The melting point (284–285° dec.) of the methiodide derivative was not depressed on admixture with a sample of the methiodide described in part A, and the infrared spectra of the two samples are identical.

1-Propyl-1,2,3,4-tetrahydronaphthalene. (A) From 1-Methyloctahydrobenzo[f]quinoline Methiodide by Hofmann's Exhaustive Methylation Procedure.—A solution of 10 g. of the methiodide in 100 ml. of water and 100 ml. of tetrahydrofuran was stirred overnight with neutral silver oxide freshly prepared from 10 g. of silver nitrate. The inorganic salts were filtered and the filtrate was concentrated under reduced pressure at a bath temperature below 70° to give a residue which was heated at 100° (0.5 mm.) for about 30 minutes before distilling the product through a semi-micro column. The yield of 1-(**3-dimethylaminopropyl**)-**3,4-dihydronaphthalene (IX)** was 4.59 g. (73%), b.p. 108–110° (0.5 mm.); λ_{max}^{EtOH} 212.5 and 218.5 (ε 18,040), 225 (ε 13,350) and 261 mμ (ε 8,750). There are no shoulders or inflections in the ultraviolet spectrum at 272.5, 282.5 or 289.5 mμ, indicating the absence of naphthalenic material at this stage.

A solution of 4.5 g. of the unsaturated amine IX and 40 ml. of absolute ethanol was shaken in the presence of 0.25 g. of Adams catalyst in a hydrogen atmosphere until one molecular equivalent of hydrogen was absorbed and the rate of hydrogenation slowed. Filtration of the mixture and concentration of the filtrate gave a residue which was diluted with ether and treated with excess methyl iodide to give 6.8 g. of material which on recrystallization from ethanol–ether afforded 6.22 g. (84%) of 1-(**3-dimethylamino**)-**1,2,3,4-tetrahydronaphthalene methiodide**, m.p. 170–172°; λ_{max}^{EtOH} 218 (ε 23,000), 265.5 (ε 1,280) and 273 mμ (ε 1,100). The molar extinction coefficients of the product are not characteristic of a 1,2,3,4-tetrahydronaphthalene chromophore and indicate the presence of a small amount of a 1-substituted 3,4-dihydronaphthalene or 1-substituted naphthalene impurity.

The above methiodide (6.2 g.) was dissolved in 200 ml. of a 1:1 ethanol–water mixture and stirred overnight with silver oxide prepared from 6 g. of silver nitrate. The mixture was filtered and the filtrate concentrated and finally heated at 120° (15 mm.) for 30 minutes before distilling the product, b.p. 120–130° (15 mm.), mostly at 126–127°. To remove traces of amine, the distillate was diluted with ether and washed with dilute hydrochloric acid and water. The ether solution was dried and concentrated under reduced pressure to give 1.86 g. (63%) of crude 1-allyl-1,2,3,4-tetrahydronaphthalene which was used directly in the next step; λ_{max}^{EtOH} 214.5 (ε 11,900), 218 (ε 12,300), 266 (ε 937), 273 (ε 1,180), 282.5 (ε 684) and 289 mμ (ε 493) with an inflection at 223.5 mμ (ε 9,370) and a shoulder at 292 mμ (ε 480); $\nu_{max}^{CCl_4}$ 1640(m), 993(m) and 913(s) cm.$^{-1}$ (vinyl grouping). The ultraviolet absorption bands at 223.5, 282.5, 289 and 292 mμ in the crude product undoubtedly are due to the presence of about 7–10% 1-propylnaphthalene (see below).

A solution of 1.72 g. of 1-allyl-1,2,3,4-tetrahydronaphthalene and 30 ml. of absolute ethanol was hydrogenated in the presence of 0.1 g. of Adams catalyst until one molecular equivalent of hydrogen was absorbed and the rate of hydrogenation slowed. After removal of the catalyst and solvent, the residue (1.68 g., 96%) was distilled through a semi-micro column to give 1.44 g. of 1-propyl-1,2,3,4-tetrahydronaphthalene, b.p. 105–126° (14 mm.), $n^{25}D$ 1.5254–1.5278. The bulk of the product had b.p. 126° (14 mm.), $n^{25}D$ 1.5278; λ_{max}^{EtOH} 214.5 (ε 11,200), 218 (ε 11,650), 223.5 (ε 8,800), 266 (ε 835), 273 (ε 1,000), 282.5 (ε 623), 289 (ε 427) and 292 mμ (ε 412). From a comparison of molar extinction coefficients of pure 1-propyl-1,2,3,4-tetrahydronaphthalene (part B) and β-(1-naphthyl)-propionic acid,[17] it was estimated that the product was contaminated with about 7% 1-propylnaphthalene. The infrared spectrum of the product did not show the presence of this impurity.

In a different experiment, 16.2 g. of 1-methyloctahydrobenzo[f]quinoline methiodide was carried through two successive Hofmann exhaustive methylation reactions without hydrogenation of the intermediate 1-(3-dimethylaminopropyl)-3,4-dihydronaphthalene (IX). Distillation of the product from the second Hofmann reaction gave 2.74 g. (34%) of crude 1-propylnaphthalene, b.p. 123–126° (12 mm.); λ_{max}^{EtOH} 223.5 (ε 82,500), 272.5 (ε 5,970), 282.5 (ε 6,970), 289.5 (ε 4,790), 292.5 (ε 4,640). Attempted hydrogenation of this material using Adams catalyst in ethanol resulted in a negligible uptake of hydrogen.

