[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE]

The Synthesis of L-Leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-arginine, an Insulin Fragment with Strepogenin Activity[1]

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RECEIVED JULY 22, 1959

The disulfide of L-leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-arginine was synthesized and shown to be identical with the same peptide previously isolated from enzymic digests of insulin. This peptide had high strepogenin activity. Carbobenzoxy-L-leucyl-L-valine was coupled with S-benzyl-L-cysteinylglycine ethyl ester by the dicyclohexylcarbodiimide method, and the resulting ester was converted into the hydrazide and azide. The latter was allowed to react with γ-ethyl-L-glutamyl-L-arginine methyl ester hydrochloride. The protecting groups were removed by saponification and treatment with sodium in liquid ammonia and the free peptide purified by chromatography on Dowex-50 and Dowex-2. L-Leucyl-L-valyl-L-cysteinylglycyl-L-glutamic acid and L-cysteinylglycyl-L-leucyl-L-valyl-L-glutamic acid were also prepared by several routes and shown to be less potent in the strepogenin assay than the hexapeptide. A Curtius rearrangement to yield a substituted urea rather than the expected peptide was observed in the reaction between carbobenzoxy-L-leucyl-L-valine azide and S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester.

The isolation of leucylvalylcysteinylglycylglutamylarginine from insulin digested with pepsin, trypsin and chymotrypsin was recently reported.[2] This peptide possessed considerable strepogenin activity[3] (200 units per mg.). To confirm the structure and to obtain greater amounts, the chemical synthesis of this peptide was undertaken. This has been completed and a comparison of the synthetic with the natural peptide has revealed no chemical or biological difference between the two. Both promoted the growth of *Lactobacillus casei* in the strepogenin assay to the same extent, *i.e.*, 200 units per mg. ±5%, which is the experimental error of the assay. The proof of structure of the isolated material has thus been completed. It was assumed that the peptide occurs in nature with all amino acids in the L-configuration. Hence, only L-amino acids were used in the synthesis and methods of coupling were chosen which are known to lead to minimal racemization.

The reactions used for the synthesis are outlined below. Carbobenzoxy-S-benzyl-L-cysteinylglycine ethyl ester was treated with hydrogen bromide in acetic acid to remove the carbobenzoxy group and the free amine (I) coupled to carbobenzoxy-L-leucyl-L-valine by the dicyclohexylcarbodiimide method. The resulting tetrapeptide ester (II) was converted into the hydrazide (III) and azide (IV). Carbobenzoxy-γ-ethyl-L-glutamate was condensed with L-nitroarginine methyl ester by the mixed anhydride method to form carbobenzoxy-γ-ethyl-L-glutamyl-L-nitroarginine methyl ester (V), and the latter was hydrogenated in the presence of an excess of hydrogen chloride to give the dipeptide dihydrochloride (VI). The latter was treated with one mole triethylamine to give the monohydrochloride (VII), which was allowed to react with the tetrapeptide azide (IV). Since the guanidine group of arginine is a stronger base by several orders of magnitude than the α-amino group,[4] the guanidine grouping should remain as the hydrochloride. This technique of masking the guanidine group of arginine as the hydrochloride in an azide condensation was used by Katsoyannis, *et al.*, in their synthesis of peptides in the arginine vasopressin series.[5] The resulting protected hexapeptide hydrochloride (VIII) was purified by countercurrent distribution and freed from protecting groups by saponification and sodium in liquid ammonia reduction. Pure peptide (IX) was obtained by chromatography on Dowex-50 and Dowex-2 under conditions identical to the ones employed in the isolation of the peptide from digests of insulin. Weighable quantities of pure material were thus obtained.

The hexapeptide (IX) synthesized in the present work represented the largest piece of the insulin molecule to be made synthetically thus far. The material finally obtained was the symmetrical disulfide which was a dodecapeptide. In the accepted structure of insulin,[6] the symmetrical disulfide does not appear, but rather one sees the hexapeptide sequence attached to a dissimilar peptide by a disulfide bond. The sulfhydryl containing hexapeptide, and not the symmetrical disulfide, was therefore the piece of insulin that has been synthesized.

The initial plan for the synthesis of the hexapeptide called for the use of protecting groups which could be removed with sodium in liquid ammonia[7] thus avoiding the difficulties involved in removing by saponification a γ-ester of glutamic acid. Carbobenzoxy-L-glutamic acid-γ-benzyl ester was coupled to L-nitroarginine benzyl ester with dicyclohexylcarbodiimide. The carbobenzoxy group was removed with hydrogen bromide in acetic acid and the free amine mixed with the tetrapeptide azide. This fully protected hexapeptide was more easily purified than the one described above, but it was not possible to remove satisfactorily the nitro-group from the arginine residue by use of sodium in liquid ammonia. Although catalytic hydrogenation has been reported as usable with cystine containing peptides,[8] this method failed here. Some removal of the nitro group was achieved with tin in

(1) This investigation was supported in part by a grant from the U. S. Public Health Service.

(2) G. L. Tritsch and D. W. Woolley, THIS JOURNAL, **80**, 1490 (1958).

(3) H. Sprince and D. W. Woolley, *J. Expt. Med.*, **80**, 213 (1944).

(4) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, p. 465.

