolated by precipitation with anhydrous ether from a dimethylformamide solution. The amorphous precipitate was washed with acetone, to yield 600 mg. (46%). This material was redissolved in dimethylformamide, and, upon slow evaporation of the solution, peptide II crystallized in the form of fine needles. It was recrystallized from 90% aqueous dioxane; m.p. 193–195° with sintering at 190°, $[\alpha]^{26}D$ −19° (*c* 1, dimethylformamide). In paper chromatography, R_f SBA = 0.44.

Anal. Calcd. for $C_{85}H_{74}N_{12}O_{13}S$ (1299.44): C, 62.9; H, 5.74; N, 12.9. Found: C, 62.8; H, 5.87; N, 12.9.

Nα-Carbobenzoxy-α-benzyl-L-glutamyl-Im-benzyl-L-histidyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophyl-glycyl-N^ϵ-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-N^ϵ-tosyl-L-lysyl-N^ϵ-tosyl-L-lysyl-N^G-tosyl-L-arginyl-N^G-tosyl-L-arginyl-L-proline (III).—Nα-Carbobenzoxy-N^ϵ-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-N^ϵ-tosyl-L-lysyl-N^ϵ-tosyl-L-lysyl-N^G-tosyl-L-arginyl-N^G-tosyl-L-arginyl-L-proline (I) (1.01 g.) was dissolved in methanol (30 ml.) and hydrogenated for 2 hours in the presence of palladium black (freshly prepared from 2 g. of $PdCl_2$). The catalyst was removed by filtration and the solvent evaporated *in vacuo*, leaving an oil that was then dissolved in 6 ml. of dimethylformamide, which was 0.2 molar with respect to triethylamine. The hexapeptide derivative II, Nα-carbobenzoxy-α-benzyl-L-glutamyl-Im-benzyl-L-histidyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophyl-glycine (564 mg.), was dissolved in dimethylformamide (4.0 ml.); this solution was cooled to −10° prior to the addition of a 0.2 *M* solution of triethylamine in dimethylformamide (2.2 ml.) and a 0.2 *M* solution of isobutyl chloroformate[45] in dimethylformamide (2.2 ml.). The mixture was stirred for 15 minutes at −10°, and the ice-cold dimethylformamide solution of the hydrogenated nonapeptide I was added over a period of 2 minutes, and this mixture then was stirred for 30 minutes at −10° and for 15 hours at room temperature. The product then was precipitated by the addition of ether. The crude material was subjected to countercurrent distribution in the solvent system consisting of chloroform–toluene–methanol–water (5:5:8:2) for 242 transfers. From the main peak, which distributed with a K value of 0.34, 472 mg. (35%) of material was isolated, m.p. 135–140°, $[\alpha]^{25}D$ −25.3° (*c* 0.5, dimethylformamide).

Anal. Calcd. for $C_{150}H_{190}O_{32}N_{30}S_6$ (3117.6): C, 57.6; H, 6.14; N, 13.5. Found: C, 57.4; H, 6.13; N, 13.5.

Carbobenzoxy-L-seryl-L-methionine Methyl Ester.—This protected dipeptide ester was obtained by the reaction of L-methionine methyl ester with carbobenzoxy-L-serine[49,50] using the dicyclohexylcarbodiimide procedure; yield 91%, $[\alpha]^{25}D$ −22.7° (*c* 1, methanol), m.p. 101–102°; lit.[19]: 65%, m.p. 101–102°, no optical rotation given.

L-Seryl-L-methionine Methyl Ester.—Carbobenzoxy-L-seryl-L-methionine methyl ester (4.7 g.) was dissolved in 150 ml. of methanol containing 1 ml. of concd. HCl, and 23 g. of the Pd–$BaSO_4$ catalyst[51] was added; hydrogen was passed through under vigorous vibration.[43] After 8 hours, the catalyst was recovered by filtration and washed with 50-ml. portions of hot methanol. The methanol then was removed by evaporation *in vacuo* and the oily residue was put under 100 ml. of ice-cold ethyl acetate, and 20 ml. of 50% K_2CO_3 solution was added with cooling. With shaking and stirring, the free ester dissolved slowly in the ethyl acetate. After 10 minutes, the ethyl acetate layer was separated and the water phase was extracted with another 50 ml. of ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4 and the ethyl acetate was evaporated *in vacuo*. The L-seryl-L-methione methyl ester crystallized during this process in the form of platelets; yield 2.1 g. (70%), m.p. 85–87°. A small sample was washed well with a little ice-cold ethyl acetate and much ether; m.p. 86–88°, $[\alpha]^{24}D$ −21° (*c* 1, *N* HCl). In paper chromatography, R_f BAW = 0.33.