(B) From 1-Tetralone.—A solution of 23.0 g. of 1-tetralone[18] and 100 ml. of anhydrous ether was added slowly with stirring and under a nitrogen atmosphere to the Grignard reagent prepared from 4.0 g. of magnesium and 21.3 g. of *n*-propyl bromide in 150 ml. of ether. When the addition was completed, the ether solution was refluxed for 2 hours before being washed with cold dilute hydrochloric acid and water. The dried ether solution was concentrated and the residue of crude 1-hydroxy-1-propyl-1,2,3,4-tetrahydronaphthalene was distilled slowly in the presence of a few crystals of potassium bisulfate to give 15.6 g. (58%) of a mixture of olefins, b.p. 124–126° (10 mm.). A portion (4.0 g.) of the olefinic mixture in 50 ml. of glacial acetic acid was hydrogenated in the presence of 1.0 g. of a 10% palladium-on-charcoal catalyst. The crude product was chromatographed on alumina to remove traces of oxygen-containing impurities and then distilled through a semi-micro column to give 1.9 g. of 1-propyl-1,2,3,4-tetrahydronaphthalene, b.p. 125° (13 mm.), $n^{25}D$ 1.5225 (lit.[19] $n^{20}D$ 1.5229); λ_{max}^{EtOH} 213 (ε 9,000), 266 (ε 521) and 273 mμ (ε 545). The infrared spectra of this product and the same product from part A are identical.

(17) M.p. 156.5–157.5°; λ_{max}^{EtOH} 224.5 (ε 89500), 271.5 (ε 6860), 282 (ε 8140), 289 (ε 5620) and 292.5 mμ (ε 5560) with an inflection at 264 mμ (ε 4540).

(18) C. E. Olson and A. R. Bader, *Org. Syntheses*, **35**, 95 (1955).

(19) Z. J. Vejdelek and B. Kakac, *Chem. Listy*, **48**, 1215 (1954).

CAMBRIDGE 39, MASS.

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Synthesis of Peptides with Strepogenin Activity[1]

BY R. B. MERRIFIELD AND D. W. WOOLLEY

RECEIVED JUNE 2, 1958

Four new pentapeptides and one new tetrapeptide have been synthesized. The peptides were L-seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine, L-cysteinyl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid (disulfide), L-leucyl-L-cysteinyl-L-leucyl-L-valyl-L-glutamic acid (disulfide), L-seryltriglycyl-L-glutamic acid and L-seryl-L-leucyl-L-valyl-L-glutamic acid. These were all closely related to the strepogenin-active peptide L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid. Comparison of their activities in the growth of *Lactobacillus casei* was made, and some general conclusions about the structural features needed for this kind of biological action were discussed.

The recent isolation, determination of structure and synthesis of a peptide with high strepogenin activity in the stimulation of growth of *Lactobacillus casei*[2,3] has allowed the problem of how it functions to be approached. The active peptide was L-

(1) Supported in part by grant A 1260 from the U. S. Public Health Service.

(2) R. B. Merrifield and D. W. Woolley, THIS JOURNAL, **78**, 358 (1956).

(3) R. B. Merrifield and D. W. Woolley, *ibid.*, **78**, 4646 (1956).

seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid (SHLVG). One of the first steps in the understanding of its mechanism of action should involve a determination of the relationship of structure to activity. This was especially true with this growth factor because it was already known that certain other naturally-occurring peptides (*e.g.*, oxytocin[4] and some of its relatives[5] or several peptides isolated from insulin[4,6]) also showed high activity even though they were rather dissimilar in chemical structure from SHLVG. The object of the present work therefore was to synthesize and test peptides closely related in structure to SHLVG. In this way one might learn which parts were essential and which were dispensable. Thus, for example, we wished to make and test the cysteine analog of SHLVG because existing evidence had suggested that one point in common among all peptides with high activity was the presence of either a cysteine or serine residue. It was of interest also to make replacements of other single amino acid residues and to note the effect on potency. In addition it was desirable to maintain the same amino acids as in SHLVG, but to alter their sequence, and to note the effect of this on biological activity.

Five new pentapeptides and one tetrapeptide were studied. L-Seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine, L-cysteinyl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid (disulfide), L-leucyl-L-cysteinyl-L-leucyl-L-valyl-L-glutamic acid (disulfide), L-seryltriglycyl-L-glutamic acid and L-seryl-L-leucyl-L-valyl-L-glutamic acid were synthesized by variations of the general methods used previously[3] for SHLVG. The syntheses involved coupling of suitably protected amino acids or peptides by either the azide, mixed anhydride or carbodiimide methods. The analytically pure protected peptides then were converted by appropriate hydrolytic or reductive procedures to the free tetra- or pentapeptides. The other peptide, valylhistidylglutamylserylleucine, was isolated from ribonuclease digests by Hirs, *et al.*[7] The structure recently has been determined by Dr. Hirs who kindly gave us samples of the peptide.