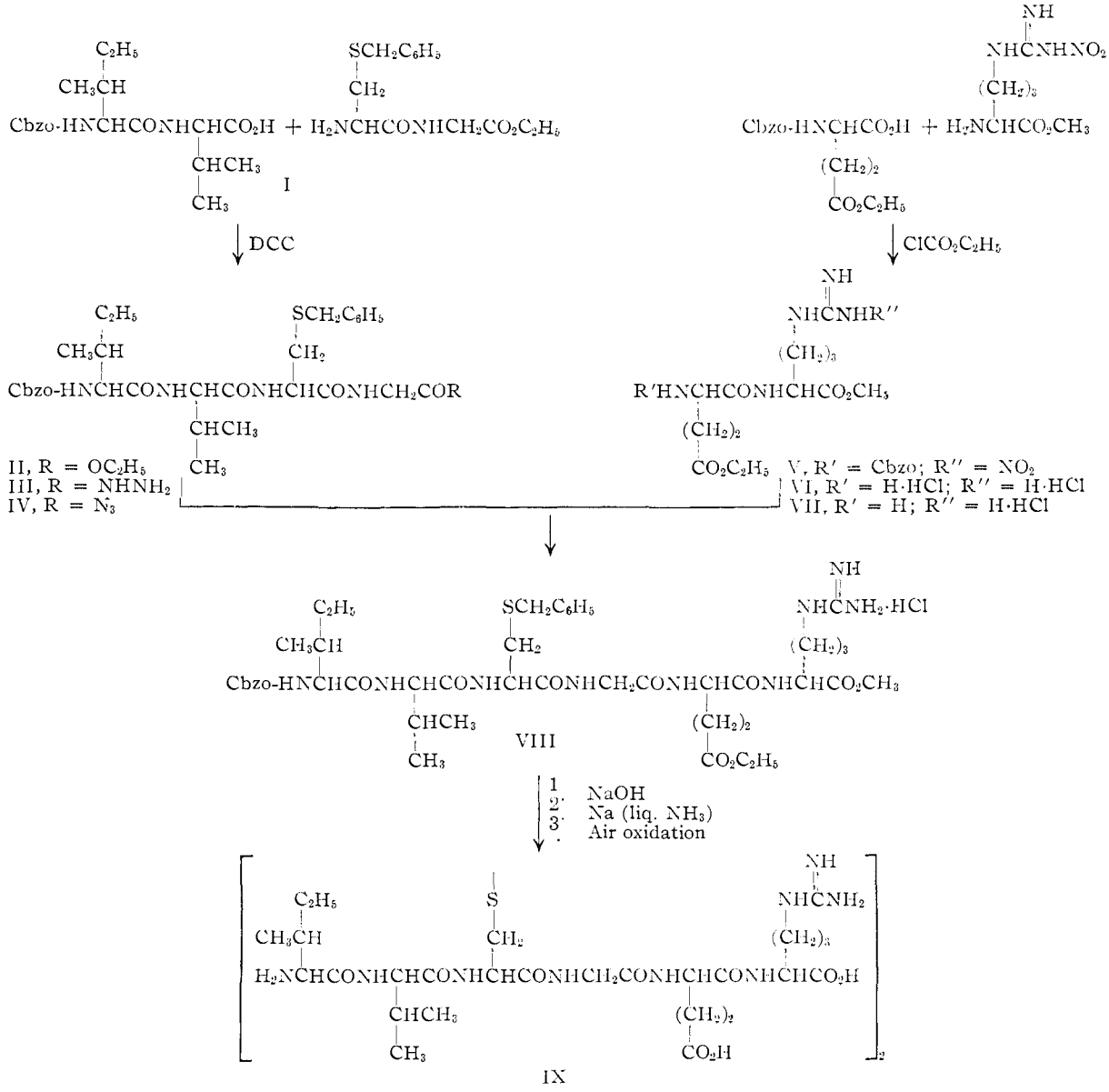
(5) P. G. Katsoyannis, D. T. Gish, G. P. Hess and V. du Vigneaud, THIS JOURNAL, **80**, 2558 (1958).

(6) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

(7) R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, **36**, 875 (1953).

(8) L. Zervas and D. M. Theodoropoulos, THIS JOURNAL, **78**, 1359 (1956).

Cbzo = $C_6H_5CH_2OCO$–
DCC = Dicyclohexylcarbodiimide



hydrochloric acid but the yields of desired peptide and of strepogenin activity were so low that this approach had to be abandoned.

Since a peptide consisting of a sequence of the first five amino acids of the peptide in question would be similar to the pentapeptides with strepogenin activity synthesized by Merrifield and Woolley,[9,10] the synthesis of leucylvalylcysteinylglycylglutamic acid was attempted. This was accomplished by three different methods, but the resulting activity seemed rather low, *i.e.*, 10–15 units/mg. Because the intermediates were available, cysteinylglycylleucylvalylglutamic acid was also prepared, and, in keeping with the predictions of Merrifield and Woolley,[10] this peptide with cysteine N-terminal had very low activity, *i.e.*, 1 unit/mg.

(9) R. B. Merrifield and D. W. Woolley, THIS JOURNAL, **78**, 4646 (1956).
(10) R. B. Merrifield and D. W. Woolley, *ibid.*, **80**, 6635 (1958).

The reaction sequences used for the pentapeptide syntheses were as follows: Carbobenzoxy-S-benzyl-L-cysteinylglycine hydrazide was converted into the azide and coupled with L-glutamic acid dibenzyl ester to give carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester. The carbobenzoxy group was removed with hydrogen bromide in acetic acid and the resulting tripeptide coupled with carbobenzoxy-L-leucyl-L-valine by the mixed anhydride or dicyclohexylcarbodiimide procedure to give the desired pentapeptide. This was also accomplished by coupling carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine azide with L-glutamic acid dibenzyl ester. However, treatment of carbobenzoxy-L-leucyl-L-valine azide with S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester always resulted in a Curtius rearrangement to give the substituted urea in spite of the fact that the temperature was rigorously kept at 0°. The product of this reaction had

an appreciably higher m.p. than the protected pentapeptide obtained from the other reactions and a markedly different optical rotation. Acid hydrolyzates of this rearrangement product did not contain any valine, and elementary analysis agreed well with the postulated structure. Furthermore, removal of its protecting groups with sodium in liquid ammonia gave rise to an activity of only 1 unit/mg. whereas the authentic pentapeptide made by the successful routes and similarly reduced always possessed 10–15 units/mg. The desired pentapeptide from each of the three successful routes had the same optical rotation. This suggested that racemization had probably not occurred extensively in any of the methods.

Experimental

General Comments.—Optical rotations were measured in 1 dm. tubes in a Rudolph photoelectric spectropolarimeter. Microanalyses were carried out by Mr. T. Bella; nitrogen analyses were by the Dumas procedure. The following buffers were used for paper electrophoresis: *p*H 6 buffer contained 25 ml. of pyridine and 2.5 ml. of glacial acetic acid, in 4 l., for *p*H 3, 0.1 *M* acetic acid was used, and for *p*H 4.0, for the separation of the various esters of glutamic acid, 0.1 *M* acetic acid containing 5 ml. of pyridine per 3 l. was used. Mobilities indicated by (−) represent migration to the cathode, (+) to the anode. All m.p. were determined in capillaries, or, where stated, on a hot stage microscope with rotatable polaroids. All compounds were recrystallized to constant m.p. (at least twice) from the solvents indicated. Paper chromatography was performed by the ascending method with Whatman No. 1 paper, and unless otherwise stated, the solvent system was sec. butanol–formic acid–water (100:20:13.3). Spots were visualized with ninhydrin, and arginine was located by Sakaguchi's reagents[11] streaked, not sprayed, on the paper. Whenever petroleum ether was used, it was material boiling between 30 and 60°. The reductions with sodium in liquid ammonia were performed essentially as described by du Vigneaud, *et al.*,[12] with careful attention to the time of persistence of the blue color before neutralization with ammonium chloride.

