Synthesis of a Protected C-Terminal δ-Aminovaleric Acid Analog of the Peptide Sequence Occurring at Positions 11–19 in Adrenocorticotropins[1a]

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The protected nonapeptide, N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester, has been synthesized by a stepwise procedure. The nonapeptide corresponds to the sequence of amino acids occurring at positions 11–19 in the adrenocorticotropic hormone (ACTH) molecule, but possesses the δ-aminovaleric acid as its terminal residue instead of the proline residue occurring at position 19 in the native hormone.

Recent studies carried out in this laboratory[2] revealed that exposure of certain esters of proline, which are commonly used in peptide synthesis, to treatment with sodium in liquid ammonia, can lead to cleavage of the proline ring with the formation of the corresponding δ-aminovaleric acid derivative, provided that the proline imino group is acylated as in the case of peptides containing a proline ester at the C terminus. Insofar as this reaction can provide a novel route for the preparation of peptides containing a C-terminal δ-aminovaleric acid residue from the corresponding proline ester analogs, it was considered of importance to undertake the direct synthesis of such peptides which can serve as reference compounds in planned future studies on this aspect of the sodium–liquid ammonia procedures. Being part of a series of synthetic studies[3–5] on the N-terminal portion of the ACTH molecule, this paper describes the synthesis of the protected nonapeptide N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester (IX, Figure 1).[6] In this peptide which corresponds to the sequence of amino acid residues occurring at positions 11–19 in ACTH, δ-aminovaleric acid residue is substituted for the proline residue occurring at position 19 in the native hormone.

δ-Aminovaleric acid hydrochloride (I) was readily converted into the corresponding crystalline methyl ester II by means of 2,2-dimethoxypropane in the presence of concentrated hydrochloric acid.[7] Compound II was coupled in two consecutive steps with N^{α}-carbobenzoxy-N^{G}-tosylarginine[8] using N-ethyl-5-phenylisoxazolium-3′-sulfonate[9] for activation of the carboxyl group in both steps. The fully protected tripeptide N^{α}-carbobenzoxy-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester (IV) obtained in this manner, was purified by means of extraction and countercurrent distribution in the toluene system (K = 1.04). After catalytic removal of the α-carbobenzoxy group from IV, the resulting tripeptide

