[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Synthesis of L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid, a Peptide with Strepogenin Activity

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The pentapeptide L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid was synthesized by condensation of carbobenzoxy-L-seryl-L-histidine azide with L-leucyl-L-valyl-L-glutamic acid diethyl ester, followed by removal of the protecting groups. Conditions for preventing self-condensation of the histidine azide derivative were devised. The protecting groups were removed from the pentapeptide with concentrated HCl at 37°. Good yields of the pure pentapeptide were obtained. The synthetic peptide had the same biological activity in the strepogenin test with *Lactobacillus casei* as did the same peptide previously isolated from acid digests of insulin. The structure and biological activity of this isolated peptide were thus confirmed.

In the course of work on the growth factor strepogenin an active peptide was recently isolated from a partial hydrolysate of insulin and its structure established as serylhistidylleucylvalylglutamic acid.[1,2] Since the biological activity of the purified, isolated sample might have been due to minute amounts of highly active contaminants the synthesis of this compound was advisable. This has now been completed and a comparison of the natural and synthetic peptides showed them to be indistinguishable both chemically and biologically. The strepogenin activities measured with *Lactobacillus casei*[3] were 80 units per mg. for the natural and 84 units per mg. for the synthetic peptide (47 and 49 units per μmole, respectively). The assay was not precise enough to distinguish between these two values. The synthetic peptide was prepared from amino acids all of the L-configuration since the naturally occurring one presumably was of this character. The methods of coupling chosen were those previously shown to avoid racemization.

The reaction sequences were the following. Carbobenzoxy-L-leucyl-L-valine was prepared from carbobenzoxy-L-leucine and L-valine by the mixed anhydride method[4] and converted to carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid diethyl ester by a similar procedure. The tripeptide ester, L-leucyl-L-valyl-L-glutamic acid diethyl ester hydrochloride was obtained by catalytic hydrogenation. The protected dipeptide carbobenzoxy-L-seryl-L-histidine methyl ester was prepared from carbobenzoxy-L-serine hydrazide[5] and L-histidine methyl ester by the azide method under conditions similar to those reported for other histidine peptides.[6,7] It was also prepared by condensation of carbobenzoxy-L-serine[8] with L-histidine methyl ester by the carbodiimide method according to the general method of Sheehan and Hess.[9] This protected dipeptide was converted through the hydrazide to the azide which was condensed with the above tripeptide ester to give carbobenzoxy-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester in 54% yield. The desired pentapeptide was obtained after removal of the protecting groups.

The protection of the imidazole ring in histidine during the synthesis was not found necessary. However, a special procedure was required in the preparation and handling of the carbobenzoxy-L-seryl-L-histidine azide. This compound was formed in acid solution from the hydrazide but was not extracted as the free base into an organic solvent before uniting it with the tripeptide ester. When this was done the azide reacted with itself (presumably by attack on the imidazole nitrogen) and was largely decomposed within a few minutes at 4° as judged by the disappearance of the Pauly test[10] for histidine. To avoid this self condensation the L-leucyl-L-valyl-L-glutamic acid diethyl ester hydrochloride was first added to the acid solution of the azide. The free bases were then liberated with potassium carbonate, extracted together into ethyl acetate and allowed to react. This procedure gave satisfactory yields of the desired compound.

A novel method was employed for removal of the carbobenzoxy and ethyl ester protecting groups from the pentapeptide. This was needed because the usual removal by saponification and hydrogenation or by treatment with hydrogen bromide in acetic acid failed with this compound. The carbobenzoxy peptide ester was dissolved in concentrated hydrochloric acid and held at 37° for 85 minutes. No detectable cleavage of the peptide chain occurred in this procedure and the yields were good.

In a similar manner the corresponding pentapeptide containing D-serine was also synthesized. For this purpose carbobenzoxy-DL-serine azide was condensed with L-histidine methyl ester and carbobenzoxy-D-seryl-L-histidine hydrazide was isolated by fractional crystallization of the diastereomers. The resulting pentapeptide was only 11% as active as the "all L" peptide in the strepogenin assay with *L. casei*. Even this small activity may have been due to a failure to remove all of the L-serine derivative during the fractional crystallization of the diastereomers.

The free dipeptide L-seryl-L-histidine and tripeptide L-leucyl-L-valyl-L-glutamic acid, and the pentapeptide diethyl ester were prepared from the corresponding protected peptides just described.

(1) R. B. Merrifield and D. W. Woolley, *Arch. Biochem. Biophys.*, **56**, 265 (1955).

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

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

(4) J. R. Vaughan, Jr., and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).