Results and Discussion

The growth factor activities of the new peptides for *Lactobacillus casei*, which are summarized in Table I, allow some clarification of the question of what is required for this type of biological activity. The sequence of the amino acid residues in the peptide was not crucial for the maintenance of activity because the random rearrangement of the residues of SHLVG as in valylhistidylglutamylserylleucine (peptide 2) gave a compound which retained high activity.

The original suggestion[8] that a glutamic acid-containing peptide was of importance seems no longer tenable because replacement of the C-terminal glutamic acid residue of SHLVG by phenylalanine gave a compound (peptide 3) of high potency.

TABLE I

STREPOGENIN ACTIVITY OF SYNTHETIC PEPTIDES

No.	Peptide[a]	Activity (units/mg.)
1	Ser—his—leu—val—glu	85
2	Val—his—glu—ser—leu	200
3	Ser—his—leu—val—phe	165
4	Thr—his—leu—val—glu	0 (inhib.)
5	Cys—his—leu—val—glu[b]	17
6	Leu—cys—leu—val—glu[b]	400
7	Ser—gly—gly—gly—glu	0
8	Ser—gly—gly—gly—glu $(OEt)_2$	0
9	Ser—leu—val—glu	29

[a] Abbreviations are according to E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946). [b] The peptides containing cysteine were aerated and presumably were present as disulfides when assayed. They are shown as half-cystine residues in the table.

Substitution of N-terminal serine by threonine has been shown previously[9] to abolish activity and in fact to lead to a compound which was a competitive inhibitor of SHLVG. This and other facts[9] indicated that serine was of importance in the peptide requirement of *L. casei*. However, results with oxytocin[4] and a series of peptides related to it[5] and with some peptides isolated from insulin hydrolysates[4,6] have shown that peptides containing cysteine, but no serine, can have high activity. Perhaps the cysteine in the peptide is convertible to serine, or *vice versa*. The mere presence of cysteine, however, was not sufficient because some cysteine-containing peptides have shown a very low potency. Possibly this was because they had the amino group of the cysteine free (N-terminal). Thus L-cysteinyl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid (disulfide) (peptide 5) had only 17 units per mg., and L-cysteinyl-L-prolyl-L-leucylglycine amide (disulfide)[5] was inactive. By contrast, however, L-leucyl-L-cysteinyl-L-leucyl-L-valyl-L-glutamic acid (disulfide) (peptide 6), in which the cysteine was not N-terminal, was highly active (400 unit/mg.). In fact this pentapeptide was the most active compound of known structure ever encountered in the strepogenin work. With it 0.2 microgram per ml. of culture medium was sufficient for half-maximal growth. The results with peptides 1 and 2 might suggest that a serine peptide also may have greater potency if the serine is not N-terminal.

An important point which emerges from Table I is that all serine-containing pentapeptides are not active. While peptide-serine (or cysteine) appears to be essential for activity it is not in itself sufficient to ensure it. Thus neither L-seryltriglycyl-L-glutamic acid (peptide 7) nor its diethyl ester (peptide 8) had detectable activity (<0.5 unit per mg.). A serine-containing tetrapeptide, L-seryl-L-leucyl-L-valyl-L-glutamic acid (peptide 9) was also of very low potency. The lack of histidine in this tetrapeptide probably cannot explain its low potency because several histidine-free peptides are known to be highly active.

The size of the peptide seemed of importance for high activity. No compound smaller than a pentapeptide has thus far been found to have high potency.

(4) D. W. Woolley and R. B. Merrifield, THIS JOURNAL, **76**, 316 (1954).

(5) D. W. Woolley, R. B. Merrifield, C. Ressler and V. du Vigneaud, *Proc. Soc. Exptl. Biol. Med.*, **89**, 669 (1955).

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

(7) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **221**, 151 (1956).

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

(9) R. B. Merrifield, *ibid.*, **232**, 43 (1958).

Hexa,[5,6] hepta-[2,5] and even nonapeptides[4] have been very active but compounds as large as proteins[10] have not. The low activity of L-seryl-L-leucyl-L-valyl-L-glutamic acid may thus be due to its small size. Possibly the low molecular weight of L-seryltriglycyl-L-glutamic acid contributed to its low activity. The probable importance of the size factor in other biologically active peptides can be noted from the recent findings on the oxytocin activities of the two hypertensins.[11]

There is some evidence that another factor may be associated with high activity, namely, the presence of non-polar or lipophylic side chains. All highly active compounds have contained either leucine, isoleucine or phenylalanine which have lipophylic side chains. Furthermore, increasing the non-polar character of a single side chain has increased the potency. Thus, peptides containing phenylalanine in place of glutamic acid (3 *vs.* 1), or leucine instead of histidine (6 *vs.* 5) were increased in activity.

The relative effects of the changes in the peptide structures on such factors as stability in the medium, penetration into the cell, or utilization within the cell have not been studied here. Conclusions concerning the effects of the various structural features on strepogenin activity refer to their over-all influence on the system.