S-Benzyl-L-cysteinylglycine Ethyl Ester Hydrobromide.—Carbobenzoxy-S-benzyl-L-cysteinylglycine ethyl ester[13] (11.0 g., 25.5 mmoles) was dissolved in 60 ml. of glacial acetic acid in a flask protected from moisture by a calcium chloride tube, 20 ml. of 30% hydrogen bromide in acetic acid was added and the mixture allowed to stand at room temperature for half an hour with occasional swirling. The dipeptide hydrobromide was precipitated by pouring the mixture into 600 ml. of anhydrous ether. The resulting hydroscopic oil was dissolved in chloroform and reprecipitated with ether to give an 84% yield of the hydrobromide (8.08 g.). From the mother liquors 240 mg. of starting material was recovered.

Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine Ethyl Ester (II).—The foregoing ester hydrobromide was dissolved in 50 ml. of methylene chloride and 3.02 ml. triethylamine was added. The precipitated triethylamine hydrobromide (1.14 g.) was filtered off and 7.84 g. (21.6 mmoles) of carbobenzoxy-L-leucyl-L-valine[9] in 60 ml. of methylene chloride was added. The clear solution was cooled to 0° and 4.50 g. (21.8 mmoles) of dicyclohexylcarbodiimide in 15 ml. of methylene chloride was added. Precipitation was virtually complete in 5 minutes. After standing at room temperature for 24 hr., 1 drop of glacial acetic acid was added to decompose any unreacted diimide, and, after half an hour, 4.72 g. of dicyclohexylurea (21.0 mmoles) was filtered off. The methylene chloride solution was cooled and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate, and water, and dried with magnesium sulfate. Concentration *in vacuo* to a small volume, followed by addition of petr. ether gave a 66% yield (9.1 g.) of tetrapeptide ester, the melting point of which was unchanged by additional recrystallization from the same solvents. On a hot stage microscope it became birefringent at 170° and melted without decomposition at 208°, $[\alpha]^{23}D$ −26.0° (*c*, 1.74 in methylene chloride).

Anal. Calcd. for $C_{33}H_{46}N_4O_7S$ (mol. wt. = 642.80): C, 61.66; H, 7.21; N, 8.72. Found: C, 61.69; H, 7.11; N, 8.56.

Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine Hydrazide (III).—Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine ethyl ester (8.72 g., 13.6 mmoles) was dissolved in 120 ml. of absolute ethanol, 20 ml. of 100% hydrazine hydrate added and refluxed 5 hr. on the steam-bath. The solution was cooled to room temperature and water added until turbid. After standing in the cold, the hydrazide was filtered off, washed with water and dried. The dry powder was dissolved in a 1:1 mixture of ethyl acetate and chloroform and precipitated with petroleum ether to give a 90% yield (7.72 g.) of the desired hydrazide, m.p. 202° without decomposition on a microscope equipped with a hot stage. At 160° the powder became birefringent. The m.p. was unchanged by further recrystallization from these solvents, $[\alpha]^{23}D$ −15.6° (*c*, 0.63, in 1:1 ethanol–ethyl acetate).

Anal. Calcd. for $C_{31}H_{44}N_6O_6S$ (mol. wt., 628.79): C, 59.22; H, 7.05; N, 13.37. Found: C, 59.39; H, 7.46; N, 13.47.

L-Nitroarginine Benzyl Ester Hydrochloride.—L-nitroarginine[14] (10 g., 45 mmoles) was suspended in 200 ml. of benzyl alcohol which was heated, stirred and saturated with dry hydrogen chloride. The addition of dry hydrogen chloride was stopped, about 10 ml. of benzene was added and water was removed by azeotropic distillation under reduced pressure with the aid of a Dean–Stark trap. Hydrogen chloride again was passed through the warm solution for 1 hr. The solvents were then removed by high vacuum distillation. The residue was dissolved in cold methanol and quickly precipitated with anhydrous ether to yield 11.6 g. of strongly birefringent crystals, m.p. 128° with decomposition and loss of birefringence on a hot stage microscope.

Anal. Calcd. for $C_{13}H_{20}N_5O_4Cl$ (mol. wt., 345.79): C, 45.3; H, 5.82. Found: C, 45.7; H, 6.19.

On paper electrophoresis at *p*H 6, unesterified nitroarginine could be detected. This small amount of impurity was permissible because it was removed when the protected dipeptide made from the ester was washed with bicarbonate as described below. The material was hydroscopic and tended to decompose to yield nitroarginine on storage.

Preparation and Purity of the Mono-esters of Glutamic Acid.—In order to prepare suitably protected glutamylarginine, carbonbenzoxy-L-glutamic acid with esterified γ-carboxyl was needed, and it was necessary that any α-monoester be avoided. Peptides involving the γ- rather than the α-carboxyl of glutamic acid in peptide linkage are known to differ in biological activity.[15,16] An unambiguous synthesis of glutamic acid γ-benzyl ester in which the α-carboxyl was shielded as the copper salt was carried out as recorded in the literature.[17] The α-benzyl ester was prepared by treating the dibenzyl ester with hydrogen iodide.[18] After γ-ethyl-L-glutamate was prepared as described in the literature[19] by a brief treatment of glutamic acid in ethanol with anhydrous hydrogen chloride, it was desirable to assess its purity, *i.e.*, the relative amount of α-carboxyl esterified. Since the *pK* of the reaction COOH → COO^- of γ-ethylglutamate is 2.15, and of α-ethyl glutamate 3.85,[20] electrophoresis at *p*H 3 seemed the obvious condition for the separation of these materials. This was found to be unsatisfactory. However, it was found empirically that electrophoresis at *p*H 4.0 separated the two esters from each other and from free glutamic acid and the diester. Such electrophoretic analysis of the γ-ethyl-L-glutamate preparation (800 v., 2 hr., 25°) revealed about 5% glutamic acid (−2 cm.), 20% glutamic

(11) C. J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).

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(17) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

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(19) B. Hegedüs, *Helv. Chim. Acta*, **31**, 737 (1948).

(20) A. Neuberger, *Biochem. J.*, **30**, 2085 (1936).

acid diethyl ester (−24 cm.) and a trace (less than 5%) of glutamic acid-α-ethyl ester (−17 cm.) in addition to the desired glutamic acid-γ-ethyl ester (−9 cm.). Under the above conditions, the various benzyl esters had the same mobilities as the corresponding ethyl esters.

The mixture of glutamic acid ethyl esters was carbobenzoxylated.[18] During this process, carbobenzoxy-glutamic acid diethyl ester was removed by ether extraction from alkaline solution, and carbobenzoxy-glutamic acid was removed when the protected dipeptide made from it was washed with bicarbonate as described below. The small amount of α-ester was a slight, and the only, contaminant.