(5) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(6) R. W. Holley and E. Sondheimer, THIS JOURNAL, **76**, 1326 (1954).

(7) R. F. Fischer and R. R. Whetstone, *ibid.*, **76**, 5076 (1954).

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

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

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

These were also tested for their biological activities. The tripeptide and its diethyl ester were completely inactive in the strepogenin assay, while the dipeptide L-seryl-L-histidine had the small, but real, activity of 0.5 unit per μmole (2.2 units per mg.). These results confirm the earlier conclusions drawn from data on isolated small peptides[2] that such compounds were of low or negligible potency. It is clear, then, that the activity of the pentapeptide is not a summation of the separate activities of the smaller constituent peptides. Nor can this activity be produced by the simultaneous presence of both the di- and tripeptides. That is, the mere presence of all five of the amino acids in a peptide linkage is not sufficient. A more specific arrangement is required. The free amino group was required for activity in the pentapeptide, but the carboxyl groups could be esterified without loss of activity. These observations are in accord with data concerning terminal substitutions of other active peptides.[11,12] Table I summarizes these activities.

TABLE I

Compound	Strepogenin activity[1] (units/μmole)
Ser-his-leu-val-glu (natural)	47
L-Ser-L-his-L-leu-L-val-L-glu (synthetic)	49
D-Ser-L-his-L-leu-L-val-L-glu	5
L-Ser-L-his-L-leu-L-val-L-glu diethyl ester	46
Cbzo-L-ser-L-his-L-leu-L-val-L-glu diethyl ester	<0.5
L-Leu-L-val-L-glu diethyl ester	<0.3
L-Leu-L-val-L-glu	<0.3
L-Ser-L-his[a]	0.5
L-Ser-L-his + L-leu-L-val-L-glu (1:1)	0.5

[a] Obtained from carbobenzoxy-L-seryl-L-histidine methyl ester by removal of the protecting groups with 12 *N* hydrochloric acid at 37° and crystallization from aqueous ethanol. It was electrophoretically homogeneous.

Experimental

Carbobenzoxy-L-seryl-L-histidine Methyl Ester.—A. Azide Method.—Carbobenzoxy-L-serine hydrazide (m.p. 176°)[13] was prepared by the method of Fruton[5] and converted to the azide by the following modification of his method. A solution of 0.89 g. (3.5 mmoles) of the hydrazide in 10 ml. of *N* hydrochloric acid was cooled to −5° and one equivalent of cold aqueous sodium nitrite solution was added all at once with stirring. The white, oily precipitate was extracted into chloroform. The aqueous phase was made alkaline with potassium carbonate and extracted again with chloroform. The combined extracts were washed with bicarbonate and water, dried over magnesium sulfate for 15 minutes and filtered.

L-Histidine methyl ester was made immediately before preparation of the above azide. L-Histidine methyl ester dihydrochloride (2.6 g., m.p. 198–199°), was dissolved in 3 ml. of water, 30 ml. of chloroform was added, the mixture was cooled in an ice–salt-bath and 2 equivalents of 10 *N* sodium hydroxide plus 15 g. of potassium carbonate were added. The mixture was shaken vigorously and quickly filtered into a flask containing magnesium sulfate. The residue was re-extracted twice with chloroform. Evaporation of the combined extracts gave an oil which crystallized in rosettes of needles; yield 1.63 g. (90%); m.p. 62–66°, lit.[7] 64–66°.

A solution of 0.59 g. (3.5 mmoles) of L-histidine methyl ester in 40 ml. of chloroform was added to the solution of carbobenzoxy-L-serine azide prepared as described above and the clear solution allowed to stand at room temperature for 24 hr. The solvent was removed and the residue distributed for 50 transfers in a countercurrent machine between 0.5 *M* sodium phosphate, *p*H 6.3, and ethyl acetate. Both ultraviolet absorption and Pauly tests showed one major component with a peak at tube 26. In addition there was Pauly-positive material in tubes 1 to 5 and ultraviolet absorbing material in tubes 45 to 50. The contents of tubes 13 to 31 were combined, adjusted to *p*H 7 and extracted several times with ethyl acetate. Evaporation of the extract to a small volume and cooling gave 0.62 g. (45%) of colorless crystals, m.p. 121–122°, $[\alpha]^{27}D$ −12.0 ± 0.5° (*c* 2, acetic acid), −4.7° (*c* 2, methanol).

Anal.[14] Calcd. for $C_{18}H_{22}N_4O_6$: C, 55.4; H, 5.7; N, 14.4. Found: C, 55.5; H, 5.6; N, 14.4.

Crystalline material could not be obtained without the countercurrent purification step.