From existing data the tentative conclusion seems to be that for high strepogenin potency a peptide should contain at least five amino acid residues, one of which should be either serine or cysteine, preferably not at the N-terminal end. In addition leucine or isoleucine or possibly other of the lipophylic amino acid residues seem to be associated with high activity. There are several peptides which incorporate these features and which have strepogenin activity even though they differ considerably in exact chemical structure. Although this lack of structural specificity required for strepogenin activity is clear, one must not forget that just any peptide will not do. Many are known which are inactive. Furthermore, it may be of interest to note that there is also a lack of specificity being found among peptides which show other biological actions[12] as, for example, the oxytocin activity of hypertensin.[11]

Experimental[13]

Microbiological Assay.—The strepogenin assay procedure with *Lactobacillus casei* was as previously described.[5,10,14] The activities were relative to a standard liver extract which was assigned an activity of 1 unit per mg. The activities of peptides 1, 2, 4, 5, 6 and 9 were based on weighed samples of the purified peptides. The concentration of L-seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine (peptide 3) was calculated from a quantitative Pauly test[15] for histidine, which had been shown to give reliable results with SHLVG and other histidine-containing peptides. The activities of peptides 7 and 8 were based on the weight of the pure protected peptide. A quantitative yield was assumed during the removal of protective groups.

Paper Electrophoresis.—This was performed as described by Kunkel and Tiselius,[16] using a pyridine acetate buffer, *p*H 5.0, 0.1 *M* in acetate. The runs were made at room temperature at 17.5 volt/cm. for 2 hr. The peptides were detected by ninhydrin or diazotized sulfanilic acid (Pauly test) sprays. Observed distances of migration were corrected for endosmotic flow (with leucine as reference) and have been expressed as U_{his} = mobility of the sample divided by the mobility of histidine. A negative value means the compound possessed a net negative charge at *p*H 5.0.

Carbobenzoxy-L-seryl-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—Carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid diethyl ester was prepared from carbobenzoxy-L-leucyl-L-valine[3] (10.9 g.) and diethyl L-glutamate hydrochloride (7.9 g.) by the dicyclohexylcarbodiimide method according to the general procedure of Sheehan and Hess.[17] After recrystallization from ethyl acetate the yield was 61%, m.p. 177–178°, $[\alpha]^{27}D$ −43.3° (*c* 2.6, acetic acid). This compared well with the same compound from the mixed anhydride procedure.[3] The product was converted to diethyl L-leucyl-L-valyl-L-glutamate as before,[3] and 0.87 g. then was coupled with 0.50 g. of carbobenzoxy-L-serine[3] by the carbodiimide procedure in 20 ml. of methylene chloride. In a few minutes at 25° the mixture set to a solid. After 24 hr. 100 ml. of ethyl acetate was added and the solution was washed with HCl, bicarbonate and water and evaporated to dryness. The crude product was crystallized from 25 ml. of methanol, yield 0.76 g. (57%), m.p. 201–203°. Two more recrystallizations from methanol gave 0.28 g., m.p. 212–214°, $[\alpha]^{25}D$ −46.9° (*c* 1, ethanol).

Anal. Calcd. for $C_{31}H_{48}N_4O_{10}$: C, 58.5; H, 7.6; N, 8.7. Found: C, 58.6; H, 7.6; N, 8.7.

L-Seryl-L-leucyl-L-valyl-L-glutamic Acid.—Carbobenzoxy-L-seryl-L-leucyl-L-valyl-L-glutamic acid diethyl ester (200 mg.) was suspended in 2 ml. of concentrated hydrochloric acid and held at 37° for 90 min. The acid was removed *in vacuo* exactly as described for the preparation of SHLVG.[3] A solution of the product in water was neutralized with ammonia, extracted with ethyl acetate and evaporated to 1 ml. Addition of 25 ml. of ethanol gave 132 mg. (94%) which was recrystallized from 5 ml. of 80% ethanol, yield 90 mg. Paper electrophoresis showed a single ninhydrin-positive spot, U_{his} −0.18. It was necessary to dry the sample for 8 hr. at 100° to remove water of crystallization.

Anal. Calcd. for $C_{19}H_{34}N_4O_8$: C, 51.1; H, 7.7; N, 12.6. Found: C, 50.9; H, 7.7; N, 12.4.

Carbobenzoxy-S-benzyl-L-cysteinyl-1(or 3)-benzyl-L-histidine Hydrazide.—1(or 3)-Benzyl-L-histidine[18] was converted to the methyl ester in the usual way. The sirupy ester (9.0 g.) was condensed with 12.9 g. of carbobenzoxy-S-benzyl-L-cystine[19] in 150 ml. of methylene chloride containing 7.1 g. of dicyclohexylcarbodiimide. After 18 hr. at 25° a nearly quantitative yield of dicyclohexyl urea was filtered off. The solution was washed with bicarbonate and water and dried. Evaporation gave an oil which could not be crystallized. This ester was dissolved in 200 ml. of methanol containing 7 ml. of hydrazine hydrate and refluxed for 1 hr. Slow cooling gave 16.4 g. (81%) of crystalline product, m.p. 174°. Recrystallization from methanol gave tufts of needles, m.p. 174–175°, $[\alpha]^{20}D$ −42.7° (*c* 1.6, acetic acid).

Anal. Calcd. for $C_{31}H_{34}N_6O_4S$: C, 63.5; H, 5.8; N, 14.3. Found: C, 63.8; H, 6.0; N, 14.3.