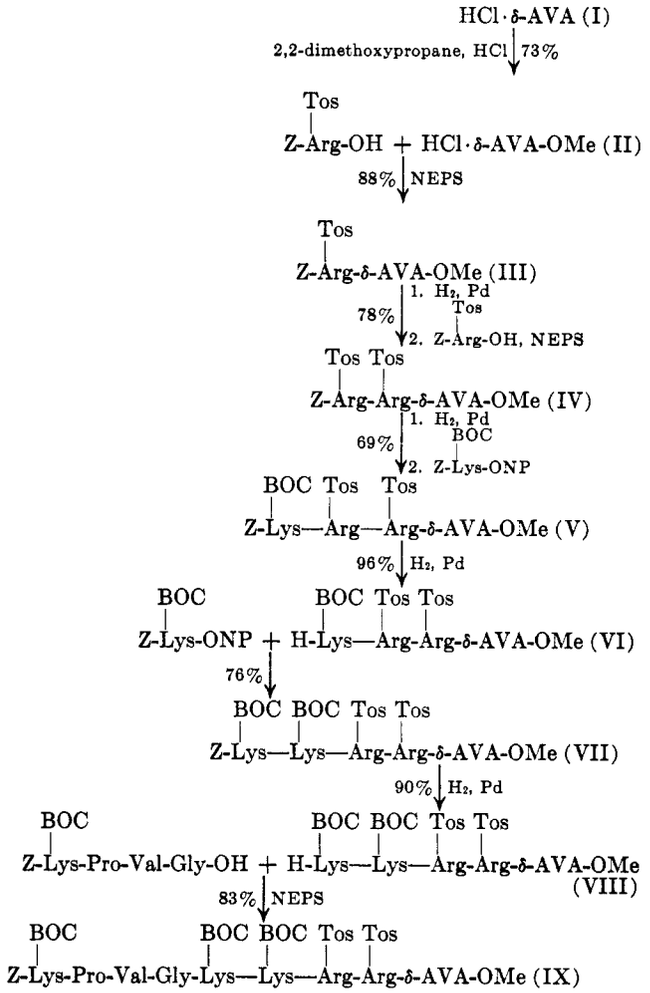

Figure 1.—Outline of the synthesis of the fully protected nonapeptide N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester: δ-AVA, δ-aminovaleric acid; OMe, methoxy; Z, carbobenzoxy; Tos, *p*-toluenesulfonyl; NEPS, N-ethyl-5-phenylisoxazolium-3′-sulfonate; BOC, *t*-butyloxycarbonyl; ONP, *p*-nitrophenyl.

free base was coupled with N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[10] in two subsequent steps to give the fully protected pentapeptide N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester (VII). Peptide VII, which was obtained in amorphous form, behaved as a single component (K = 0.56) during countercurrent distribution in the toluene system and was also homogeneous by the criteria of paper and thin layer chroma-

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tography in several solvent systems, both before and after removal of the carbobenzoxy group. For the condensation of the pentapeptide free base N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric acid methyl ester (VIII) with the tetrapeptide acid N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycine,[11] N-ethyl-5-phenylisoxazolium-3′-sulfonate was employed once again as carboxyl activating agent to give the fully protected nonapeptide IX. Compound IX was obtained in crystalline form and was homogeneous by the criteria of countercurrent distribution and thin layer chromatography in several solvent systems.

Experimental Section[12-15]

δ-Aminovaleric Acid Methyl Ester Hydrochloride (II).—δ-Aminovaleric acid hydrochloride (I) (4.61 g, 30 mmol) was suspended in 200 ml of 2,2-dimethoxypropane, and 15 ml of concentrated HCl was added while stirring at room temperature. Stirring was continued at room temperature for a total period of 17 hr. The intensely colored solution was then evaporated *in vacuo* to produce a dark crystalline mass, which was washed with portions of ether until free of any color. The white crystalline product (5 g) revealed in paper chromatography in the BPAW system the presence of one major spot (R_f 0.67) accompanied by a trace of δ-aminovaleric acid (R_f 0.48). The product was dissolved in 40 ml of hot absolute alcohol, and, while still hot, 80 ml of ethyl acetate were added. Upon slowly being cooled to room temperature, the ester (II) separated from the solution in the form of long needles. After a few hours at room temperature, 3.65 g (73%) of crystals was obtained: mp 145–146°; R_f BAW 0.44, R_f BPAW 0.67 in paper chromatography.

Anal. Calcd for $C_6H_{14}O_2NCl$ (167.6): C, 42.90; H, 8.41; N, 8.35. Found: C, 42.93; H, 8.60; N, 8.13.

N^{α}-Carbobenzoxy-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (III).—N^{α}-Carbobenzoxy-N^{G}-toslyarginine[8] (6.01 g, 13 mmol) was dissolved with slight warming in 130 ml of acetonitrile. After the solution was cooled in an ice bath, 1.82 ml (13 mmol) of triethylamine, followed by 3.63 g (14.3 mmol) of N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward reagent K),[9] was added while stirring. The mixture was stirred for 1.5 hr at 0°, and then 2.18 g (13 mmol) of compound II, followed by another 1.82 ml of triethylamine, was added. Stirring was continued for another 6 hr in the cold before the temperature of the bath was allowed to increase to room temperature. After a total reaction period of 23 hr, the solvent was removed *in vacuo*, and the resulting syrup was dissolved in 200 ml of moist ethyl acetate. The solution was extracted twice with 80-ml portions of water, followed by successive extractions with similar portions of 0.1 *N* HCl, water, 5% $NaHCO_3$ solution, water, and saturated NaCl solution. After the solution was dried over anhydrous Na_2SO_4, the ethyl acetate was removed *in vacuo* and the product was dried over P_2O_5 to give 6.57 g (88%) of a glassy material: mp 40–45°; $[\alpha]^{25}D$ −1.72° (*c* 4, methanol). The product was homogeneous in paper chromatography in four systems (R_f BAW 0.84, R_f SBA 0.86, R_f BPAW 0.82, R_f nBA 0.81) as well as in four thin layer systems[16] [R_f BAW 0.70, R_f SBA 0.64, R_f BPAW 0.75, R_f CM (8:2) 0.71].