Carbobenzoxy-γ-benzyl-L-glutamyl-L-nitroarginine Benzyl Ester.—To 4.5 g. (13 mmoles) of L-nitroarginine benzyl ester hydrochloride in 40 ml. of pyridine at 0° was added 1.3 ml. cold 10 *N* sodium hydroxide and the mixture shaken vigorously and immediately treated with 20 g. potassium carbonate and filtered without delay into a precooled flask containing magnesium sulfate. The drying agent was removed and 4.8 g. carbobenzoxy-L-glutamic acid γ-benzyl ester in 30 ml. pyridine was added and the mixture cooled. Dicyclohexylcarbodiimide (2.68 g.) in 15 ml. of pyridine was added and the mixture allowed to stand at room temperature overnight and filtered. The solvent was removed completely *in vacuo*, and the residue dissolved in benzene which was washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate, and water, and dried with magnesium sulfate. Filtration followed by concentration to a small volume and addition of petr. ether yielded the dipeptide, which was recrystallized from ethanol–water, 4.8 g. (56%), m.p. 87–88°, unchanged by further recrystallization; $[\alpha]^{25}D$ 8.16° (*c*, 0.613 in chloroform).

Anal. Calcd. for $C_{33}H_{38}N_6O_9$ (mol. wt. = 662.68): C, 59.81; H, 5.78; N, 12.68. Found: C, 59.57; H, 5.90; N, 12.83.

Carbobenzoxy-γ-ethyl-L-glutamyl-L-nitroarginine Methyl Ester (V).—L-Nitroarginine methyl ester hydrochloride, m.p. 155°, was prepared according to van Orden and Smith.[21] Paper electrophoresis at *p*H 6 revealed about 5% unesterified nitroarginine. The procedure of Hofmann, *et al.*,[14] resulted in a larger proportion of unesterified nitroarginine. The carbobenzoxy-L-glutamic acid γ-ethyl ester prepared as described in a previous section (5.90 g., 19 mmoles) in 80 ml. of chloroform was cooled to 0° and 2.68 ml. of triethylamine added. To the clear solution, 2.28 ml. of ethylchlorocarbonate was added and stirred for 10 minutes. A slurry of 7.8 g. (29 mmoles) of nitro-L-arginine methyl ester hydrochloride in 80 ml. of chloroform containing 4 ml. of triethylamine was cooled to 0° and added with vigorous stirring to the mixed anhydride. The mixture was stirred for 18 hr. at 4°, and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate, and water, and dried with magnesium sulfate. Evaporation *in vacuo* to a small volume, followed by addition of anhydrous ether and recrystallization from ethyl acetate–petr. ether gave 8.50 g. (86% based on carbobenzoxy-L-glutamic acid γ-ethyl ester) of amorphous carbobenzoxy-γ-ethyl-L-glutamyl-L-nitroarginine methyl ester, m.p. 58° (unchanged by further recrystallization), $[\alpha]^{22}D$ −16.5° (*c*, 1.8 in chloroform).

Anal. Calcd. for $C_{22}H_{32}N_6O_9$ (mol. wt. = 524.53): C, 50.37; H, 6.15; N, 16.02. Found: C, 50.07; H, 6.32; N, 16.14.

The dicyclohexylcarbodiimide reagent, which worked so well for the preparation of the protected dipeptide dibenzyl ester, did not work at all here.

γ-Ethyl-L-glutamyl-L-arginine Methyl Ester Dihydrochloride (VI).—Carbobenzoxy-γ-ethyl-L-glutamyl-L-nitroarginine methyl ester (8.50 g., 16 mmoles) was dissolved in 250 ml. of anhydrous methanol and 27 ml. of 1.5 *M* anhydrous hydrogen chloride in methanol added. A slurry of 2.0 g. 5% Pd on charcoal in a little methanol was added and the mixture hydrogenated at 4 atm. of hydrogen for 18 hr. The catalyst was filtered off. Sakaguchi analysis of an aliquot of the filtrate indicated that 100% of the guanidine group of arginine had been liberated. The alcoholic solution was concentrated *in vacuo* and anhydrous ether added to precipitate an hygroscopic powder, which was stored over phosphorus pentoxide and used within one to two days for the next condensation. Paper electrophoresis (*p*H 6, 600 v., 25°, 1.5 hr.) showed a strong spot at −12 cm. and a weak spot at −7 cm. The weak spot was probably carbobenzoxy-γ-ethyl-L-glutamyl-L-arginine methyl ester because it reacted with Sakaguchi's reagents but gave no ninhydrin reaction. The main spot gave a positive reaction in both these tests. Analysis of this hygroscopic, impure material showed C, 40.68; H, 6.67; N, 17.47. (Calcd. for $C_{14}H_{29}N_5O_5Cl_2$, mol. wt. 418.32; C, 40.10; H, 6.99; N, 16.80.) The impurity did not couple with the azide in the next step and was subsequently removed in the purification of the protected hexapeptide.

Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-γ-ethyl-L-glutamyl-L-arginine Methyl Ester Hydrochloride (VIII).—All reactions were carried out at 4° or below. Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine hydrazide (III) (4.3 g. 6.9 mmoles) was dissolved in 260 ml. of glacial acetic acid and 65 ml. of 2 *N* hydrochloric acid and cooled to −2°. The clear solution was stirred and 520 mg. (10% excess) of sodum nitrite in 20 ml. of water added as fast drops. The mixture was stirred for 10 minutes at −2° and then 1.3 l. of ice water was added slowly. The precipitated azide (IV) was filtered on a large Büchner funnel and washed with ice water until the washings were at about *p*H 5. The precipitate was then washed with cold 1 *M* sodium bicarbonate and again with ice water until the washings were neutral. The azide (m.p. 82°) was dried briefly in a precooled desiccator over potassium hydroxide and dissolved in 260 ml. of cold N,N-dimethylformamide and added to a solution of 4.20 g. (10.0 mmoles) of γ-ethyl-L-glutamyl-L-arginine methyl ester dihydrochloride in 65 ml. of cold N,N-dimethylformamide containing 1.06 ml. of triethylamine (7.55 moles). The reaction mixture was stirred at 4° overnight and 3 hr. at room temperature. The crude protected hexapeptide was precipitated by addition of ether until the solution was turbid, followed by 500 ml. of petr. ether. It was reprecipitated from methanol with ethyl acetate to yield 3.5 g. Chromatography in *n*-butanol–water–ethanol (4:4:2) revealed spots of R_f = 0.5, 0.6, and 1.0. The first two reacted with ninhydrin and Sakaguchi's reagents, the last only with Sakaguchi's reagents and was the desired compound.

