Synthesis of 3β-Acetoxy-17β-(L-arginyl-L-arginyl-L-prolyl)amino-5α-androstane[1,2]

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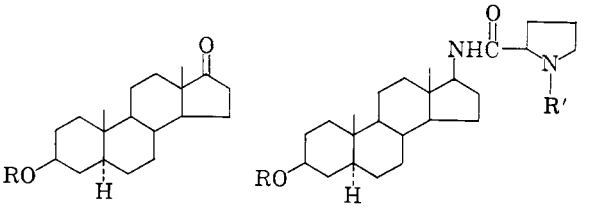
Received August 4, 1966

A steroidal peptide based on the 17–19 unit sequence of β-corticotropin has been synthesized. Construction of the title substance (tripeptide IIIe) was achieved starting from 3β-hydroxy-17β-amino-5α-androstane. The phenylisoxazolium method was used for peptide bond formation and a combination of acetyl (for the steroid nucleus), carbobenzoxy, and nitro (for arginine) protecting groups were employed. Peptide IIIe was characterized as the triacetate derivative and the assigned structure received additional support from results of an amino acid analysis.

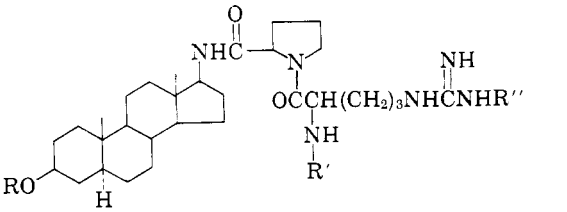
Steroid hormones of the adrenal cortex, for example, corticosterone and aldosterone, maintain life by regulating carbohydrate and electrolyte metabolism, influencing muscular efficiency, and protecting against stresses such as cold and heat. Other steroid hormones influence growth and some have been implicated in the aging process.[4] The anterior pituitary adrenotropic hormone, in turn, regulates production of steroids in the adrenal cortex. The role of human adrenocorticotropic hormone[5] (ACTH) in elaboration of corticosteroids is of particular interest with respect to steroid biosynthesis[6] and practical medical problems. At present all adrenocorticotropic hormones[7] of established structure contain an L-lysyl-L-lysyl-L-arginyl-L-arginine[8] segment which seems to be a necessary requirement for high corticotropic activity. The double arginine segment of β-corticotropin[9] is bound on the C terminus to proline; proline, in turn, is also a common constituent of other pituitary hormones.[10]

The thesis that steroidal peptides based on the arginine (both) and adjacent[1] portions of ACTH might alter or otherwise interfere with established hormone production led us to consider preparing a steroid containing an L-arginyl-L-arginyl-L-proline unit corresponding to the 17–19 sequence of β-corticotropin. Also, the possibility that such steroidal peptides might provide leads to treatment of hormone-based medical problems and/or prove useful in future steroid–protein binding studies reinforced our interest.[11] Accordingly, a study, begun in 1959 concerned with arginine-linked steroids related to the steroidal toad poison, bufotoxin, was extended in 1962 to include L-arginyl-L-arginyl-L-proline-containing steroidal peptides. The first objective in this area was synthesis of 3β-hydroxy-17β-(L-arginyl-L-arginyl-L-prolyl)amino-5α-androstane or (if found necessary) its 3β-acetoxy (IIIe) derivative. Meanwhile, results from other aspects of our steroidal peptide pro-



Ia, R = H
b, R = $COCH_3$

IIa, R = R′ = H
b, R = H; R′ = $CO_2CH_2C_6H_5$
c, R = $COCH_3$; R′ = $CO_2CH_2C_6H_5$
d, R = $COCH_3$; R′ = H



IIIa, R = H; R′ = $CO_2CH_2C_6H_5$; R″ = NO_2
b, R = $COCH_3$; R′ = $CO_2CH_2C_6H_5$; R″ = NO_2
c, R = $COCH_3$; R′ = H; R″ = NO_2
d, R = $COCH_3$; R′ = Arg–NO_2; R″ = NO_2
(Cbz)
e, R = $COCH_3$; R′ = Arg; R″ = H

(1) Structural Biochemistry. III. Part II: G. R. Pettit, A. K. Das Gupta, and R. L. Smith, *Can. J. Chem.*, **44**, 2023 (1966). The preceding contribution may also be considered Part XXXV of the series "Steroids and Related Natural Products." The present paper is based on part of the Ph.D. dissertation submitted by R. L. Smith to the Graduate School, University of Maine, Aug 1965.

(2) This investigation was aided in part by National Science Foundation Research Grants GB-249 and GB-4939 and American Cancer Society Grants No. T-79D to T-79G.

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(4) For example, see: "Hormones and the Aging Process," E. T. Engle and G. Pincus, Ed., Academic Press Inc., New York, N. Y., 1956.

(5) T. H. Lee and A. B. Lerner, *J. Am. Chem. Soc.*, **81**, 6084 (1959).