Anal. Calcd. for $C_9H_{18}N_2O_4S$ (250.31): C, 43.2; H, 7.25; N, 11.2. Found: C, 43.3; H, 7.29; N, 11.1.

Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methioninhydrazide (IV).—Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methionine methyl ester[19] (4.8 g.) was dissolved in 75 ml. of hot 95% aqueous methanol and 0.5 ml. of hydrazine was added to the still hot solution. After 30 minutes a gel started to precipitate; the reaction mixture was allowed to stand at room temperature for 2 hours and then for 10 hours in the refrigerator. The gel then was filtered off and washed thoroughly with ethyl acetate, methanol, and water. The yield of dry material was 3.5 g. (73%). An additional 0.5 g. of the product (10%) was obtained from the mother liquors after further standing. The hydrazide was dissolved in 90% aqueous dioxane and stored in the cold, resulting in an amorphous product, m.p. 195–210°. Repeated treatment with the same solvent finally led to crystallization of the hydrazide in the form of fine needles; yield 1.9 g. (40%). From the mother liquors, another 800 mg. (17%) was obtained. The hydrazide melted at 245–246° dec., $[\alpha]^{24}D$ −12° (*c* 0.5, dimethylformamide), $[\alpha]^{25}D$ −15° (*c* 0.5, glacial acetic acid). Lit:[19] melting point and optical rotation were not given; analyses for C and H were 52.9 and 5.4, respectively, no value for N.

Anal. Calcd. for $C_{28}H_{38}N_6O_9S$ (634.69): C, 53.0; H, 6.03; N, 13.2. Found: C, 53.2; H, 6.22; N, 13.0.

Carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-N^G-tosyl-L-arginyl-L-tryptophyl-glycyl-N^ϵ-tosyl-L-lysyl-L-prolyl-L-valyl-glycyl-N^ϵ-tosyl-L-lysyl-N^ϵ-tosyl-L-lysyl-N^G-tosyl-L-arginyl-N^G-tosyl-L-arginyl-L-proline.—The protected pentadecapeptide III (460 mg.) was dissolved in dimethylformamide (10 ml.) and methanol (10 ml.), and 1 *N* hydrochloric acid (0.2 ml.) was added. Hydrogenation was carried out for 5.5 hours in the presence of palladium black (freshly prepared from 1 g. of $PdCl_2$). The catalyst was then filtered off and the solvent removed by evaporation *in vacuo*. The residual oil was dissolved in dimethylformamide (5 ml.) which was 0.2 molar with respect to triethylamine.

Peptide IV, Nα-carbobenzoxy-L-seryl-L-tyrosyl-L-seryl-L-methioninhydrazide (255 mg.), was dissolved in 3.0 ml. of 2 *N* HCl and 1.5 ml. of acetic acid. The solution was cooled to −5° prior to the addition, with stirring, of 31 mg. of $NaNO_2$. The azide then was extracted into two 3-ml. portions of ethyl acetate; after the ethyl acetate extract had been washed thoroughly at 0° with 10% $NaHCO_3$ and water, it was dried over Na_2SO_4. The azide solution then was added to the above hydrogenated pentadecapeptide III in the dimethylformamide–triethylamine solution. An amorphous precipitate formed which again dissolved when the ethyl acetate was removed by evaporation *in vacuo*. The solution was kept at 0° for 2 days. The reaction mixture then was poured into 200 ml. of ethyl ether, and the precipitated product was filtered and precipitated again from dimethylformamide–ethyl ether.

(47) (a) R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958); (b) M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).