For purification, the crude protected hexapeptide was subjected to countercurrent distribution in 1 g. batches, for ten transfers in a system consisting of *n*-butanol–0.16 *M* acetic acid, and ethanol (10:10:3), ten ml. each phase. The lead tubes formed stable emulsions which had to be centrifuged to separate the layers. On the basis of the Sakaguchi color reaction as shown in Fig. 1, two peaks were obtained with *K* of 0.25 and 4.0. Hydrolysis in 6 *N* hydrochloric acid and chromatography of some of the material in tubes 0 to 3 revealed only glutamic acid and arginine, while hydrolyzates of the contents of tubes 5 to 10 contained all six required amino acids.

During early experiments, the azide condensation yielding the protected hexapeptide was performed using equimolar amounts of tetrapeptide azide and dipeptide. Under these conditions, the unwanted peak with a *K* of 0.25 was about ten times greater than the desired peak with a *K* of 4.0. This low yield of hexapeptide was probably due to inhomogeneity of the dipeptide after hydrogenation. If a significant amount of carbobenzoxy–dipeptide had been present, the addition of one mole of triethylamine might have neutralized some guanidine hydrochloride and the basic guanidine grouping would interfere in the azide reaction. It was for this reason that only 0.75 mole of triethylamine was added to one mole of dipeptide dihydrochloride and only 0.7 mole of azide was used. In this manner some dipeptide was wasted, but the tetrapeptide, which was more difficult to prepare, was more efficiently utilized as shown in Fig. 1. It was difficult to estimate the amount of carbobenzoxy–dipeptide in the γ-ethyl-L-glutamyl-L-arginine methyl ester preparation.

The contents of tubes 5 to 10 were evaporated on the rotary evaporator[22] (40°) until the protected hexapeptide just began to precipitate. At this point, almost all of the alcohol had evaporated and the liquid foamed excessively. The remainder of the solvents was removed by lyophilization. Absence of the slow-moving material was shown by paper chromatography in *n*-butanol–water–ethanol (4:4:2) which revealed only material of R_f 1.0. The yield of purified pro-

(21) H. O. van Orden and E. L. Smith, *J. Biol. Chem.*, **208**, 751 (1954).

(22) L. C. Craig, J. C. Gregory and W. Hallsmann, *Anal. Chem.*, **22**, 462 (1950).

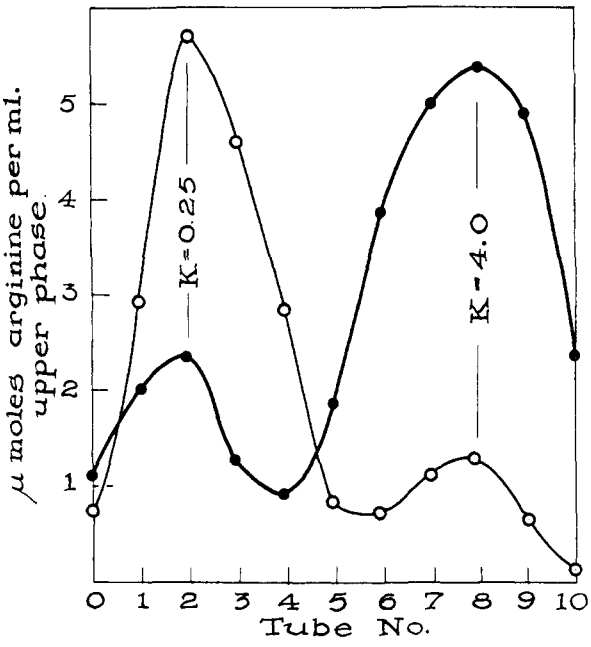

Fig. 1.—Countercurrent distribution of the protected hexapeptide (VIII). ●, product from 0.7 mole IV and 1 mole VII. O, product from 1 mole IV and 1 mole VII. (For details see text.)

tected hexapeptide from three such countercurrent distributions was 2.90 g. (2.96 mmoles), (43% yield based on tetrapeptide hydrazide) of amorphous powder, m.p. 128° on a hot stage microscope, $[\alpha]^{22}D$ −26.1° (*c*, 1.6 in methanol).

Anal. Calcd. for $C_{45}H_{68}N_9O_{11}SCl$ (mol. wt. = 978.59): C, 55.40; H, 7.02; N, 12.90; arginine by Sakaguchi, 16.0. Found: C, 55.40; H, 7.21; N, 12.53; arginine by Sakaguchi, 15.6.

Disulfide of L-leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-arginine (IX).—One gram carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-γ-ethyl-L-glutamyl-L-arginine methyl ester hydrochloride was dissolved in 20 ml. of methanol and 4.0 ml. of 1.0 *N* sodium hydroxide added. The solution was allowed to stand 1 hr. at room temperature and then adjusted to *p*H 6 with 1.5 ml. of 1 *N* hydrochloric acid. The alcohol was removed *in vacuo* and when foaming became excessive, the remaining solvent was removed by lyophilization. The dried residue was dissolved in about 100 ml. of dried and redistilled ammonia, and small pieces of sodium (about 200 mg. total) added to the boiling solution until the blue color persisted for 5 minutes. A few crystals of ammonium chloride were added and the ammonia allowed to evaporate. After drying over sulfuric acid, the residue was dissolved in 30 ml. of cold 0.1 *M* acetic acid and the *p*H adjusted to 6. Chromatography in 4:4:2 *n*-butanol:water:ethanol gave a spot of R_f 0.4 reacting with both ninhydrin and Sakaguchi's reagents (the desired peptide) and spots of R_f 0.5 and 0.7 reacting with Sakaguchi's reagents only. A total of 90,000 units of strepogenin activity were obtained from three such experiments. Pure peptide was obtained by ion exchange chromatography based on the scheme used for the isolation of naturally occurring peptide from insulin digests.[2] The crude material containing the 90,000 strepogenin units in 100 ml. of water at *p*H 4 was poured on a column of Dowex-50 × 4, 3 × 15 cm., which had been equilibrated with *p*H 4.9 sodium acetate buffer, 0.1 *M* in Na^+. The column was then washed with 2 l. of *p*H 4.9 buffer. After this, a 250 ml. mixing flask was attached by means of which gradient elution from *p*H 4.9 to *p*H 6.0 was carried out. The desired peptide emerged at *p*H 5.5. Another peak reacting with ninhydrin and Sakaguchi's reagents appeared at *p*H 6 but this material was practically inactive in the strepogenin test. Subsequently, the column was washed with 0.1 *N* NaOH to remove all remaining material. Figure 2 shows the elution pattern and Table I the recovery of material.