B. **Carbodiimide Method.**[9]—A solution of 1.20 g. (5.0 mmoles) of carbobenzoxy-L-serine, m.p. 117–118° (prepared by the method of Baer and Maurukas[8]) in 5 ml. of dry tetrahydrofuran was mixed with a solution of 0.85 g. (5.0 mmoles) of L-histidine methyl ester in 5 ml. of tetrahydrofuran and 1.13 g. (5.5 mmoles) of dicyclohexylcarbodiimide in 5 ml. of tetrahydrofuran was added. Dicyclohexylurea (3.4 mmoles) was filtered off after 4 hr. The remaining reagent was decomposed with 1 ml. of acetic acid and 0.94 mmole additional of the urea was recovered. The solvent was replaced with ethyl acetate, which was extracted with dilute hydrochloric acid. From the organic phase a side product was obtained as 440 mg. (1.0 mmole) of silky needles, m.p. 163°. This analyzed for N-(carbobenzoxy-L-seryl-)-N,N′-dicyclohexylurea, a by-product to be expected in this type of synthesis.[15]

Anal. Calcd. for $C_{24}H_{35}N_3O_5$: C, 64.7; H, 7.9. Found: C, 64.8; H, 7.8.

These two products thus accounted for all of the diimide reagent. The desired product was extracted from the aqueous phase at *p*H 6.5 with ethyl acetate. The extract was washed with water, dried and evaporated; yield 0.96 g. (49%). The product was purified by countercurrent distribution in which it migrated at the same rate as the compound from the azide reaction. The crystalline material so obtained showed $[\alpha]^{27}D$ −12.4 ± 0.5° (*c* 2, acetic acid).

The carbobenzoxy-L-seryl-L-histidine methyl ester was prepared by these two routes in order to determine whether racemization had occurred in either route. The optical rotation of the ester was the same when it was made by the azide or carbodiimide method. Consequently either no racemization had occurred or (less likely) the extent of racemization was the same in both procedures. This conclusion is justified because the diastereomers carbobenzoxy-L-seryl-L-histidine methyl ester and carbobenzoxy-D-seryl-L-histidine methyl ester were found to be inseparable by the countercurrent distribution system used.

Carbobenzoxy-L-seryl-L-histidine Hydrazide.—Carbobenzoxy-L-seryl-L-histidine methyl ester, 1.2 g. (3.1 mmoles) and 0.3 ml. of hydrazine hydrate in 10 ml. of methanol were refluxed for 30 min. and then allowed to stand at 25° for 24 hr. The mixture was evaporated *in vacuo* over sulfuric acid, and the residue crystallized from 15 ml. of hot methanol. Recrystallization of the somewhat waxy product from 5 ml. of water yielded 0.95 g. (79%), m.p. 178–179°. A second crystallization from methanol gave a product with m.p. 178–179°, $[\alpha]^{27}D$ −18.0° (*c* 2, methanol).

Anal. Calcd. for $C_{17}H_{22}N_6O_5$: C, 52.3; H, 5.7; N, 21.5. Found: C, 52.2; H, 5.8; N, 21.6.

Carbobenzoxy-D-seryl-L-histidine Hydrazide.—Carbobenzoxy-DL-serine azide (10 mmoles) was coupled with L-histidine methyl ester as previously described for the L-serine derivative. The product was purified by countercurrent distribution. The ester (1.2 g.) was converted to the hydrazide as described above and this was dissolved in 15 ml. of hot methanol. Cooling gave 0.23 g., m.p. 195°. Recrystallization from 10 ml. of boiling methanol gave 0.17 g. of needles, m.p. 195–196°, unchanged by recrystallization from ethanol or water; $[\alpha]^{27}D$ +5.0° (*c* 2, methanol).

(11) D. W. Woolley, *J. Biol. Chem.*, **171**, 443 (1947).

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

(13) All melting points were determined in capillaries and are uncorrected.

(14) The elementary analyses were done by Mr. T. Bella.

(15) H. G. Khorana, *Chemistry and Industry*, 1087 (1955).

Anal. Calcd. for $C_{17}H_{22}N_6O_5$: C, 52.3; H, 5.7; N, 21.5. Found: C, 52.5; H, 5.6; N, 22.9.

Although the nitrogen analysis indicated the presence of some impurity, paper electrophoresis in 0.1 *M*, *p*H 5.0 pyridine acetate revealed a single component by the Pauly test which migrated at the same rate as the corresponding compound containing L-serine (mobility relative to histidine 0.61). There was no detectable ninhydrin-positive material. Concentration of the mother liquors from the 0.23 g. obtained above gave 0.54 g. of crude mixed isomers, m.p. 163–166°.