(48) One of these minor impurities was identified and shown to be the debenzylated hexapeptide derivative, K = 0.34.

(49) E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 25 (1955).

(50) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1859 (1958).

(51) R. Mozingo, S. A. Harris, D. E. Wolf, C. E. Hoffhine, Jr., N. R. Easton and K. Folkers, *J. Am. Chem. Soc.*, **67**, 2092 (1945).

The weight of the crude product was 520 mg., m.p. 137–145°. This material was dissolved in boiling methanol (250 ml.), filtered free from a trace of insoluble material, and precipitated by the addition of ethyl acetate (1500 ml.); yield of the protected nonadecapeptide V: 300 mg. (56%), m.p. 167–170°, $[\alpha]^{26}D$ −25.0° (*c* 0.5, dimethylformamide).

Anal. Calcd. for $C_{156}H_{212}O_{39}N_{34}S_7$ (3412.2). C, 54.9; H, 6.26; N, 14.0. Found: C, 54.5; H, 6.36; N, 13.7.

Removal of Protecting Groups and Purification of the Nonadecapeptide V.—The protected nonadecapeptide V (200 mg.) was dissolved in liquid ammonia (800 ml.) which had been distilled from sodium. Sodium was added in small pieces over an interval of 30 minutes until a blue color persisted (approximately 50 mg. of sodium required). Most of the ammonia was allowed to evaporate spontaneously and the rest (20 ml.) was removed by lyophilization. The residue was dissolved in 0.1 *N* acetic acid (20 ml.) and the solution was passed through an Amberlite-cation exchanger, IRC-50 (XE-64), for desalting; the column (0.9 × 10 cm.) was washed with 0.25% acetic acid (250 ml.) and water (20 ml.) and the peptide was then eluted with a pyridine–acetic acid–H_2O solution (30:4:66 by volume). The eluate was lyophilized to yield a white powder; yield of crude nonadecapeptide V, 160 mg. (92%).

Purification by Countercurrent Distribution.—The crude nonadecapeptide (130 mg. with an ACTH potency of 7.3 U.S.P. units/mg. by the *in vitro* method) was submitted to countercurrent distribution in the system[52a] consisting of 0.1% acetic acid–1-butanol–pyridine (11:5:3); an automatic all-glass apparatus[52b] consisting of 240 tubes was employed. After a forerun which was carried out until all the tubes in the train contained both upper and lower phase (5 ml. for each), the peptide material was introduced into the apparatus and upper phase then was recycled for the distribution of this material. After 240 transfers, aliquots (0.1 ml.) of the lower phase from every fourth tube were removed, and the peptide content of these samples was determined on the basis of Folin–Lowry color.[53]

There were present a main peak with a *K* value of 0.08 and two small peaks with $K = 1.00$ and 1.96, respectively. The contents of the tubes in the train (41–240) were removed; these tubes were refilled with fresh lower and upper phase, and further distribution was carried out for 1188 transfers. The contents of tubes 55–117 then were pooled and isolated by evaporation *in vacuo;* yield 52 mg., ACTH potency 20.9 units per mg. The product was subjected to further countercurrent distribution in the system consisting of 2-butanol–aqueous 0.5% trichloroacetic acid for 214 transfers; the main component with $K = 0.58$ was isolated from tubes 69–95 (yield 26 mg., ACTH potency 39.8 units per mg.) and shown to be the desired nonadecapeptide V. Redistribution of peptide V in 2-butanol–aqueous 0.5% trichloroacetic acid for 116 transfers gave a distribution pattern which follows closely the theoretically calculated curve of $K = 0.58$.