Carbobenzoxy-S-benzyl-L-cysteinyl-1(or 3)-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—Carbobenzoxy-S-benzyl-L-cysteinyl-1(or 3)-benzyl-L-histidine hydrazide (0.50 g.) was dissolved in 4.25 ml. of 0.4 *N* HCl and 4.25 ml. of acetic acid, cooled to −10° and converted to the azide by dropwise addition of 60 mg. of sodium nitrite in 1 ml. of water with stirring. Good cooling was very important here to avoid rearrangement. After 5 min. 0.38 g. of diethyl-L-leucyl-L-valyl-L-glutamate hydrochloride,[3] 40 ml. of cold chloroform and 10 ml. of cold water were added directly to the flask containing the precipitated

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

(11) H. Schwarz, F. N. Bumpus and I. H. Page, THIS JOURNAL, **79**, 5697 (1957).

(12) D. W. Woolley and R. B. Merrifield, *Science*, **128**, 238 (1958).

(13) All melting points were determined in capillaries and were uncorrected. The elementary analyses were done by Mr. T. Bella.

(14) Commercial casein hydrolysate of General Biochemicals Inc. was used. Certain other brands were unsatisfactory.

(15) E. Jorpes, *Biochem. J.*, **26**, 1507 (1932).

(16) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(17) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

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

(19) S. Goldschmidt and C. Jutz, *Chem. Ber.*, **86**, 1116 (1953).

azide. A cold 1:1 mixture of triethylamine in chloroform was then added dropwise with cooling and stirring until the *p*H reached 8. After 45 min. at 0° the layers were separated and the chloroform solution was washed with 0.1 *N* HCl and water and dried over magnesium sulfate. The filtrate was evaporated to dryness and the product crystallized from 20 ml. of ethanol, yield 0.48 g. (58%), m.p. 183–184°. Recrystallization from ethanol gave a product which first melted sharply at 166° but which resolidified and then remelted at 187–188°. This behavior was observed with several other preparations of this material.

Anal. Calcd. for $C_{51}H_{67}N_7O_{10}S$: C, 63.2; H, 7.0; N, 10.1. Found: C, 62.9; H, 6.9; N, 10.0.

L-Cysteinyl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid (Disulfide).—This was prepared in two ways: (A) by saponification followed by reduction with sodium in liquid ammonia (B) by hydrolysis with concentrated hydrochloric acid, followed by sodium reduction.

A.—Carbobenzoxy-S-benzyl-L-cysteinyl-1(or 3)-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester (250 mg.) was dissolved in a mixture of 5 ml. of pyridine, 2.5 ml. of ethanol and 1.0 ml. of water, and 0.56 ml. of *N* NaOH was added. After 30 min. at 25° the solution was adjusted to *p*H 7 with HCl and evaporated to dryness. The residue was extracted with water and the solution was filtered and evaporated to dryness. The residue was dissolved in 30 ml. of redistilled ammonia and sodium was added with stirring until the deep blue color remained for 5 min. An amount of NH_4Cl equivalent to the sodium used was added and the ammonia was evaporated. The tan residue was dissolved in 5 ml. of cold 0.1 *N* acetic acid and adjusted to *p*H 7. The solution was aerated for 30 min., extracted with benzene and filtered. A Pauly test indicated 204 mg. (83%) of deprotected peptide. The solution was desalted by adsorption on a 1 × 10 cm. column of 4% Dowex-2, OH form, and elution with acetic acid. A 75% recovery of chloride-free material was obtained. This was then distributed in a counter current machine for 60 transfers in *n*-butanol–water–acetic acid (4:4:1). Tubes 3 to 9 were combined and evaporated to dryness. The residue was dissolved in water and precipitated with ethanol, yield 20 mg., m.p. 210° dec.

Anal. Calcd. for $C_{50}H_{80}N_{14}O_{16}S_2$: C, 50.2; H, 6.7; N, 16.4. Found: C, 50.2; H, 7.0; N, 16.1.

Paper electrophoresis showed a single spot (U_{his} 0.12) by the ninhydrin, Pauly and NaCN-nitroprusside tests. At *p*H 5.5 the sample behaved like an uncharged molecule. Ascending paper chromatography in propanol–water (2:1) also showed a single spot, R_f 0.26.

B.—The protected pentapeptide (240 mg.) was incubated at 37° for 90 min. with 10 ml. of concentrated hydrochloric acid. A small insoluble residue was removed and the solution was evaporated to dryness *in vacuo* as before.[3] The product migrated during paper electrophoresis in *p*H 6.9, 0.05 *M*, phosphate buffer as a single ultraviolet-absorbing, ninhydrin-positive spot with a rate 0.66 relative to glutamic acid. The product was reduced with sodium in liquid ammonia and aerated. The resulting pentapeptide disulfide behaved during electrophoresis as the compound from method A (U_{his} 0.12). The peptide was precipitated as the mercuric salt from 0.1 *N* acetic acid. This was washed with water and decomposed with H_2S, yield 83 mg. (56%) by the Pauly test. The free peptide was precipitated with ethanol, dissolved in water, filtered and reprecipitated, yield 35 mg.

Anal. Found: C, 49.9; H, 6.6.

Both preparations showed the same strepogenin activity.