Anal. Calcd for $C_{27}H_{37}O_7N_5S$ (575.7): C, 56.32; H, 6.48; N, 12.16; S, 5.57. Found: C, 56.08; H, 6.28; N, 12.15; S, 5.72.

N^{α}-Carbobenzoxy-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (IV).—A solution of the protected dipeptide (III) (3.0 g, 5.2 mmol) in absolute methanol (60 ml) was hydrogenolyzed for 5 hr in the presence of a Pd catalyst freshly prepared from 1 g of $PdCl_2$. The resulting solution was immediately investigated by means of thin layer and paper chromatography, which revealed the presence of a single ninhydrin-positive component in several systems (R_f BAW 0.28, R_f SBA 0.31, R_f CM 0.22 in thin layer chromatography, and R_f BAW 0.60, R_f SBA 0.68, R_f BPAW 0.67 on paper). As soon as it was evident from the CM thin layer system that the hydrogenolysis reaction was complete, the catalyst was removed by filtration and the methanol removed *in vacuo*.

A solution of the resulting syrup in 15 ml of acetonitrile was added without delay[17] to a mixture which was prepared in the following manner. N^{α}-Carbobenzoxy-N^{G}-tosylarginine[8] (2.41 g, 5.2 mmol) was dissolved in 35 ml of acetonitrile; triethylamine (0.73 ml, 5.2 mmol) was added, followed by 1.453 g (5.72 mmol) of Woodward reagent K[9] at 0°, and the solution was stirred in the cold for 1.5 hr. After addition of the hydrogenolysis product, stirring was continued for another hour at 0° and then for 24 hr at room temperature.

At the end of the reaction period, the mixture so obtained was evaporated *in vacuo* to a syrup, the latter was dissolved in 200 ml of moist ethyl acetate, and the solution was extracted twice with 80-ml portions of water and then with portions (80 cc) of 0.1 *N* HCl, water, 5% $NaHCO_3$ solution, water, and saturated NaCl solutions.[18] After the solution was dried over anhydrous Na_2SO_4, the solvent was removed *in vacuo;* the thoroughly dried product was dissolved in methanol (15 ml) and precipitated from anhydrous ether (600 ml). The precipitate (3.9 g) was subjected to countercurrent distribution for 100 transfers in the toluene system. From the peak obtained (K = 1.04), 3.59 g (78%) of compound IV was recovered: mp 87–91°; $[\alpha]^{24}D$ −6.22° (*c* 3, methanol), Peptide IV was homogeneous in paper chromatography in three solvents (R_f BAW 0.88, R_f SBA 0.85, R_f BPAW 0.91) as well as in three thin layer systems [R_f BAW 0.66, R_f SBA 0.63, R_f CM (8:2) 0.65].

Anal. Calcd for $C_{40}H_{55}O_{10}N_9S_2$ (886.0): C, 54.30; H, 6.26; N, 14.23; S, 7.24. Found: C, 54.20; H, 6.01; N, 14.50; S, 7.15.