(6) An interesting and useful review of corticosteroid and androgen biosynthesis has been prepared by R. I. Dorfman and D. C. Sharma, *Steroids*, **6**, 229 (1965).

(7) K. H. Hofmann, *Pure Appl. Chem.*, **6**, 245 (1963).

(8) K. H. Hofmann, T. Liu, H. Yhaima, M. Yamaihara, and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961); and, F. Šorm, *Collection Czech. Chem. Commun.*, **26**, 1180 (1961). Recently, replacement of arginine in this segment by ornithine gave a tetrapeptide with similar biological properties: G. I. Tesser and R. Schwyzer, *Helv. Chim. Acta*, **49**, 1013 (1966).

(9) An elegant total synthesis of the complete 39-unit amino acid sequence of porcine ACTH has been accomplished by R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **49**, 134 (1966). See also a new synthesis of the first 26 amino acid sequences: J. Ramachandran and C. H. Li, *J. Am. Chem. Soc.*, **87**, 2691 (1965), and J. Ramachandran, D. Chung, and C. H. Li, *ibid.*, **87**, 2696 (1965).

(10) *Cf.*, E. Schröder and R. Hempel, *Experientia*, **20**, 529 (1964); C. H. Li, W-K. Liu, and J. S. Dixon, *J. Am. Chem. Soc.*, **88**, 2050 (1966).

(11) For a summary of steroid–protein binding studies see: G. W. Oertel, K. Groot, and P. Brühl, *Z. Physiol. Chem.*, **341**, 10 (1965); "Biological Activities of Steroids in Relation to Cancer," G. Pincus and E. P. Vollmer, Ed., Academic Press Inc., New York, N. Y., 1960; J. A. Schellman, R. Lumry, and L. T. Samuels, *J. Am. Chem. Soc.*, **76**, 2808 (1954); U. Westphal, *Endocrinology*, **57**, 456 (1955); A. A. Sandberg, W. R. Slaunwhite, Jr., and H. N. Antoniades, *Recent Progr. Hormone Res.*, **13**, 209 (1957). Noteworthy in this regard are the experiments of Erlanger, Liberman, and colleagues concerned with preparing steroids covalently linked to proteins. For example, testosterone has been attached (predominantly) by a carbamate linkage involving C-17 and the N^{ϵ}-lysyl positions to bovine serum albumin. The resulting protein (antigen with a steroid heptan) was found capable of stimulating formation of steroid-specific antibodies. For leading references consult: P. E. Zimmering, S. M. Beiser, and B. F. Erlanger, *J. Immunol.*, **95**, 262 (1965); R. O. Neri, S. Tolksdorf, S. M. Beiser, B. F. Erlanger, F. J. Agate, Jr., and S. Lieberman, *Endocrinology*, **74**, 593 (1964); and a review by S. Lieberman, B. F. Erlanger, S. M. Beiser, and F. J. Agate, Jr., *Recent Progr. Hormone Res.*, **15**, 165 (1959).

gram[1,12] allowed selection of the general method outlined below for obtaining tripeptide IIIe.

Synthesis of tripeptide IIIe was achieved as follows. Conversion of 3β-hydroxy-17-oxo-5α-androstane (Ia) to L-prolyl amide IIa was accomplished as previously described.[1] Next, amine IIa was condensed with N^{α}-carbobenzoxy-N^{G}-nitro-L-arginine in the presence of Woodward's reagent K (WRK).[1,13] The resulting protected dipeptide (IIIa) was purified by column chromatography on neutral alumina. By conducting the peptide-forming reaction in acetonitrile solution (3 days at room temperature), arginine[14] peptide IIIa was obtained in reasonable yield (52%). Shortening the reaction period to 24 hr reduced the yield (of peptide IIIa) to 39%. When attempts to selectively remove the carbobenzoxy protecting group from peptide IIIa by catalytic hydrogenolysis (at atmospheric pressure in the presence of palladium black) proved to be unrewarding, the HBr–glacial acetic acid[15] cleavage was next considered. To ascertain what effect, aside from acetylation, this reagent might have on the unprotected 3β-hydroxyl group, reaction between alcohol Ia and 2 *N* HBr–acetic acid was attempted. After a 2-hr period at room temperature, acetate Ib was isolated in 53% yield accompanied by a less polar (on a thin layer chromatogram) side product and unreacted starting material. Results of the preceding experiment clearly demonstrated that protection of the 3β-hydroxyl group would be desirable. Thus, dipeptide IIIa was treated with acetic anhydride–pyridine at room temperature for 20 hr. The corresponding 3β-acetoxy derivative (IIIb) was obtained in nearly quantitative yield.