Carbobenzoxy-L-leucyl-L-valyl-L-glutamic Acid Dibenzyl Ester.—Carbobenzoxy-L-leucyl-L-valine[3] (2.2 g.), dibenzyl L-glutamate hydrochloride[20] (2.2 g.) and triethylamine 0.84 ml. were dissolved in 50 ml. of methylene chloride and condensed by the dicyclohexylcarbodiimide method. After working up as in the preceding examples, three volumes of petroleum ether were added to a methylene chloride solution, yield 2.6 g. (64%). The product was recrystallized from ethanol, m.p. 153–155°, $[\alpha]^{27}D$ −18.6° (*c* 2, ethyl acetate).

Anal. Calcd. for $C_{38}H_{47}N_3O_8$: C, 67.7; H, 7.0; N, 6.2. Found: C, 67.5; H, 7.1; N, 6.3.

(20) H. Sachs and E. Braun, THIS JOURNAL, **75**, 4610 (1953).

Carbobenzoxy-L-leucyl-S-benzyl-L-cysteine.—Carbobenzoxy-L-leucine (2.1 g.) was dissolved in 40 ml. of tetrahydrofuran containing 8.1 g. of triethylamine and treated at −5° with 0.87 g. of ethyl chlorocarbonate. After 15 min. a solution of 1.7 g. of S-benzyl-L-cysteine[21] in 16 ml. of 0.5 *N* NaOH was added. After 1 hr. at 25° the solution was acidified and evaporated to an oil. The oil was dissolved in 50 ml. of ethyl acetate, washed with HCl and water and dried over $MgSO_4$. Two volumes of petroleum ether were added and crystallization began in a few minutes, yield 1.7 g. (47%), m.p. 154–156°. Recrystallization twice from ethyl acetate gave fine needles, m.p. 159–160°, $[\alpha]^{27}D$ −40.8° (*c* 2, ethanol).

Anal. Calcd. for $C_{24}H_{30}O_5N_2S$: C, 62.9; H, 6.6; N, 6.1. Found: C, 62.8; H, 6.7; N, 6.3.

Carbobenzoxy-L-leucyl-S-benzyl-L-cysteine Ethyl Ester.—S-Benzyl-L-cysteine ethyl ester hydrochloride[22] (1.05 g.) in 20 ml. of methylene chloride was treated with 0.53 ml. of triethylamine. Carbobenzoxy-L-leucine (1.00 g.) was added followed by 0.78 g. of dicyclohexylcarbodiimide. After 3 hr., the reaction mixture was worked up in the usual way. Fine needles, 0.97 g. (55%), were obtained by addition of 10 ml. of petroleum ether to an ethyl acetate solution (5 ml.), m.p. 104–105°. Two recrystallizations from ethyl acetate–petroleum ether raised the m.p. to 114°, $[\alpha]^{20}D$ −58.9° (*c* 2, methanol).

Anal. Calcd. for $C_{26}H_{34}N_2O_5S$: C, 64.2; H, 7.0; N, 5.8. Found: C, 63.9; H, 7.0; N, 6.0.

Carbobenzoxy-L-leucyl-S-benzyl-L-cysteine Hydrazide.—The foregoing ester (0.73 g.) was refluxed for 1 hr. with 0.5 ml. of hydrazine hydrate in 5 ml. of methanol. The solution was evaporated to dryness over sulfuric acid. The residue was recrystallized twice from 50% methanol, yield 0.60 g., m.p. 124–126°, $[\alpha]^{20}D$ −36.2° (*c* 2, methanol).

Anal. Calcd. for $C_{24}H_{32}N_4O_4S$: C, 61.0; H, 6.8; N, 11.9. Found: C, 61.3; H, 7.0; N, 11.9.

Carbobenzoxy-L-leucyl-S-benzyl-L-cysteinyl-L-leucyl-L-valyl-L-glutamic Acid Dibenzyl Ester.—The carbobenzoxy group was removed from 0.56 g. of carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid dibenzyl ester by treatment with 6.0 ml. of 10% HBr in acetic acid for 1 hr. at 25°.[23] The mixture was added dropwise to 200 ml. of cold *M* bicarbonate from which the product was extracted with two portions of methylene chloride. The extract was concentrated *in vacuo* to 40 ml. Carbobenzoxy-L-leucyl-S-benzyl-L-cysteine (0.38 g.) was added and when dissolved 0.27 g. of dicyclohexylcarbodiimide was added. After 20 hr. the solution was evaporated to dryness under reduced pressure, and the residue was triturated with 10 ml. of ethyl acetate. Dicyclohexylurea (0.24 g.) was filtered off and washed. The ethyl acetate solution was washed with HCl, bicarbonate and water and dried. Addition of petroleum ether gave 0.37 g. (45%). Recrystallization twice from ethanol gave 0.12 g., m.p. 217–218°, $[\alpha]^{28}D$ −32.7° (*c* 2, chloroform).

Anal. Calcd. for $C_{54}H_{69}O_{10}N_5S$: C, 66.2; H, 7.1; N, 7.2. Found: C, 66.4; H, 7.2; N, 7.3.