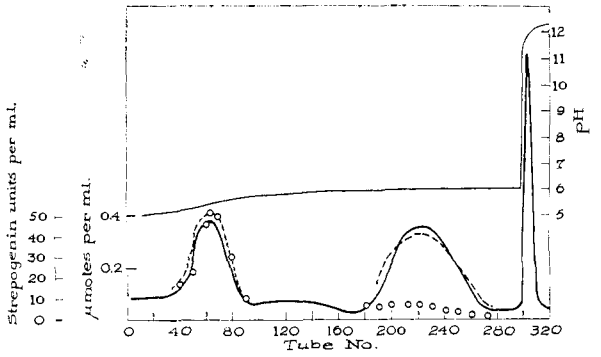

Fig. 2.—Fractionation of the disulfide of the hexapeptide by chromatography on Dowex-50. Ten ml. (0.5 hr.) fractions were collected. O, strepogenin activity; ———, Sakaguchi; – – – –, ninhydrin.

Much strepogenin activity appeared in the fractions emerging earlier and later than the desired hexapeptide (Table I). The more alkaline fraction (tubes 300–315) could have been the hexapeptide in which the glutamic acid residue was replaced by the γ-ethyl ester of glutamic acid or by glutamine. The relative difficulty of saponification of the γ ester of glutamic acid residues is well known.

The material eluting at *p*H 5.5 was desalted with Dowex-2 as described earlier,[2] the ammonium acetate removed by sublimation, and the residue precipitated from water with ethanol to yield 50 mg. (0.067 mmole), $[\alpha]^{23}D$ −21.7° (*c* 0.147 in water). It chromatographed on paper in 4:4:2 *n*-butanol:water:ethanol as a spot with an R_f of 0.4 and was thus indistinguishable from the naturally occurring peptide. It reacted with nitroprusside only when CN^- was added, and the red color developed fairly slowly, indicating that the peptide was the disulfide which was difficult to reduce. When dried for 2 hr. *in vacuo* at 100°, analysis showed 16.51% nitrogen and 20.1% arginine by Sakaguchi's reaction. Theory for the disulfide of the hexapeptide diacetate dihydrate ($C_{58}H_{108}N_{18}O_{24}S_2$ mol. wt. = 1505.70) is 16.74% nitrogen and 20.8% arginine. Hydrolysis in 6 *N* hydrochloric acid followed by paper chromatography showed approximately equal amounts of leucine, valine, cystine, glycine, glutamic acid and arginine. The relative amounts were judged in the usual way by comparison of the intensity of color (after ninhydrin) of spots from a series of dilutions of unknown with known amino acids run alongside.

TABLE I

RECOVERY OF PEPTIDES FROM DOWEX-50 CHROMATOGRAPHY

	Sakaguchi (mmoles)	Ninhydrin	Total units
Sample on column	3.09		90,000
Elution at *p*H 4.9	2.50	2.07	17,800
Tubes 40–90	0.12	0.12	16,000
Tubes 180–270	.26	.20	1,800
Tubes 300–315	.06	.33	5,300
	2.94	2.72	40,900

The identity of this synthetic peptide with the one isolated from insulin digests was indicated by (a) the same point of emergence on chromatography on Dowex-50, (b) the same R_f on paper chromatography in *n*-butanol–water–ethanol (4:4:2), (c) the same rate of migration in paper electrophoresis at *p*H 3, and (d) the same strepogenin activity (see below).

Carbobenzoxy-L-leucyl-L-valine Hydrazide.—Carbobenzoxy-L-leucine (1.02 g., 3.9 mmoles) in 5 ml. of methylene chloride was mixed with 0.7 g. L-valine methyl ester hydrochloride[23] and 0.60 ml. of triethylamine in 10 ml. of methylene chloride, and 0.9 g. of dicyclohexylcarbodiimide in 5 ml. methylene chloride was added. Precipitation of dicyclohexylurea was virtually complete in 15 minutes. After 4 hr. 0.8 g. (3.6 mmoles) of the urea was filtered off.

(23) E. L. Smith, D. H. Spackman and W. J. Polglase, *J. Biol. Chem.*, **199**, 801 (1952).

Addition of 1 drop of glacial acetic acid gave no further precipitate. The solution was cooled and extracted with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water, and dried with magnesium sulfate. After filtration, the solvent was removed *in vacuo*, and the residue dissolved in 2 ml. of absolute ethanol and 1.0 ml. (5 fold excess), of hydrazine hydrate added. Crystals formed at once. After standing at room temperature overnight, the precipitate was filtered off and recrystallized from ethyl acetate–petr. ether to give a 60% yield (based on carbobenzoxy-leucine) (0.87 g.) of carbobenzoxy-L-leucyl-L-valine hydrazide, m.p. 163° (unchanged by further recrystallization), $[\alpha]^{25}D$ −43.4° (c, 0.24 in ethanol). It gave a good silver mirror with ammoniacal $AgNO_3$.

Anal. Calcd. for $C_{19}H_{30}N_4O_4$ (mol. wt. = 378.46): C, 60.29; H, 7.99; N, 14.8. Found: C, 60.17; H, 7.94; N, 14.9.

Carbobenzoxy-L-leucyl-L-valyl methyl ester was obtained as an oil by Smith, *et al.*,[23] who used the azide rather than the diimide method.

Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-glutamic Acid Dibenzyl Ester.—L-Glutamic acid dibenzyl ester hydrochloride[18] (1.5 g., 4.1 mmoles) was suspended in 5 ml. of water and 25 ml. of ether at 0° in a separatory funnel and shaken with 0.6 ml. of triethylamine. The ether layer was separated and dried with magnesium sulfate without allowing the temperature to rise above 4°. Carbobenzoxy-S-benzyl-L-cysteinylglycine hydrazide[13] (1.07 g., 2.57 mmoles) was dissolved in 16 ml. of glacial acetic acid and 2 ml. of water and 1 ml. of 1 *N* hydrochloric acid and cooled to 2°. Fast drops of 2.14 ml. of 10% sodium nitrite were added. After stirring for 5 minutes the azide was taken up in 400 ml. of cold ether and the aqueous layer removed. The ether was washed with cold 1 *M* sodium bicarbonate and ice water and dried with magnesium sulfate all at 4°. The filtered solutions of azide and dibenzyl-L-glutamate were mixed and allowed to stand at 4° for 5 hr. and at room temperature overnight. The precipitate was filtered off and dissolved in ethyl acetate, cooled and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water and dried with magnesium sulfate. Filtration followed by concentration *in vacuo* and addition of petr. ether yielded the crude protected tripeptide. This was recrystallized from acetone–water to give 1.48 g., m.p. 75°, which was unchanged by additional recrystallizations from ethanol–water, acetone–water or ethyl acetate–petr. ether, $[\alpha]^{23}D$ −4.6 (*c*, 0.26 in ethanol), +28.1 (*c*, 0.313 in chloroform).