In order to assess utility of acetylation at an earlier stage, 3β-hydroxy-17β-(N^{α}-carbobenzoxy-L-prolyl)amino-5α-androstane (IIb)[1] was prepared and allowed to react with acetic anhydride–pyridine. The corresponding 3β-acetate (IIc) was obtained in good yield (80% after purification) and then subjected to reaction with 2 *N* HBr–glacial acetic acid at room temperature for 1 hr. Amide IId was obtained in 89% yield. As the free base readily formed solvates (for example, with chloroform), characterization was easier using the hydrochloride derivative. Treating amine IId with the active ester formed from N^{α}-carbobenzoxy-N^{G}-nitro-L-arginine and WRK (with triethylamine) provided a fair amount (52%) of dipeptide IIIb. A comparison thin layer chromatogram of dipeptide IIIb prepared by the alternate route and a sample obtained by acetylating dipeptide IIIa displayed identical mobilities. Of both pathways, acetylation at the dipeptide stage proved experimentally more convenient. Both synthetic routes from carbamate IIb consist of three steps and give comparable over-all yields but the exposed 3β-hydroxy group method involves intermediates easier to purify by column chromatography.

Applying the HBr cleavage step to protected dipeptide IIIb and subsequent treatment of the free base with HCl gave the hydrochloride derivative of peptide IIIe as a fine powder melting at 193–194°. Since dipeptide IIIe had quite limited solubility in acetonitrile, the next peptide bond forming sequence was conducted in acetonitrile–dimethylformamide. By this means protected diarginine peptide IIId was isolated in reasonable yield (72%). Based on a number of preliminary experiments, the following technique for removing protecting groups from tripeptide IIId and characterizing the resulting steroidal peptide was found satisfactory. A solution of protected tripeptide IIId in methanol–glacial acetic acid[16] containing suspended palladium black was hydrogenated (slight positive pressure) at room temperature 24 hr. The product, tripeptide IIIe, was characterized as the triacetate salt (54% yield): a microcrystalline compound melting at approximately 154°. The structure assigned peptide IIIe received further support from results of an amino acid analysis. The ratio of arginine to proline (for example, 0.96:0.51) was in good agreement with expected values.

In our experience, the phenylisoxazolium peptide bond forming sequence has proved useful for synthesis of steroidal peptides.[1,12] Experimental convenience combined with minimal racemization[13] and safe application in the presence of exposed hydroxyl groups allows the WRK technique to be recommended for synthesis of steroidal peptides. In general, relatively small steroidal peptides of the type reported here may also prove useful for greatly enhancing the hydrophilic properties of certain steroids. The rapid increase in water solubility from amide IIa to tripeptide IIIe deserves comment.

At present, endocrine evaluation data are available for protected peptides IIb, IIc, IIIb, and IIIe in the levator ani, seminal vesicle, and ventral prostate bioassay systems. In each case the substance was administered (over the range 0.2–4.2 mg/kg) for a period of 7 days to the male rat with testosterone as standard

(12) A preliminary communication by G. R. Pettit, A. K. Das Gupta, H. Klinger, and J. Occolowitz, *Experientia*, **20**, 545 (1964), reports the first synthesis of a steroidal dipeptide; synthesis of 3β-hydroxy-17β-(L-prolyl-L-prolyl)amino-5α-androstane has recently been summarized (see ref 1). Prior to 1964, only peptides of the bile acid type had been prepared: see, for example, A. F. Hofmann, *Acta Chem. Scand.*, **17**, 173 (1963). In 1964 M. M. Dhar and K. L. Agarwal, *Steroids*, **3**, 139 (1964), reported preparation of α-poly-γ-(3β-hydroxycholest-5-ene)-and α-poly-γ-(3β-hydroxy-24-ethylcholest-5-ene)-L-glutamate but exact structures for these polymers have not been determined. Later, N. D. Tam, *Compt. Rend.*, **260**, 717 (1965), reported synthesis of 3α-(L-phenylalanyl)amino- and 3α-(glycylglycyl)-amino-20-oxo-5α-pregnane by stepwise peptide formation. The potentially important field of steroidal peptide chemistry represents an essentially unexplored area.

(13) In addition to ref 1 and citations contained therein, refer to F. Weygand, A. Prox, and W. König, *Ber.*, **99**, 1451 (1966), and C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963), for other examples involving Woodward's reagent K.