The same peptide was synthesized by the mixed anhydride procedure with ethyl chlorocarbonate but in only 20% yield. The conditions were similar to those used for the synthesis of carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid diethyl ester described previously.[3]

L-Leucyl-L-cysteinyl-L-leucyl-L-valyl-L-glutamic Acid (Disulfide).—The preceding protected pentapeptide (109 mg.) was suspended in 30 ml. of redistilled ammonia at −72° and reduced with sodium until the blue color remained for 2 min. Ammonium chloride equivalent to the sodium was added. The product was present as a fine white precipitate which dissolved on warming the solution to its boiling point. The ammonia was removed *in vacuo*, and the residue was dissolved in 0.5 *N* HCl and the solution was filtered. The filtrate was adjusted to *p*H 3.5. The white precipitate was centrifuged and washed 5 times with 3-ml. portions of water, yield 18 mg. (28%). Despite the repeated washing the peptide still contained ash (4–12% in various preparations). The presence of ash may have accounted for the failure to

(21) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939).

(22) C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 417 (1944).

(23) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

obtain good analytical figures. The isolated peptide accounted for 76% of the strepogenin activity in the reduction mixture. The peptide gave a negative nitroprusside test for sulfhydryl and a positive test for disulfide. Paper chromatography of an acid hydrolysate showed leucine, valine, glutamic acid and cystine in the ratios 2:1:1:1 (visual comparison with standards).

An effort was also made to prepare this peptide by the azide procedure from carbobenzoxy-L-leucyl-S-benzyl-L-cysteine hydrazide and diethyl L-leucyl-L-valyl-L-glutamate. When the reaction was allowed to proceed at 4° for 20 hr. a small yield of carbobenzoxy-L-leucyl-S-benzyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester was obtained. When, however, the azide and ester were mixed at 0° and immediately allowed to warm to room temperature a rearrangement product invariably resulted, the analyses of which agreed with the urea resulting from an isocyanate rearrangement, followed by coupling with the tripeptide ester. The protected peptide obtained by the cold azide coupling procedure was converted to the free peptide by concentrated HCl followed by sodium reduction in liquid ammonia. This yielded strepogenin activity equal to that obtained from reduction of the benzyl ester described above. An analytically pure sample was not isolated.

Carbobenzoxy-L-serylglycylglycylglycylglycine Ethyl Ester.—Glycylglycylglycine ethyl ester hydrochloride[24] (4.0 g.) was dissolved in 50 ml. of water and converted at 0° to the free amine by dropwise addition of *N* NaOH to *p*H 9.0. The solution was concentrated at 25° to 5 ml., diluted with absolute ethanol and evaporated to dryness. The residue was dissolved in chloroform and dried over $MgSO_4$, yield 2.2 g. This procedure was used because the amine could not be extracted into chloroform from aqueous solution. A chloroform solution containing 2.1 g. of carbobenzoxy-L-serine azide[3] was mixed with the triglycine ester and held at 0° for 1 hr. and at 25° for 72 hr. The precipitate was filtered off and dried, yield 3.2 g. (92%). Recrystallization from ethanol gave a product melting at 176–178°, unchanged after recrystallization from chloroform–petroleum ether, $[\alpha]^{20}D$ −9.3° (*c* 2, acetic acid).

Anal. Calcd. for $C_{19}H_{26}O_8N_4$: C, 52.0; H, 6.0; N, 12.8. Found: C, 51.8; H, 6.0; N, 12.9.

Carbobenzoxy-L-serylglycylglycylglycylglycine Hydrazide.—Carbobenzoxy-L-serylglycylglycylglycylglycine ethyl ester (3.2 g.) was dissolved in 250 ml. of hot ethanol and 5.0 ml. of hydrazine hydrate was added. The solution was cooled to 25° and the product filtered off after 24 hr., yield 2.2 g., (67%), m.p. 214–216°. Recrystallization from water raised the m.p. to 217–219°, $[\alpha]^{27}D$ −9.6° (*c* 2, *N* HCl).

Anal. Calcd. for $C_{17}H_{24}O_7N_6$: C, 48.1; H, 5.7; N, 19.8. Found: C, 47.7; H, 5.6; N, 19.8.

Carbobenzoxy-L-seryltriglycyl-L-glutamic Acid Diethyl Ester.—Carbobenzoxy-L-serylglycylglycylglycine hydrazide (1.0 g.) was dissolved in 8 ml. of *N* HCl, diluted to 40 ml. with water and converted to the azide at 0° by addition of 2 ml. of a 10% aqueous solution of sodium nitrite. The granular precipitate of the azide was filtered, washed and dissolved in 5 ml. of cold dimethylformamide. To this was added a freshly prepared solution of 0.56 g. of diethyl L-glutamate in 2 ml. of dimethylformamide. After 24 hr. at 25° the solvent was removed *in vacuo* at 40°. The product was dissolved in warm chloroform and washed with *N* HCl. Petroleum ether was added and after 24 hr. the white, granular precipitate was filtered, yield 0.51 g. (37%). Recrystallization from 15 ml. of hot water gave 0.39 g. of white beads, m.p. 138–140°, $[\alpha]^{27}D$ −9.6° (*c* 2, ethanol).

Anal. Calcd. for $C_{26}H_{37}N_5O_{11}$: C, 52.4; H, 6.3; N, 11.8. Found: C, 52.4; H, 6.5; N, 12.2.

Hydrolysis and paper chromatography showed serine, glycine and glutamic acid in the ratios 1:3:1.