This same hydrazide was also prepared by the mixed anhydride procedure starting with carbobenzoxy-DL-serine and L-histidine methyl ester by use of the general methods described above. The melting points and optical rotations were the same for the hydrazides prepared by the two different procedures. The purity of the D-diastereomer was demonstrated by constancy of melting point on recrystallization. Both the melting point and the rotation showed it to be different from the L-diastereomer.

Carbobenzoxy-L-leucyl-L-valine.—A solution of 20.4 g. (77 mmoles) of carbobenzoxy-L-leucine[16] and 7.77 g. (77 mmoles) of triethylamine in 180 ml. of tetrahydrofuran was cooled to −5°. To this clear solution 8.35 g. (77 mmoles) of cold ethyl chlorocarbonate was added with stirring. After 10 min. a solution of 9.0 g. (77 mmoles) of L-valine in 77 ml. (1 equiv.) of *N* sodium hydroxide was added with vigorous stirring. The mixture was warmed to room temperature over a period of 30 min., acidified with 84 ml. of *N* hydrochloric acid, and concentrated under reduced pressure to 50 ml. The solution was extracted three times with 75-ml. portions of ethyl acetate. The extracts were combined, decolorized with carbon and dried with magnesium sulfate. Addition of petroleum ether gave 13 g. (46%) of colorless needles, m.p. 108–110°. Recrystallization from ethyl acetate–petroleum ether gave 11.5 g. (41%), m.p. 109–110°, $[\alpha]^{25}D$ −18.7° (*c* 4, ethanol).

Anal. Calcd. for $C_{19}H_{28}N_2O_5$: C, 62.6; H, 7.7; N, 7.7. Found: C, 62.5; H, 7.6; N, 7.9.

This compound was prepared by Smith, *et al.*,[17] *via* the azide route, who found m.p. 108–109°, $[\alpha]^{22}D$ −15.0° (*c* 1, ethanol).

Carbobenzoxy-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—A solution of 8.4 g. (23 mmoles) of carbobenzoxy-L-leucyl-L-valine and 2.32 g. of triethylamine in 75 ml. of tetrahydrofuran was cooled to −5° and 2.53 g. of cold ethyl chlorocarbonate was added with stirring. After 10 min., 100 ml. of tetrahydrofuran containing 4.7 g. (23 mmoles) of freshly prepared L-glutamic acid diethyl ester was added. The latter was liberated from the hydrochloride in the manner described above for histidine methyl ester. After 10 min. of stirring in the cold the mixture was heated rapidly to boiling and refluxed for 2 min. according to the general procedure of Vaughan and Eichler.[4] The contents of the flask were quickly cooled and poured into 1 liter of 3% sodium bicarbonate. The resulting precipitate was filtered and washed. The solid was dissolved in 250 ml. of ethyl acetate which was then washed several times with dilute hydrochloric acid and water. The solution was evaporated to dryness and the crystals dissolved in 60 ml. of hot ethanol. After addition of 15 ml. of water and slow cooling tufts of fine needles appeared; yield 5.6 g. (44%), m.p. 177–178°, $[\alpha]^{25}D$ −43.0° (*c* 3, acetic acid).

Anal. Calcd. for $C_{28}H_{43}N_3O_8$: C, 61.2; H, 7.9; N, 7.7. Found: C, 61.1; H, 7.8; N, 7.7.

L-Leucyl-L-valyl-L-glutamic Acid Diethyl Ester Hydrochloride.—A suspension of 5.0 g. (9.1 mmoles) of the preceding compound in 200 ml. of *N* ethanolic hydrogen chloride was hydrogenated at atmospheric pressure in the presence of 200 mg. of 5% palladium on carbon at 25° for 4 hr. The catalyst was filtered and washed with ethanol. The filtrate and washings were concentrated *in vacuo* and the product crystallized by addition of ether; yield 3.78 g. (92%), m.p. 238–239°. Recrystallization from ethanol did not change the melting point; $[\alpha]^{25}D$ −25.5° (*c* 2, ethanol).

Anal. Calcd. for $C_{20}H_{37}N_3O_6 \cdot HCl$: C, 53.2; H, 8.5; N, 9.3. Found: C, 53.1; H, 8.4; N, 9.5.

(16) Mann Research Laboratories, New York.