(14) For a summary of recent advances in the chemistry of arginine consult: M. Bodanszky and M. A. Ondetti, *Chem. Ind.* (London), 26 (1966); St. Guttmann and J. Pless, *Acta Chim. Acad. Sci. Hung.*, **44**, 23 (1965); *Chem. Abstr.*, **63**, 13396 (1965); Z. Paulay and S. Bajusz, *Acta Chim. Acad. Sci. Hung.*, **43**, 147 (1965); *Chem. Abstr.*, **63**, 5738 (1965); V. G. Debabov and V. D. Davydov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 203 (1965); *Chem. Abstr.*, **62**, 11901 (1965); R. E. Plapinger, M. M. Nachlas, M. L. Seligman, and A. M. Seligman, *J. Org. Chem.*, **30**, 1781 (1965); P. M. Scopes, K. B. Walshaw, M. Welford, and G. T. Young, *J. Chem. Soc.*, 782 (1965); M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, and P. L. Thomas, *J. Am. Chem. Soc.*, **86**, 4452 (1964); C. H. Li, D. Chung, and J. Ramachandran, *J. Am. Chem. Soc.*, **86**, 2715 (1964); K. Sturm, R. Geiger, and W. Siedel, *Ber.*, **97**, 1197 (1964); G. Harris and I. C. MacWilliam, *J. Chem. Soc.*, 5552 (1963); R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **46**, 1550 (1963); K. Hofmann, R. D. Wells, H. Yajima, and J. Rosenthaler, *J. Am. Chem. Soc.*, **85**, 1546 (1963); C. H. Li, J. Ramachandran and D. Chung, *ibid.*, **85**, 1895 (1963); K. Sato and S. Abe, *J. Org. Chem.*, **28**, 1928 (1963); R. Geiger, K. Sturm, and W. Siedel, *Ber.*, **96**, 1080 (1963); M. Bodanszky, J. T. Sheehan, M. A. Ondetti, and S. Lande, *J. Am. Chem. Soc.*, **85**, 991 (1963); R. Paul, G. W. Anderson, and F. M. Callahan, *J. Org. Chem.*, **26**, 3347 (1961); L. Zervas, T. T. Otani, M. Winitz, and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959); L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957); and a review by R. Schwyzer, *Pure Appl. Chem.*, **6**, 265 (1963).

(15) See *e.g.*, P. G. Katsoyannis, K. Fukuda, and A. Tometsko, *J. Am. Chem. Soc.*, **85**, 1681 (1963), and a review by E. Schröder and K. Lübke, *Peptides*, **1**, 28 (1965).

(16) M. Brenner and H. C. Curtius, *Helv. Chim. Acta*, **46**, 2126 (1963).

inhibitor. Employing these procedures, no significant activity was noted.

Experimental Section

Acetonitrile, dimethylformamide (DMF), and triethylamine (from KOH) were redistilled and stored over Molecular Sieve type 4-A. Arginine was used as obtained from Henley and Co., New York, N. Y. Other reagents were purchased as noted in the preceding contribution.[1] All solvent extracts containing reaction products were dried ($MgSO_4$). Neutral alumina (E. Merck, A.G. Darmstadt) and silica gel (E. Merck, 0.05–0.2 mm) were used for column chromatography. Thin layer chromatograms (R_f values were recorded for analytical samples) were prepared on microscope slides using silica gel G (E. Merck) and plates were activated by heating at 110° for 0.25 hr. Subsequently, the plates were stored over $CaCl_2$ in a desiccator and both elution and development (I_2, H_2SO_4 or phosphomolybdic acid) steps were conducted as summarized in the previous paper.[1] Chromatography solvents were redistilled.

All analytical samples exhibited a single spot on a thin layer chromatogram and melting points were recorded using a Kofler melting point apparatus. Mixture melting points were determined in open Kimble glass capillary tubes using a silicone oil bath. The infrared (in KBr) and pmr (in $CDCl_3$ unless otherwise noted) spectra were recorded by R. A. Hill and P. A. Whitehouse, University of Maine. The pmr spectra were determined using a Varian Model A-60 spectrometer (Me_4Si as internal standard). Elemental microanalytical data were provided by Dr. A. Bernhardt, Max-Planck Institut, Mülheim, Germany, and optical rotations (at 20°) by Dr. P. Demoen, Analytical Department, Janssen Labs., Beerse, Belgium. We are also grateful to Professor R. G. Hiskey, Department of Chemistry, University of North Carolina, for performing a quantitative amino acid analysis (using a Phoenix Model VB-6000 instrument) of tripeptide IIIe.

N^G-Nitro-L-arginine.—A sample of L-arginine (30.0 g) was allowed to react with fuming HNO_3 (40 ml) and fuming H_2SO_4 (25 ml, 30% SO_3) essentially as described by Hofmann.[17] The colorless crystalline product (27.0 g, 72%) decomposed at 250–252°. Two recrystallizations from water afforded an analytical sample as crystal clusters: dec pt 252° (sintering from 246°); $[\alpha]_D$ +24.2° (*c* 1.03, 2 *N* HCl); ν_{max} 3340, 3130, 2900 (broad), 1654, 1600 (broad, shoulder at 1551), 1518, 1461, 1450, 1320 (broad) and 1243 (broad) cm^{-1}.

Anal. Calcd for $C_6H_{13}N_5O_4$: C, 32.87; H, 5.98; N, 31.95. Found: C, 33.02; H, 6.01; N, 32.16.

Decomposition points of 251–252 and 263° as well as optical rotation values of $[\alpha]^{25}D$ +24.3° and $[\alpha]^{19}D$ +20.8° have been reported[17,18] for nitroarginine.