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(12) Melting points were determined in a Fischer-Johns melting block apparatus and are uncorrected. Microanalyses were performed in the Microanalytical Laboratory of the Department of Chemistry, University of California at Berkeley. Samples for microanalyses were dried for *ca.* 16 hr in an Abderhalden drying pistol with P_2O_5 under reduced pressure at 77 or 40° depending on the melting point of a particular sample. For paper chromatography the descending method on Whatman No. 1 filter paper was used. The solvents employed were 1-butanol–acetic acid–water (BAW) in a ratio of 4:1:1, 1-butanol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24, 2-butanol–10% aqueous ammonia (SBA) in a ratio of 85:15, and 1-butanol saturated with 0.1% aqueous ammonia (nBA), using the lower phase for saturation of the atmosphere in the tank and the upper phase for the development of chromatograms. Thin layer chromatography was carried out according to the procedure of Stahl.[13] The plates were prepared by mixing 30 g of silica gel G with 100 mg each of luminescent zinc cadmium sulfide and zinc orthosilicate (Du Pont) in 65 ml of water for 30 sec at high speed in a Waring Blendor, and pouring the resulting suspension as a uniform layer (250 μ thick) on glass plates (20 × 5 cm) by means of an adjustable applicator (Desaga/Brinkmann Instrument Co., Inc., N. Y.). After 1 hr at room temperature the plates were kept at 100° for 1 hr and stored over anhydrous $CaSO_4$ ("Drierite"). In addition to the BAW, BPAW, and SBA solvent systems used in paper chromatography a system consisting of chloroform–methanol mixed in a ratio of 8:2 (CM), was also employed for development of thin layer plates; thin layer chromatograms were revealed by means of the ninhydrin reagent and the chlorine procedure,[14] and also by means of ultraviolet fluorescence quenching on thin layer plates. Hydrogenolytic operations were performed in the presence of an excess of Pd catalyst prepared freshly[15] from $PdCl_2$. A vibro-mixer (Model E1, A. G. Fuer Chemie Apparatebau, Zurich) was employed for mixing and the progress of the reaction was followed by testing for CO_2 in the outlet gas stream. The toluene system (chloroform–toluene–methanol–water mixed in a ratio of 5:5:8:2), was employed for countercurrent distribution and the distribution patterns were determined from the dry weight of material present in aliquots taken at regular intervals over the length of the distribution train.

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(16) During a repetition of this synthesis, the CM thin layer system revealed the presence of a trace of slower moving, ninhydrin-negative, chlorine-positive material. This contaminant could be separated from the material in the main peak (K = 0.82) during a 100-transfer countercurrent distribution in the toluene system.

(17) During preliminary experiments it was observed that, when chromatographed immediately after the completion of hydrogenolysis, the dipeptide free base behaved as a single ninhydrin-positive component in paper and thin layer systems. When the solution obtained after hydrogenolysis was stored at 0°, however, the appearance of 2 minor, faster moving ninhydrin-positive spots was noted after thin layer chromatography in the CM system and in particular in the SBA system, whereas the appearance of faster moving ninhydrin-negative material became evident in paper chromatography in the BAW and BPAW systems. The identity of these materials is not known at present.