NH_2-Terminal Residue Analysis.—The nonadecapeptide (2.0 mg.) was dissolved in 0.5 ml. of 5% $NaHCO_3$ and 5% dinitrofluorobenzene[23] (1.0 ml.) in ethanol was added; the mixture was shaken at room temperature for 4 hours. The solvents were removed *in vacuo*, and the residue was again suspended in water and extracted three times with ethyl ether. The water suspension of the dinitrophenylated (DNP-) nonadecapeptide was then acidified to *p*H 3.0 and extracted with three portions of ethyl ether to remove the dinitrophenol. The DNP-peptide was isolated by centrifugation, washed with water and then dried over P_2O_5. There was no color in the water fraction. Glass-distilled constant boiling HCl (0.5 ml.) was added to the DNP-peptide; hydrolysis was performed for 16 hours at 110° in a sealed, evacuated tube. The hydrolysate was diluted with water and then extracted with ethyl ether. When the ethyl ether and the aqueous fractions were submitted to chromatography[24] on paper, DNP-serine was found together with traces of DNP-glutamic acid and bis-DNP-lysine.

Amino Acid Analysis.—Peptide V (2.0 mg.) was dissolved in 0.5 ml. of glass-distilled constant-boiling HCl and hydrolyzed for 24 hours at 110° in a sealed, evacuated tube. One portion of the hydrolysate was analyzed by the paper–DNP method[24] and another by the chromatographic method of Spackman, Stein and Moore.[25] The results are, in moles residue per mole peptide

Paper-DNP method:

$$Ser_{1.8}Tyr_{0.6}Met_{0.8}Glu_{0.7}His_{1.1}Phe_{1.2}Arg_{3.1}Gly_{1.8}Lys_{2.9}Pro_{2.2}Val_{1.2}$$

Chromatographic method:

$$Ser_{1.6}Tyr_{1.0}Met_{1.1}Glu_{0.9}His_{1.0}Phe_{1.0}Arg_{3.1}Gly_{2.0}Lys_{3.1}Pro_{2.1}Val_{1.0}$$

The intact peptide was found to contain tyrosine and tryptophan in a molar ratio of one to one, as determined by the method of Goodwin and Morton.[26]

Enzymatic Digest.—The synthetic nonadecapeptide (1.0 mg.) was dissolved in 0.5 ml. of tris-(hydroxymethyl)-aminomethane (TRIS) buffer, *p*H 8, containing 0.002 *M* $MgCl_2$. To this solution was added 0.1 ml. of an enzyme solution containing 2.0 mg. of trypsin (Worthington Biochemical, Lot #590) and 2.0 mg. of chymotrypsin (Armour Lot #381092) in 1.0 ml. of *p*H 8 TRIS buffer. The solution was allowed to stand at room temperature for 24 hours and then immersed in a boiling water-bath for 15 minutes and, finally, was cooled slowly to room temperature to denature the enzymes. Then 2.0 mg. of leucine aminopeptidase (Worthington Biochemical, Lot #LAP 5808B) was added and the solution again was allowed to stand at room temperature for 24 hours. The solution then was analyzed for free amino acids by the paper–DNP method.[24] The results shown as molar ratios are

$$Ser_{2.1}Tyr_{0.4}Met_{0.6}Glu_{1.3}His_{1.0}Try_{0.8}Phe_{0.9}Arg_{2.6}Gly_{1.7}Lys_{2.8}Pro_{0.4}Val_{1.0}$$

The low value for proline indicates that the arginylproline linkage is not attacked by the enzymes. The paper chromatographic behavior of DNP-Arg-Pro-OH in the solvent system employed[24] is identical with that of DNP-Arg-OH; hence, the arginine content (2.6) as estimated by the paper–DNP method is greater than two. Enzymatic degradation studies (unpublished) on the pentapeptide H-Lys-Lys-Arg-Arg-Pro-OH also shows the arginylproline bond to be resistant to trypsin and leucine aminopeptidase.

Biological Assays.—The *in vitro* steroidogenesis assay for adrenocorticotropic activity was carried out essentially as described by Saffran and Schally[38] with slight modifications. Adrenals of 40-day old rats of the Long–Evans strain removed one day after hypophysectomy were used. A four-point design with duplicate observations was employed and the relative potency was computed by the variance analysis method,[54a] using an ACTH preparation[54b] with a potency

(52) (a) L. S. Craig and W. Konigsberg, *J. Org. Chem.*, **22**, 1345 (1957); (b) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *Anal. Chem.*, **23**, 1276 (1951).