N^α-Carbobenzoxy-N^G-nitro-L-arginine.—Carbobenzoxy chloride (30.3 g used as received from Mann Research Laboratories, New York, N. Y.) was allowed to react with nitroarginine (25.0 g) as previously described.[18] Following recrystallization from EtOH–H_2O, a pure sample (9.2 g, 23%) was obtained as fine needles melting at 135–136° (sintering from 130°); $[\alpha]_D$ −2.2° (*c* 2.06, MeOH); ν_{max} 3320–3280 (doublet), 3140, 2900, 1728 (shoulder at 1718), 1686, 1655, 1600, 1542, 1450, 1428, 1322–1268 (broad), 1243, 1200, 1113, and 1074 cm^{-1}.

Anal. Calcd for $C_{14}H_{19}N_5O_6$: C, 47.60; H, 5.41; N, 19.82. Found: C, 47.79; H, 5.48; N, 19.67.

Melting point and optical rotation values of 134–136° (and 126°) and $[\alpha]^{27}D$ −3.5° have been reported[17,18] for carbobenzoxynitroarginine.

3β-Hydroxy-17β-(N^α-carbobenzoxy-N^G-nitro-L-arginyl-L-prolyl)amino-5α-androstane (IIIa).—To a rapidly stirred cold solution (ice bath) of N^α-carbobenzoxy-N^G-nitro-L-arginine (1.2 g) in acetonitrile (60 ml) was added Woodward's reagent K (0.85 g) and triethylamine (0.3 g). Before adding 3β-hydroxy-17β-(L-prolyl)amino-5α-androstane (IIa, 1.3 g),[1] cooling and stirring were continued 2 hr. Following a 70-hr reaction period at room temperature, solvent was removed *in vacuo*, and the residue was extracted ($CHCl_3$, 150 ml). The pale yellow extract was washed successively with water (50 ml), two 50-ml portions of 5% HCl, water (50 ml), two 50-ml portions of 5% $NaHCO_3$, and six 50-ml portions of water. During the initial aqueous wash, a pale yellow viscous oil collected on the separatory funnel walls and this material was extracted with a second 150-ml portion of chloroform. The latter $CHCl_3$ solution was treated as just described and evaporated to dryness. The combined residue from both $CHCl_3$ extracts weighed 1.9 g. A solution of the residue in $CHCl_3$ (5 ml) was chromatographed on neutral alumina (50 g). Elution with the same solvent (200 ml) gave a 0.07-g fraction of side product which was discarded. Continued elution with 19:1 $CHCl_3$–MeOH (75 ml) afforded a colorless foam (1.3 g, 52%) which crystallized from $CHCl_3$–ethyl acetate as microcrystals melting at 214–215° dec (softening at 210°). Recrystallization from methanol followed by two recrystallizations from MeOH–H_2O (2:1) yielded a solvate as microcrystalline clusters slowly melting from 141.5–148°: R_f 0.29 in $CHCl_3$–MeOH (9:1); $[\alpha]_D$ −49.5° (*c* 2.47, $CHCl_3$); ν_{max} 3380, 2900 (shoulder at 2830), 1706, 1628, 1530, 1442, 1330, 1258, 1150 (weak), 1077 (weak), and 1040 (weak) cm^{-1}.

Anal. Calcd for $C_{38}H_{57}N_7O_7$: C, 63.06; H, 7.94; N, 13.54. Found: C, 63.27; H, 8.12; N, 13.47.

Reaction of 2 *N* HBr–Acetic Acid with 3β-Hydroxy-17-oxo-5α-androstane (Ia).—A solution of ketone Ia (1.0 g) in 2 *N* HBr–glacial acetic acid (14 ml) was stirred at room temperature 2 hr. Solvent was removed (*in vacuo*) at 50° and the resulting yellow oily residue was dissolved in chloroform. The $CHCl_3$ solution was washed (H_2O, 5% $NaHCO_3$, H_2O). Following evaporation of solvent, a solution of the residue in 1:1 hexane–benzene was chromatographed on neutral alumina (20 g). Fractions eluted with the same solvent and benzene gave a colorless oil (0.7 g) which crystallized from hexane as crystal clusters weighing 0.6 g (52%) and melting at 104.5–106°. Three recrystallizations from hexane afforded a pure sample of 3β-acetoxy-17-oxo-5α-androstane (Ib) as needles: R_f 0.77 in diethyl ether–benzene (3:2), mp 106–106.5°, and pmr response δ 0.84 (singlet, 6 methyl protons) and 2.01 (singlet, 3 acetyl protons). Acetate Ib was found identical[19] with an authentic sample prepared (98% yield) by treating alcohol Ia with acetic anhydride–pyridine.