Anal. Calcd. for $C_{39}H_{41}N_3O_8S$ (mol. wt. 711.81); C, 65.8; H, 5.80; N, 5.90. Found: C, 65.7; H, 5.92; N, 5.79.

Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-L-glutamic Acid Dibenzyl Ester. A. Mixed Anhydride Method (Dipeptide + Tripeptide).—Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester (0.414 g.) in 2.1 ml. of acetic acid was treated with 0.7 ml. of 30% hydrogen bromide in acetic acid. After 1 hr. at room temperature the solution was poured into anhydrous ether and centrifuged. The residue was dissolved in 4 ml. of chloroform and the free amine liberated with 0.1 ml. of triethylamine. The mixed anhydride was prepared by treating 0.212 g. of carbobenzoxy-L-leucyl-L-valine in 2 ml. of chloroform and 0.1 ml. of triethylamine with 0.08 ml. of ethylchloroformate for 5 minutes at 0°. The free amine was then added, and the mixture stirred at room temperature 3 hr. and then cooled and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water and dried with magnesium sulfate. Filtration followed by concentration *in vacuo* and addition of petr. ether precipitated the desired product which was recrystallized from chloroform by addition of petr. ether. The yield was 60 mg. (12%) of protected pentapeptide, m.p. 129°, $[\alpha]^{25}D$ −31.2° (*c* 1.5 in chloroform).

Anal. Calcd. for $C_{50}H_{61}N_5O_{10}S$ (mol. wt., 924.09): C, 64.98; H, 6.65. Found: C, 65.08; H, 6.83.

B. Diimide Method. (Dipeptide + Tripeptide.)—Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester (0.570 g., 0.8 mmole) was decarbobenzoxylated with hydrogen bromide in acetic acid as in (A) above and the precipitated hydrobromide dissolved in 10 ml. of an equal mixture of methylene chloride and acetonitrile and treated with 0.12 ml. of triethylamine. The insoluble triethylamine hydrobromide was filtered off, and 0.290 g. of carbobenzoxy-L-leucyl-L-valine in 4 ml. of methylene chloride–acetonitrile added, followed by 0.18 g. of dicyclohexylcarbodiimide in 1 ml. of methylene chloride. This solution became turbid in 10 minutes. After 4 hr. the yellow solution was filtered. Addition of 1 drop of glacial acetic acid gave no further precipitate in 1 hr. The clear solution was cooled and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water, and dried, and worked up and recrystallized as in (A) above to yield 70 mg. (10%) of protected pentapeptide, m.p. 135°, $[\alpha]^{27}D$ −31.3° (*c* 1.5 in chloroform).

C. Azide Method. (Tetrapeptide + Amino Acid.)—The modified procedure[10] was used to minimize loss of amino acid ester and azide before the reaction occurred. Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine hydrazide (96 mg., 0.153 mmole) dissolved in 3 ml. of acetic acid and 1 ml. of 0.4 *N* hydrochloric acid was cooled to 0° and 0.11 ml. of 10% sodium nitrite added. After 5 minutes 70 mg. of L-glutamic acid dibenzyl ester hydrochloride in 10 ml. of chloroform and 0.2 ml. of water was added. Triethylamine (10 ml.) was mixed with 12 ml. of chloroform, cooled and was added dropwise with stirring and cooling until the *p*H was 8. The two layers were stirred at 4° overnight, separated and the organic layer washed and worked up as in (A) above. The recrystallized protected pentapeptide weighed 72 mg. (53%), m.p. 136°, $[\alpha]^{24}D$ −29.5° (*c*, 0.9 in chloroform).

Curtius Rearrangement in the Reaction of Carbobenzoxy-L-leucyl-L-valine Azide with S-Benzyl-L-cysteinylglycyl-L-glutamic Acid Dibenzyl Ester.—Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester (1.16 g., 1.63 mmoles) was decarbobenzoxylated with hydrogen bromide in acetic acid and precipitated with ether as above and the hydrobromide shaken with ice-cold 1 *M* sodium bicarbonate and ethyl acetate until it had dissolved and the bicarbonate layer extracted several times with ethyl acetate. The combined ethyl acetate solutions were washed with ice-water, dried with magnesium sulfate and the solvent replaced by ether and kept cold until ready to be added to the azide. Carbobenzoxy-L-leucyl-L-valine hydrazide (0.642 g.) in 30 ml. of 50% acetic acid and 2 ml. of 1 *N* hydrochloric acid was cooled to 0° and 1.23 ml. of 10% sodium nitrite added with stirring. The precipitated azide was dissolved in cold ether, the aqueous layer removed and the ether washed with cold 1 *M* sodium bicarbonate and water and dried with magnesium sulfate without letting the temperature rise above 4°. The azide and the free amine were filtered and mixed and allowed to stand at 4° 3 hr. and at room temperature overnight. The resulting precipitate was separated and dissolved in chloroform and washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water, and dried with magnesium sulfate. Filtration, concentration *in vacuo* to a small volume, addition of petr. ether and recrystallization of the precipitated product by addition of petr. ether to a saturated solution in chloroform–methanol (1:1) yielded 230 mg. (16%) of material, m.p. 202° (birefringent at 190°) on a hot stage microscope, $[\alpha]^{25}D$ 34.2° (*c*, 0.35 in chloroform). The rotation thus differed from that of the desired protected pentapeptide by 65°. The product was probably R–NH–C:O–NH–R′, where R = carbobenzoxy-L-leucyl—NH—CH(CH(CH$_3$)—CH$_3$)— and R′ = —CH($CH_2SCH_2C_6H_5$)—C:O-glycyl-L-glutamic acid dibenzyl ester.

Anal. Calcd. for $C_{59}H_{62}N_6O_{10}S$ (mol. wt. = 939.12): C, 63.94; H, 6.65; N, 8.95. Found: C, 63.96; H, 6.53; N, 8.97.

Hydrolysis in 6 *N* hydrochloric acid followed by chromatography revealed the presence only of leucine, S-benzylcysteine, glycine and glutamic acid. The absence of valine was consistent with the suggested structure.

This Curtius rearrangement was not avoided by use of the modified azide method as in (C) above.

Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-leucyl-L-valyl-L-glutamic Acid Dibenzyl Ester.—Carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid dibenzyl ester[10] (0.202 g., 0.3 mmole) in 1 ml. of acetic acid was treated with 0.33 ml. of 30% hydrogen bromide in acetic acid as above and converted into the free amine with 0.05 ml. of triethylamine in 0.5 ml. of water, dissolved in ether and dried. Carbobenzoxy-S-benzyl-L-cysteinylglycine hydrazide[13] was converted into the azide as above and filtered into the same flask as the amine. After standing 24 hr. at 4° and then at room tem-

perature, the precipitate was recrystallized from chloroform and petr. ether to yield 85 mg. (31%) of birefringent crystals of protected pentapeptide, m.p. 138° on a hot stage microscope (unchanged by additional recrystallization), $[\alpha]^{23}D$ 80.6° (*c*, 0.26 in methanol), $[\alpha]^{23}D$ 0° in chloroform.