(53) O. H. Lowry, J. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(54) (a) G. L. Bliss, "The Statistics of Bioassay," Academic Press, Inc., New York, N. Y., 1952, pp. 461. (b) We wish to thank Dr. E. A. Lazo-Wasem of Wilson Laboratories for the Standard ACTH preparation (Lot CN-145EI). (c) A glutaminyl analog of the nonadecapeptide also has been synthesized by similar routes and the synthetic product was found to have a partition coefficient of 0.45 after countercurrent distribution for 116 transfers in the solvent system of 2-butanol–0.5% trichloroacetic acid. The results of *in vitro* adrenal assay[38] showed the glutaminyl analog of the nonadecapeptide to have an ACTH potency of 24.7 U.S.P. units per mg., whereas the adrenal ascorbic acid depletion method[39,55] with administration by the subcutaneous route gave a potency of 20.8 U.S.P. units per mg. It is clearly evident that replacement of glutamic acid in position 5 of the nonadecapeptide by glutamine[56] caused a decrease of adrenal-stimulating activity. It may be recalled that Boissonnas, *et al.*,[57] in a preliminary note reported the synthesis of the methyl ester of an icosapeptide corresponding to the first twenty N-terminal amino acids of adrenocorticotropins which exhibited an ACTH activity of 2–3 units per mg. by the *in vitro* assay method.[38] Since no details about physicochemical data of the synthetic product were included, it is difficult to decide whether the low potency of the Boissonnas peptide is due to the esterification of the C-terminal valine or to "the possibility of a certain amount of racemization at some steps of the synthesis." In this connection, it may be noted that Hofmann[58] reported a synthetic tridecapeptide amide, Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-$CONH_2$ which "exhibited a low but reproducible *in vitro* corticotropic activity (0.05 U.S.P. units/mg.)." While this manuscript was in the hands of the Editor, Hofmann and his coworkers[59] announced the synthesis of a tricosapeptide amide which was claimed to possess essentially the full activity of the native ACTH.

of 86 U.S.P. units per mg. as the standard; by this procedure, the synthetic[54c] peptide V was found to possess an ACTH potency of 39.8 U.S.P. units per mg. with a 95% confidence limit for the assay in the range of 32.7–48.5.

The peptide V was also tested for ACTH activity by the adrenal ascorbic acid depletion method,[39] with administration by both intravenous and subcutaneous routes. In each procedure, a six-point design with six hypophysectomized rats per dose was employed. It was found[55] that with intravenous administration, a potency of 34.6 U.S.P. units per mg. was obtained for the nonadecapeptide, with a 95% confidence limit in the range of 22.3 to 55.0; whereas with subcutaneous administration, the nonadecapeptide was found to possess a potency of 74.2 U.S.P. units per mg., with a 95% confidence limit in the range of 50.6 to 100.6.

The melanocyte-stimulating activity of peptide V was determined by the method described by Shizume, *et al.*,[36] with isolated skins of *Rana pipiens;* the unit of activity used is the same as that defined by these investigators. Bioassay also was carried out with hypophysectomized *Rana pipiens* (not more than 4 days after operation) as described by Hogben and Slome.[37]

In vitro lipolytic assay[61] showed that peptide V is highly active as a lipolytic agent. In hypophysectomized rats, peptide V also is capable of eliciting an increased Fe^{59} incorporation[62] into the red cells at a rate comparable to that elicited by the native hormone. In addition, peptide V has been assayed in man and shown to possess the ACTH potency[63] predicted from the results of animal assay. Thus, the synthetic nonadecapeptide V exerts the same biological functions, namely, adrenal-stimulation, melanocyte stimulation, lipolysis and erythropoiesis as the natural ACTH molecule.[27]

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(55) We are indebted to Drs. M. L. Pabst, D. A. Harvey and M. Speeter of the Upjohn Co. for these assay data (7/13/60–9/22/60).

(56) In a private communication (11/23/60) Schwyzer and his coworkers[60] also have reported the synthesis of the glutaminyl analog of the nonadecapeptide and found it to possess an ACTH potency of 20–30 I.U. per mg. by the *in vitro* adrenal method.[38]

(57) R. A. Boissonnas, St. Guttmann, J. P. Waller and P. A. Jaquenoud, *Experientia*, **12**, 466 (1956).