3β-Acetoxy-17β-(N^α-carbobenzoxy-L-prolyl)amino-5α-androstane (IIc).—A solution composed of pyridine (25 g), acetic anhydride (11 g), and 3β-hydroxy-17β-(N^α-carbobenzoxy-L-prolyl)-amino-5α-androstane (5.2 g)[1] was stirred at room temperature 15 hr. The colorless solution was poured onto crushed ice and 5 hr later the mixture was extracted ($CHCl_3$). Successively washing the chloroform solution with 5% HCl, H_2O, 5% $NaHCO_3$, and H_2O, followed by removal (*in vacuo*) of solvent, led to a colorless foam (5.0 g). Recrystallization from MeOH–H_2O (5:1, 30 ml) gave crystal clusters weighing 4.5 g (80%) and melting at 164–167°. Three recrystallizations from the same solvent afforded a pure specimen as needle clusters: R_f 0.31 in $CHCl_3$–MeOH (49:1); mp 166–169°;[20] $[\alpha]_D$ −69.7° (*c* 1.15, $CHCl_3$); $[\alpha]_D$ −85.8° (*c* 0.47, MeOH); ν_{max} 3320, 2930, 1680 (shoulders at 1732 and 1708), 1532, 1420 (shoulder at 1450), 1356, 1245 and 1120 cm^{-1}; pmr response δ 0.55 (singlet, 3 methyl protons), 0.82 (singlet, 3 methyl protons), 2.0 (singlet, 3-acetyl protons), 5.09 (singlet, 2 benzyl protons), and 7.25 (singlet, 5 aromatic protons).

Anal. Calcd for $C_{34}H_{48}N_2O_5$: C, 72.31; H, 8.57; N, 4.96; O, 14.16. Found: C, 72.40; H, 8.35; N, 5.13; O, 14.08.

3β-Acetoxy-17β-(L-prolyl)amino-5α-androstane (IId). Method A.—A suspension of carbamate IIc (4.0 g) in freshly prepared 2 *N* HBr–glacial acetic acid (29 ml) was stirred at room temperature 1 hr. A clear solution was obtained during the first 0.5 hr. The yellow solution was concentrated *in vacuo* to approximately 15 ml and diluted with dry diethyl ether (400 ml). Approximately 0.5 hr later the amine hydrobromide (3.3 g, 92%) was collected and washed with diethyl ether. A solution of the hydrobromide in chloroform (150 ml) was washed (5% $NaHCO_3$, H_2O). Removal (*in vacuo*) of solvent gave a colorless solid, yield 2.7 g (89%), dec pt 210–213°. A solution of the base (IId) in $CHCl_3$ was chromatographed on neutral alumina (25 g). The fractions eluted with chloroform to $CHCl_3$–MeOH (19:1) weighed 2.7 g and represented nearly pure (as evidenced by thin

(17) K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

(18) M. Bergmann, L. Zervas, and H. Rinke, *Z. Physiol. Chem.* **224**, 40 (1934); *Chem. Abstr.*, **28**, 4076 (1934).

(19) The identical composition of both substances was confirmed by mixture melting point determination and infrared spectral (as well as thin layer chromatographic) comparison.

(20) In another experiment, a specimen melting at 171–174° (sintering from 164°) was obtained.

layer chromatograms) amine IId. Three recrystallizations from $CHCl_3$–hexane (1:1) afforded an analytical sample as fine needles: R_f 0.28 in $CHCl_3$–MeOH (85:15); dec pt 242° (sintering from 205°); $[\alpha]D$ −65.0° (*c* 0.69, $CHCl_3$); ν_{max} 3400, 3200, 2920 (shoulder at 2720), 1730, 1678, 1550 (broad), 1450, 1366, 1243 (broad), 1030 and 760 cm^{-1}; and pmr response δ 0.74 (singlet, 3 methyl protons), 0.80 (singlet, 3 methyl protons), 1.99 (singlet, 3 acetyl protons), and 7.16 (singlet, $CHCl_3$).

The preceding infrared and pmr data supported by a positive Beilstein test indicated that the specimen of amine IId decomposing at 242° was a chloroform solvate. Thin layer chromatographic (18:15 $CHCl_3$–MeOH mobile phase) comparison of the chloroform solvate with a specimen of amine IId prepared by method B (below) provided evidence for the identical composition of both products.

A methanol (1 ml) solution of amine IId (0.06 g) was treated with ethereal HCl (25 ml), and the hydrochloride derivative (0.04 g) which separated was recrystallized from 1:10 methanol–diethyl ether. The analytical specimen of fine needles was obtained as a partial methanol solvate decomposing at 270° (with sublimation at 215°): R_f 0.26 in $CHCl_3$–MeOH (4:1); ν_{max} 3200 (shoulder at 3360), 2950 (shoulder at 2730), 1734, 1678, 1550 (broad), 1452, 1366, 1260 (broad), and 1030 cm^{-1}.