(18) Extraction with saturated NaCl solution reduced the solubility of the peptide in the organic phase, leading to some precipitation of the product. This could be avoided by the addition of a few milliliters of methanol after extracting with the saturated NaCl solution.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (V).—Peptide IV (3.52 g, 3.97 mmol) was dissolved in absolute methanol (130 ml) and hydrogenolyzed for 4.5 hr in the presence of Pd catalyst prepared freshly from 1.5 of $PdCl_2$. The hydrogenolysis mixture was subjected immediately to thin layer chromatography, which revealed the presence of a single ninhydrin-positive spot in three solvent systems (R_f BAW 0.23, R_f SBA 0.22, R_f CM (8:2) 0.14). Paper chromatography in three solvents also revealed the presence of a single component, (R_f BAW 0.67, R_f SBA 0.70, R_f BPAW 0.87). As soon as completion of the hydrogenolysis reaction was verified by thin layer chromatography in the CM (8:2) system, the catalyst was removed by filtration and the solution was evaporated to dryness *in vacuo*. The product was dissolved directly in a mixture of dimethylformamide (2 ml) and acetonitrile (14 ml), and, while stirring at room temperature, N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[10] (2.2 g, 4.38 mmol) was added. After 48 hr at room temperature, the reaction mixture was precipitated from anhydrous ether, and the precipitate (3.76 g) subjected to a 140 transfer countercurrent distribution in the toluene system. The material recovered from the peak (K = 0.69) was dissolved in 150 ml of chloroform, and the solution extracted successively with 50-ml portions of first a 10% citric acid solution followed by water and then with a 5% $NaHCO_3$ solution until the final aqueous phase was colorless (four extractions). After the solution was extracted once with water and then with saturated NaCl solution, the organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The glassy product (3.37 g) was dissolved in methanol and precipitated from anhydrous ether (700 ml). A solution of the precipitate in 200 ml of moist ethyl acetate was extracted once more with 50 ml of 5% $NaHCO_3$ solution and then thrice with 100-ml portions of water. The organic phase was evaporated *in vacuo* and yielded after drying over P_2O_5 3.06 g (69%) of a colorless glassy product: mp 82–85°, $[\alpha]^{23}D$ −11.2° (*c* 2, methanol), homogeneous in paper chromatography in two solvents (R_f BAW 0.84, R_f BPAW 0.86) as well as in thin layer chromatography in three solvent systems [R_f BAW 0.68, R_f SBA 0.65, R_f CM (8:2) 0.61].

Anal. Calcd for $C_{51}H_{75}O_{13}N_{11}S_2$ (1114.3): C, 55.00; H, 6.79; N, 13.84; S, 5.76. Found: C, 54.83; H, 6.87; N, 13.85; S, 5.57.

N^{ϵ}-*t*-Butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (VI).—Protected tetrapeptide (V) (2.26 g, 2.03 mmol) was dissolved in 100 ml of absolute methanol and hydrogenolyzed catalytically for 4 hr in the presence of Pd freshly prepared from 1 g of $PdCl_2$. The catalyst was removed by filtration and the solvent was evaporated *in vacuo* to yield 1.925 g (96%) of compound VI in the form of a glassy product which was homogeneous in paper chromatography in two solvents (R_f BAW 0.77, R_f BPAW, 0.89) as well as in three thin layer systems [R_f BAW 0.41, R_f BPAW 0.66, R_f CM (8:2) 0.25].

The product, after countercurrent distribution for 101 transfers in the toluene system, was distributed as a single peak (K = 2.06), $[\alpha]^{25}D$ −5.02° (*c* 1.8, methanol).

Anal. Calcd for $C_{43}H_{69}O_{11}N_{11}S_2$ (980.2): C, 52.67; H, 7.10; N, 15.72. Found: C, 52.40; H, 7.10; N, 15.60.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (VII).—To a solution of 1.47 g (1.5 mmol) of tetrapeptide free base VI in 7 ml of acetonitrile, was added 0.83 g (1.65 mmol) of N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester.[10] The mixture was stirred until a clear solution was obtained and was then allowed to stand at room temperature for 72 hr. The entire reaction mixture was precipitated from anhydrous ether (500 ml) and the yellowish precipitate (1.84 g) was subjected to countercurrent distribution in the toluene system for 150 transfers. The material recovered from the peak (K = 0.56) was dissolved in 15 ml of methanol and precipitated from anhydrous ether (600 ml) to give 1.53 g (76%) of peptide VII in the form of a white amorphous powder, mp 83–88°, $[\alpha]^{26}D$ −14.3° (*c* 1.5, methanol). The product behaved as a single component in three paper chromatographic systems (R_f BAW 0.90, R_f SBA 0.88, R_f BPAW 0.92) and was homogeneous in thin layer chromatography in four solvent systems [R_f BAW 0.72, R_f SBA 0.66, R_f BPAW 0.69, R_f CM (8:2) 0.64].

Anal. Calcd for $C_{62}H_{95}O_{16}N_{13}S_2$ (1342.6): C, 55.46; H, 7.13; N, 13.56; S, 4.77. Found: C, 55.21; H, 7.20; N, 13.51; S, 4.66.