Anal. Calcd. for $C_{50}H_{61}N_5O_{10}S$ (mol. wt., 924.09): C, 64.98; H, 6.65. Found: C, 64.63; H, 6.42.

Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-γ-benzyl-L-glutamyl-L-nitroarginine Benzyl Ester.—Carbobenzoxy-γ-benzyl-L-glutamyl-L-nitroarginine benzyl ester (1.01 g., 1.52 mmoles) in 10 ml. of acetic acid was treated with 6 ml. of 30% hydrogen bromide in acetic acid for half an hour at room temperature. A crystalline, but hygroscopic hydrobromide resulted when the solution was poured into ether. Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine hydrazide (960 mg.) in 30 ml. of acetic acid and 10 ml. of 0.4 *N* hydrochloric acid was cooled to −10°, and 1.1 ml. of 10% sodium nitrite was added. After 5 minutes, the dipeptide hydrobromide in 100 ml. of chloroform and 20 ml. of water was added. A mixture of 100 ml. of triethylamine and 120 ml. of chloroform, precooled to 0°, was added until the *p*H was 8. The mixture was stirred at 4° overnight, the layers separated and the organic layer washed with cold 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and water and dried with magnesium sulfate. Filtration followed by concentration *in vacuo* to a small volume and addition of petr. ether yielded 650 mg. (38%) of protected hexapeptide, m.p. 162–164°, $[\alpha]^{22}D$ −15.3° (*c*, 1.1 in chloroform). The m.p. was not changed by additional recrystallization from chloroform–petr. ether or from ethanol by addition of ether–petr. ether (1:1).

Anal. Calcd. for $C_{58}H_{72}N_{10}O_{13}S$ (mol. wt. 1125.29): C, 59.8; H, 6.45; N, 12.45. Found: C, 60.1; H, 6.79; N, 12.67.

Various attempts were made to remove protecting groups from this peptide. Biological activity was determined by the strepogenin assay (see below), guanidine groups by quantitative Sakaguchi[11] and α-amino groups by quantitative ninhydrin.[24]

(a) One hundred mg. was treated with sodium in liquid ammonia (blue color persisted for 5 minutes). After removal of the ammonia, the residue was taken up in 15 ml. water and the *p*H adjusted to 6.5. Air had to be bubbled through the solution for 3 hr. before the nitroprusside reaction was negative. This preparation contained 8.8% of the expected strepogenin activity (1050 units instead of 12000 units) and 0.8% of the expected guanidine groups (0.72 instead of 89 μmoles).

(b) Catalytic hydrogenation liberated only 5% of the guanidine groups and 57% of the α-amino groups from the protected hexapeptide.

(c) Sodium in liquid ammonia (blue color persisted for 5 minutes) liberated 9% of the guanidine groups from carbobenzoxy-γ-benzyl-L-glutamyl-L-nitroarginine benzyl ester, while catalytic hydrogenation liberated 80%. However, if the protected dipeptide after treatment with sodium in liquid ammonia was subjected to catalytic hydrogenation, no further guanidine groups were liberated.

(d) Treatment of 5 mg. of carbobenzoxy-γ-benzyl-L-glutamyl-L-nitroarginine benzyl ester with 50 mg. $SnCl_2$ in 2.5 ml. of methanol and 2.5 ml. of 1 *N* hydrochloric acid for 5 minutes at 80° liberated 80–90% of both the guanidine and α-amino groups. Ten mg. of protected hexapeptide was treated in this manner with the liberation of 30% of the guanidine groups. However, when this material was suitably freed of reagents and treated with sodium in liquid ammonia, only 80 units of strepogenin activity (1200 units is theoretical) were formed. Since this method of removing protecting groups was not suitable, the synthesis of the hexapeptide had to be modified.

Strepogenin Activity.—The strepogenin assay with *Lactobacillus casei* was performed as previously described.[3,25,26] Wilson's soluble liver fraction L was assigned the value of 1 unit per mg. and served as the standard. One unit was sufficient to give approximately half-maximal growth in 10 ml. basal medium[26] incubated long enough at 37° for tubes containing no peptide to just show visible turbidity (usually 20 hr.). The disulfide of L-leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-arginine showed 190–200 units per mg. based on dry weight and on Sakaguchi analysis. The peptide isolated from insulin digests had the same activity, but since only minute amounts of material were available, the amount of peptide assayed was estimated only by the Sakaguchi reaction.

Assays of the pentapeptides indicated them to be less potent. The pure protected pentapeptides and the modified hexapeptide[27] were treated with sodium in liquid ammonia (blue color persisted for 5 minutes) to remove protecting groups and the reaction mixtures were assayed. Potencies (see Table II) based on expected weight of free peptide were calculated on the assumption of 100% yield in this deprotection and are thus minimal values. Actually, in the case of the hexapeptide (IX) the yield was 23%. (Protected hexapeptide (VIII) when saponified and reduced showed 45 units per mg., whereas pure hexapeptide isolated from the reaction mixture showed 200 units per mg.) However, with the protected hexapeptide an additional step (saponification) was involved.

TABLE II

STREPOGENIN ACTIVITY OF VARIOUS PROTECTED PENTA- AND HEXAPEPTIDES REDUCED BY SODIUM IN LIQUID AMMONIA

Pentapeptide: Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-L-glutamic acid dibenzyl ester	Units/mg.
(A) Di- and tripeptide condensed by azide reaction	10
(B) Di- and tripeptide condensed by diimide reacn.	15–17
(C) Tetrapeptide and amino acid condensed by azide reaction	10–12
Product of Curtius rearrangement in pentapeptide synthesis	0.8–1.2
Pentapeptide: Carbobenzoxy-S-benzyl-L-cysteinylglycyl-L-leucyl-L-valyl-L-glutamic acid dibenzyl ester	1
Modified hexapeptide (ref. 27)	17

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(24) S. Moore and W. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(25) D. W. Woolley, *ibid.*, **172**, 71 (1948).

(26) Commercial casein hydrolyzate of General Biochemicals, Inc., was used. Certain other brands were unsatisfactory.

(27) Modified hexapeptide means the product obtained by treating protected hexapeptide containing nitroarginine (carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-γ-benzyl-L-glutamyl-L-nitroarginine benzyl ester) with sodium in liquid ammonia. It may be L-leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-nitroarginine but this was not proved.