(58) K. Hofmann, in *Brook. Symp. Biol.*, **13**, 184 (1960).

(59) K. Hofmann, H. Yajima, N. Yanaihara, T. Y. Liu and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961).

(60) R. Schwyzer, W. Rittle, H. Kappeler and B. Iselin, *Angew. Chem.*, **23**, 915 (1960).

(61) We are indebted to Dr. F. L. Engel of Duke University for the lipolytic assay (11/28/60). For the assay method, see E. Lopez, J. E. White and F. L. Engel, *J. Biol. Chem.*, **234**, 2254 (1959).

(62) We wish to thank Drs. D. C. van Dyke and J. H. Lawrence of this University for the erythropoietic assay (11/22/60). For the assay method, see W. Fried, L. Plzak, L. O. Jacobson and E. Goldwasser, *Proc. Soc. Exp. Biol. Med.*, **92**, 203 (1956).

(63) It is a pleasure to thank Dr. P. Forsham of this University for his coöperation in the clinical tests of our synthetic products (10/29/60).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, EMORY UNIVERSITY, ATLANTA 22, GA.]

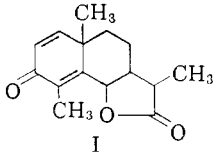
The Synthesis of Dienones Related to Santonin and ψ-Santonin via Aryl Participation

BY LEON MANDELL, DRURY CAINE AND GORDON E. KILPATRICK

RECEIVED JUNE 10, 1961

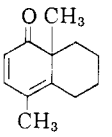
Compound VII has been synthesized and shown to undergo solvolysis with aryl participation leading to the formation of dienones II and XV.

The work of Winstein and his associates[1] has shown the feasibility of utilizing aryl participation as a means of synthesizing dienones having interesting spirane structures. Recently, Masamune[2] has applied Ar_1-5[1] participation to the synthesis of part of the ring skeleton of phyllocladene. A very common type of dienone, typified by santonin (I) has been studied with regard to synthesis[3]

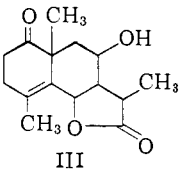


I

and its tendency to undergo the dienone–phenol[4] rearrangement. It was the object of this work to see if the aryl participation reaction could be used for the preparation of non-spirane dienones of the santonin type and also of the related dienone system II, which incorporates features of the ψ-

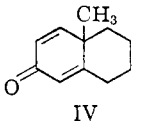


II

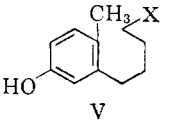


III

santonin ring structure, III. This problem became clearly defined with the failure of recent attempts[5,6] to prepare 3-keto-9-methyl-Δ-1,4-hexahydronaphthalene, IV, utilizing aryl participation. In these efforts compounds of the type V were synthesized and solvolyzed. However, cyclization occurred



IV



V

ortho to the hydroxyl resulting in the formation of a naphthalene of structure VI. It thus became

(1) S. Winstein, R. Heck, S. Lapporte and R. Baird, *Experientia*, **12**, 138 (1956); S. Winstein and R. Baird, *J. Am. Chem. Soc.*, **79**, 756 (1957).

(2) S. Masamune, *ibid.*, **83**, 1009 (1961).

(3) For syntheses of the ring system in santonin see M. Yanagita, S. Inayama, M. Hirakura, and F. Seki, *J. Org. Chem.*, **23**, 690 (1958); M. Yanagita and R. Futaki, *ibid.*, **21**, 949 (1956); P. R. Hills and F. J. McQuillin, *J. Chem. Soc.*, 4060 (1953); F. D. Gunstone and R. M. Heggie, *ibid.*, 1437 (1952).

(4) R. B. Woodward and T. Singh, *J. Am. Chem. Soc.*, **72**, 494 (1950), and references cited therein.

(5) W. E. Hill, Doctoral Dissertation, Emory University (1959).

(6) M. S. Newman and A. B. Mekler, *J. Org. Chem.*, **26**, 336 (1961).