Anal. Calcd for $C_{26}H_{43}ClN_2O_3 \cdot 0.5CH_3OH$: C, 65.89; H, 9.39; Cl, 7.34; N, 5.80. Found: C, 66.08; H, 9.43; Cl, 7.63; N, 6.08.

Method B.—A mixture of carbamate IIc (8.0 g) in methanol (300 ml) containing suspended Pd black (freshly prepared from 1 g of $PdCl_2$) was stirred in a hydrogen atmosphere for 8 hr. The hydrogenolysis experiment was conducted by passing hydrogen through the reaction mixture and exit gases into an aqueous solution of $Ba(OH)_2$. The methanol solution was filtered and concentrated (*in vacuo*) to dryness. The residue, predominantly amine IId hydrochloride (from HCl contained by the Pd catalyst) recrystallized from ethyl acetate–methanol as needles decomposing at 272–279°. The product was dissolved ($CHCl_3$) and washed (10% $NaHCO_3$, H_2O). Following concentration (*in vacuo*) to dryness, amine IId was crystallized from ethyl acetate; yield 3.8 g, mp 210–212°. Two additional recrystallizations from ethyl acetate gave a pure specimen melting at 212–214°, $[\alpha]D$ −63.6° (*c* 0.35, MeOH).

Anal. Calcd for $C_{26}H_{42}N_2O_3$: C, 72.52; H, 9.83; N, 6.51; O, 11.15. Found: C, 72.47; H, 9.83; N, 6.60; O, 11.29.

3β-Acetoxy-17β-(N$^{\alpha}$-carbobenzoxy-N^{G}-nitro-L-arginyl-L-prolyl)amino-5α-androstane (IIIb). Method A.—A suspension of dipeptide IIIa (0.6 g) in acetic anhydride–pyridine (1:5 g) was stirred at room temperature 20 hr. The resulting straw-colored solution was poured onto crushed ice. After 1 hr the solid phase was collected and washed (H_2O, 5% HCl, H_2O, 5% $NaHCO_3$, H_2O). The acetate weighed 0.6 g (99% yield) and slowly melted above 143° (softening at 140°). When repeated attempts to recrystallize the product were unsuccessful, a solution of the acetate in chloroform was chromatographed on neutral alumina (12 g). Elution with the same solvent gave 0.59 g of peptide IIIb. Two recrystallizations from MeOH–H_2O (2:1) yielded a pure sample as microcrystalline needles: R_f 0.58 in $CHCl_3$–methanol (9:1); mp 129–136° (slow melting); $[\alpha]D$ −42.8° (*c* 0.3 $CHCl_3$); ν_{max} 3280, 2930, 1724, 1630 (broad), 1534, 1450 (broad), 1246 (broad), and 1027 cm^{-1}.

Anal. Calcd for $C_{46}H_{59}N_7O_8$: C, 62.71; H, 7.77; N, 12.80. Found: C, 62.62; H, 7.87; N, 12.67.

Method B.—Carbobenzoxynitroarginine (0.35 g) was dissolved in warm acetonitrile (25 ml). After cooling the solution to ice-bath temperature, Woodward's reagent K (0.26 g) and triethylamine (0.2 ml) were added. When solution was complete (2 hr), amine IId (0.43 g) was added and stirring (with cooling) continued 30 hr. A colorless solid which separated as the reaction progressed was dissolved by adding $CHCl_3$. Following removal of solvent *in vacuo*, a chloroform extract of the residue was chromatographed on silica gel (20 g). Fractions eluted with chloroform and 9:1 $CHCl_3$–methanol corresponded to 0.64 g of nearly pure peptide IIIb. The peptide was obtained as a pale yellow foam which resisted crystallization. Thus, an analytical sample was prepared by preparative thin layer chromatography using 95:5 $CHCl_3$–MeOH as mobile phase. Before product isolation, the plate was eluted three times with the same solvent. The sample prepared by preparative thin layer chromatography displayed only one spot on a thin layer chromatogram with 9:1 $CHCl_3$–MeOH as eluent. Dipeptide IIIb, prepared by this method, exhibited $[\alpha]D$ −105.9° (*c* 0.34, MeOH) and elemental analytical values of C, 62.96; H, 7.95; N, 12.56; O, 16.59 (calcd 16.72).

Method C.—Protected dipeptide IIIb (3.1 g) was also prepared from amine IId (4.0 g) in 52% yield (after purification using the method (70-hr reaction period) described above for synthesis of dipeptide IIIa. Comparison thin layer chromatography of dipeptide IIIb samples prepared by methods A–C using 9:1 $CHCl_3$–MeOH as mobile phase showed identical R_f values (0.58) for each specimen.