N^{ϵ}-*t*-Butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (VIII).—A solution of 0.8 g (0.6 mmol) of the fully protected pentapeptide (VII) in 40 ml of methanol was hydrogenolyzed for 4 hr in the presence of a Pd catalyst prepared from 0.5 g of $PdCl_2$. After the suspension was kept at 0° overnight, the catalyst was removed by filtration, the solution concentrated to a small volume *in vacuo* at room temperature and the product precipitated from anhydrous ether to give 0.65 g (90%) of the free base VIII as an amorphous powder: mp 95–100°; $[\alpha]^{23}D$ −9.6° (*c* 1, methanol). Peptide VIII behaved as a single component in paper chromatography in two solvents (R_f BAW 0.87, R_f SBA 0.93) as well as in thin layer chromatography in three solvent systems [R_f BAW 0.53, R_f SBA 0.45, R_f CM (8:2) 0.34].

Anal. Calcd for $C_{54}H_{89}O_{14}N_{13}S_2$ (1208.5): C, 53.70; H, 7.42; N, 15.05. Found: C, 53.36; H, 7.47; N, 14.89.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylpropylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginyl-δ-aminovaleric Acid Methyl Ester (IX).—N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylpropylvalylglycine[11] (0.323 g, 0.508 mmol) was dissolved in 6 ml of acetonitrile with slight warming. While the solution was cooled at 0°, 0.072 ml (0.51 mmol) of triethylamine followed by 0.143 g (0.56 mmol) of Woodward reagent K[9] was added and the mixture was kept stirring at 0° for 1.5 hr. A solution of VIII (0.614 g, 0.508 mmol) in 10 ml of acetonitrile was added and stirring were continued at room temperature for 18 hr. Substantial amounts of crystalline material, which started to separate from the reaction mixture several minutes after the addition of VIII, were filtered off at the end of the reaction period after the mixture was left for a few hours at 0°. After the crystalline fraction was washed with some ice-cold acetonitrile, followed by ethyl ether, it was dried *in vacuo* over P_2O_5. The washing solvents and the mother liquor fraction were combined and evaporated to dryness and the resulting yellow syrup was dissolved in 70 ml of chloroform. The solution was extracted successively with 40-ml portions of water, a 10% citric acid solution, followed by water, and a saturated NaCl solution. The organic phase was dried over anhydrous Na_2SO_4 and yielded 0.174 g of material after removal of the solvent. The latter fraction was contaminated with three minor slower moving chlorine-positive components as revealed by thin layer chromatography in the CM system, and was purified by means of countercurrent distribution for 100 transfers in the toluene system. The material recovered after countercurrent distribution was combined with the crystalline fraction (0.7 g) originally obtained and subjected to another 100 transfer distribution in the toluene system. A single peak (K = 0.34) which closely approached the theoretical distribution pattern, was obtained and yielded 0.83 g of material which was dissolved in a small volume of methanol (1–2 ml) and became crystalline upon cooling and scratching. After some cold ethyl acetate was added, the crystalline product was filtered off and dried to give 0.82 g (83%) of peptide IX, mp 152–154°, $[\alpha]^{26}D$ −26.9° (*c* 1, methanol). The product was homogeneous in thin layer chromatography in four solvent systems [R_f BAW 0.67, R_f BPAW 0.73, R_f SBA 0.61, R_f CM (8:2) 0.67].

Anal. Calcd for $C_{85}H_{134}O_{22}N_{18}S_2$ (1824.2): C, 55.97; H, 7.40; N, 13.82; S, 3.51. Found: C, 55.62; H, 7.69; N, 14.08; S, 3.66.

Registry No.—II, 15764-82-6; III, 15764-83-7; IV, 15889-52-8; V, 15764-99-5; VI, 15764-84-8; VII, 15815-82-4; VIII, 15764-85-9; IX, 15889-53-9.

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