L-Seryltriglycyl-L-glutamic Acid and its Diethyl Ester.—Carbobenzoxy-L-seryl-triglycyl-L-glutamic acid diethyl ester (46 mg.) was dissolved in 6 ml. of 0.5 *N* methanolic hydrogen chloride and hydrogenated in the presence of 50 mg. of 5% palladium on carbon at 25° and 1 atmosphere for 2 hr. The theoretical amount of CO_2 (as $BaCO_3$) was obtained. After removal of the catalyst and evaporation to dryness the diethyl L-seryltriglycyl-L-glutamate hydrochloride was dissolved in water. This solution was used for microbiological assay without further purification. Paper electrophoresis showed a single ninhydrin-positive spot, U_{his} 0.57.

An aliquot of the aqueous solution of the ester was saponified in 0.07 *N* NaOH (3.6 equivalents) at 25° for 40 min. Paper electrophoresis showed that about 95% of the ninhydrin-positive material migrated with U_{his} − 0.35. Only one other product could be detected (U_{his} − 0.08) which presumably was due to a trace of the mono ester. No starting material or free amino acids were found. Longer saponification, however, gave more complex mixtures.

Carbobenzoxy-L-leucyl-L-valyl-L-phenylalanine Benzyl Ester.—L-Phenylalanine benzyl ester hydrochloride was prepared by repeated treatment at 100° of L-phenylalanine with benzyl alcohol saturated with hydrochloric acid followed by azeotropic distillation with benzene. Recrystallization from ethanol gave a 70% yield, m.p. 196°.[25] This hydrochloride (0.88 g.) was converted to the free base with triethylamine in methylene chloride and condensed by the dicyclohexylcarbodiimide method with carbobenzoxy-L-leucyl-L-valine[3] (1.09 g.). After 20 hr. at 25° the reaction mixture was worked up as usual. Addition of three volumes of petroleum ether to a methylene chloride solution gave a gel-like solid, yield 1.42 g. (79%), m.p. 153–154°. Two recrystallizations from ethyl acetate gave m.p. 158–160°, $[\alpha]^{28}D$ −11.0°, (*c* 2, ethyl acetate).

Anal. Calcd. for $C_{35}H_{43}N_3O_6$: C, 70.0; H, 7.2; N, 7.0. Found: C, 70.1; H, 7.3; N, 7.0.

Carbobenzoxy-L-seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine Benzyl Ester.—Carbobenzoxy-L-leucyl-L-valyl-L-phenylalanine benzyl ester (0.60 g.) in 4 ml. of 10% HBr in acetic acid for 1 hr. at 25° was dropped into 100 ml. of cold *M* bicarbonate. The L-leucyl-L-valyl-L-phenylalanine benzyl ester was extracted into ethyl acetate and dried. Paper electrophoresis showed a single ninhydrin-positive spot, U_{his} 0.41. Carbobenzoxy-L-seryl-L-histidine hydrazide, 0.39 g., was dissolved in 10 ml. of *N* HCl plus 3 ml. of ethanol and converted to the azide at −10°.[3] Cold ethyl acetate (25 ml.) and potassium carbonate (1.8 g. in 2 ml. of water) were added, and immediately thereafter the cold ethyl acetate solution of the freshly prepared L-leucyl-L-valyl-L-phenylalanine benzyl ester was added with shaking (*cf.* 3). The organic layer was separated immediately, anhydrous $MgSO_4$ was added, and the mixture held at 0° for 3 hr. and at room temperature for 72 hr. Ethyl acetate (100 ml.) was added, the mixture was filtered, and the product was precipitated with chloroform, yield 0.36 g., (44%), m.p. 212–214°. Recrystallization first from 50 ml. of hot ethanol and finally from 50 ml. of 60% ethanol gave 0.18 g., m.p. 213–215°, $[\alpha]^{27}D$ −43.8° (*c* 1, ethanol).

Anal. Calcd. for $C_{44}H_{56}N_7O_9$: C, 63.9; H, 6.7; N, 11.9. Found: C, 63.5; H, 6.9; N, 11.3.

A two dimensional chromatogram of an acid hydrolysate showed leucine, phenylalanine, valine, histidine and serine in approximately equimolar ratios.

L-Seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine.—Carbobenzoxy-L-seryl-L-histidyl-L-leucyl-L-valyl-L-phenylalanine benzyl ester (50 mg.) was dissolved in 10 ml. of ethanol containing 1 ml. of acetic acid and hydrogenated at 1 atm., 25°, for 2 hr. in the presence of 50 mg. of 5% palladium on carbon. The mixture was filtered, and the solution was evaporated to dryness. The product was dissolved in 5 ml. of water and filtered from a small amount of starting material. Paper electrophoresis showed a single ninhydrin- and Pauly-positive spot, U_{his} 0.44. The peptide concentration was determined by a quantitative Pauly test and the solution was used directly for microbiological assay.

NEW YORK 21, NEW YORK

(24) E. Fischer, *Ber*, **36**, 2982 (1903).

(25) B. F. Erlanger and R. M. Hall, THIS JOURNAL, **76**, 5781 (1954), obtained a similar yield using polyphosphoric acid as the dehydrating agent, m.p. 203° cor.