3β-Acetoxy-17β-(N^{G}-nitro-L-arginyl-L-prolyl)amino-5α-androstane (IIIc) Hydrochloride.—Employing the 2 *N* HBr–glacial acetic acid (17 ml) technique (see experiment IId, method A) dipeptide IIIb (3.1 g) was converted to a straw-colored foam (1.4 g, 56% yield) corresponding to amine IIIc. A solution of product in chloroform was chromatographed on neutral alumina (30 g) and three less polar impurities were eluted by $CHCl_3$ (150 ml) and 19:1 $CHCl_3$–MeOH (175 ml). Continued elution with 9:1 to 5:1 $CHCl_3$–MeOH gave colorless fractions (0.5 g total) of amine IIIc. A thin layer chromatogram (9:1 $CHCl_3$–MeOH mobile phase) of the 0.5-g sample showed only one component. However, attempts at recrystallizing the foam (from $CHCl_3$) introduced two less polar impurities. Consequently, amine IIIc in methanol was converted using ethereal HCl to the hydrochloride derivative. Repeated recrystallization from methanol–diethyl ether gave an analytical sample as a fine powder: R_f 0.17 in $CHCl_3$–MeOH (85:15); mp 193–194°; $[\alpha]D$ −65.1° (*c* 1.11, methanol); and ν_{max} 3340–3290 (doublet with shoulder at 3190), 2910 (shoulders at 2785 and 2630), 1644 (broad), 1540 (broad), 1450 (broad), 1375, and 1270 cm^{-1}.

Anal. Calcd for $C_{32}H_{54}ClN_7O_6$: C, 57.50; H, 8.14; N, 14.68. Found: C, 57.29; H, 8.33; N, 14.46.

3β-Acetoxy-17β-(N$^{\alpha}$-carbobenzoxy-N^{G}-nitro-L-arginyl-N^{G}-nitro-L-arginyl-L-prolyl)amino-5α-androstane (IIId).—Woodward's reagent K (0.04 g) was added to a cold (ice bath), rapidly stirred solution of N$^{\alpha}$-carbobenzoxy-N^{G}-nitro-L-arginine (0.06 g) and triethylamine (0.02 g) in acetonitrile (5 ml). After stirring with cooling for 3 hr, a solution of dipeptide IIIc (0.10 g) in 10:1 acetonitrile–DMF (5 ml) was added to the clear solution. Stirring at room temperature was continued 21 hr and solvent was then removed (*in vacuo* at 50°). The yellow oily residue was dissolved in chloroform (10 ml) and washed as noted for the partial purification of peptide IIIa. Removal of solvent *in vacuo* afforded a pale beige foam (0.11 g, 72% yield). Two recrystallizations from 2:1 MeOH–H_2O afforded a pure sample as microcrystalline plates: R_f 0.62 in $CHCl_3$–MeOH (25:15); mp 147–149° (slow melting); $[\alpha]D$ −62.8 and −66.0° (*c* 0.43 and 0.61, MeOH); ν_{max} 3300, 2920, 1716 (broad), 1628 (broad), 1535 (broad), 1450, 1260 (broad), 1155, 1028 cm^{-1}. The analytical sample was dried to constant weight *in vacuo* at 100°. However, the oxygen analysis suggests presence of a partial solvate or hydrate.

Anal. Calcd for $C_{46}H_{70}N_{12}O_{11}$: C, 57.12; H, 7.30; N, 17.38; O, 18.21. Found: C, 57.02; H, 7.22; N, 16.65; O, 18.96.

3β-Acetoxy-17β-(L-arginyl-L-arginyl-L-prolyl)amino-5α-androstane (IIIe) Triacetate.—A vigorously stirred solution of protected tripeptide IIId (0.10 g) in 9:1 MeOH–glacial acetic acid (25 ml) was hydrogenated (slightly positive pressure) in the presence of Pd black (0.05 g) at room temperature for 24 hr. The solution was filtered through a Celite layer and concentrated (*in vacuo* at 40°) to a glasslike residue (0.13 g). A solution of the vitreous product in methanol–anhydrous diethyl ether (2:20 ml) was stored at 0° for 10 hr. The solid which separated was collected, washed with diethyl ether, and dried to yield a tan powder (0.051 g, 54% yield) displaying one spot on a thin layer chromatogram (7:3 $CHCl_3$–MeOH mobile phase). Two additional precipitations from methanol–diethyl ether afforded an analytical sample as a microcrystalline powder: R_f 0.05 in $CHCl_3$–MeOH (7:3); mp 154° (slow melting with sintering from 147°); $[\alpha]D$ −58.3° (*c* 0.64, MeOH); ν_{max} 3300 (broad), 2930, 1738, 1655 (broad), 1555 (broad), 1452, 1404, 1250, and 1030 cm^{-1}; amino acid analysis (values expressed as μmoles/ml): $proline_{0.25}$ and 0.24; $arginine_{0.98}$ and 0.96 (calcd, $proline_{0.50}$, $arginine_{1.00}$).

Anal. Calcd for $C_{43}H_{78}N_{10}O_{11}$: C, 57.24; H, 8.52; N, 15.18. Found: C, 57.05; H, 8.39; N, 15.44.