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

L-Leucyl-L-valyl-L-glutamic Acid.—A 100-mg. sample of L-leucyl-L-valyl-L-glutamic acid diethyl ester hydrochloride was mixed with 6.65 ml. (3 equiv.) of 0.1 *N* sodium hydroxide and held at 25° for 1.5 hr. Unchanged ester was extracted with ethyl acetate, and the aqueous phase was adjusted to *p*H 5 with acetic acid. The solution was concentrated to 1 ml. and the product precipitated by the addition of ethanol. The amorphous material was dissolved in water and precipitated with ethanol two more times and finally it was crystallized as feathery clusters of needles from 2 ml. of water by the addition of 5 ml. of ethanol. The product melted at 217°.

Anal. Calcd. for $C_{16}H_{29}N_3O_6$: C, 53.5; H, 8.1. Found: C, 53.1; H, 8.4.

This product migrated as a single component during paper electrophoresis in 0.1 *M*, *p*H 5.0 pyridine acetate (mobility relative to histidine −0.29).

Carbobenzoxy-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester.—A solution of 0.250 g. (0.64 mmole) of carbobenzoxy-L-seryl-L-histidine hydrazide in 3.8 ml. of *N* hydrochloric acid was cooled to −5° and a *M* solution of sodium nitrite was added until a slight excess of nitrous acid remained (starch–iodide paper). After 15 min. the remaining nitrous acid was destroyed with ammonium sulfamate. A slurry of 0.289 g. (0.64 mmole) of L-leucyl-L-valyl-L-glutamic acid diethyl ester hydrochloride in 3 ml. of water was added and rinsed in with 2 ml. of water. Twenty-five ml. of ethyl acetate was added, followed by an ice-cold solution of 0.7 g. of potassium carbonate in 1 ml. of water. The mixture was shaken vigorously and centrifuged. The aqueous phase was reëxtracted twice with ethyl acetate and the combined extracts were washed once with water. Magnesium sulfate (3 g.) was added to the organic phase which was then held at room temperature for 24 hr. Water (25 ml.) and enough ethyl acetate (150 ml.) to dissolve the precipitated peptide were added and the aqueous phase was discarded. The organic solvent was evaporated and the residue was dissolved in 12 ml. of methanol. After several days in the cold the product crystallized as spheres composed of tightly packed needles; yield 268 mg. (54%), m.p. 211–212°. After recrystallization from 10 ml. of 50% ethanol the m.p. was 213°, $[\alpha]^{27}D$ −46.3° (*c* 3, ethanol).

Anal. Calcd. for $C_{37}H_{55}N_7O_{11}$: C, 57.4; H, 7.2; N, 12.7. Found: C, 57.6; H, 7.0; N, 12.8.

L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid.—A suspension of 100 mg. (0.13 mmole) of the above carbobenzoxy pentapeptide ester in 5 ml. of 12 *N* hydrochloric acid was placed at 37° for 85 min. After 2 min. the solution had cleared and in 30 min. a slight turbidity had appeared. At the end of the reaction period the solution was evaporated *in vacuo* to dryness at 25° and the residue was dried over potassium hydroxide in high vacuum for 3 hr. The solid was dissolved in 2 ml. of water, and the solution freed of a small amount of insoluble material, adjusted to *p*H 6 with dimethylamine and evaporated to dryness. The residue was triturated several times with absolute ethanol. The insoluble part was dissolved in 1 ml. of water and precipitated with 10 ml. of ethanol. It was then dissolved in 2 ml. of water, 4 ml. of ethanol was added and the solution was cooled until the peptide precipitated. It was dried in high vacuum at 65°; yield 57 mg. (73%), m.p. 206–207° dec. (slight browning from 200°), $[\alpha]^{25}D$ −55.8° (*c* 1, water). The analysis indicted a monohydrate.

Anal. Calcd. for $C_{25}H_{41}N_7O_9 \cdot H_2O$: C, 49.9; H, 7.2; N, 16.3. Found: C, 49.7; H, 7.0; N, 16.4.

The compound was dried further in high vacuum at 100° for 5 hr.

Anal. Calcd. for $C_{25}H_{41}N_7O_9$: C, 51.4; H, 7.1; N, 16.8. Found: C, 50.6; H, 7.0; N, 16.9.

Paper electrophoresis in 0.1 *M*, *p*H 5.0 pyridine acetate revealed a single spot when tested with the ninhydrin and with the Pauly reagents (mobility relative to histidine 0.18). Paper chromatography in *n*-propanol–water (2:1) gave one spot with R_f 0.32. These values corresponded satisfactorily with those of the natural pentapeptide although enough of the latter was no longer available for direct comparison on the same sheets of paper. Paper chromatography of an acid hydrolysate of the synthetic peptide showed approximately equimolar amounts of serine, histidine, leucine, valine and glutamic acid.

The conventional methods for removal of carbobenzoxy and ethyl ester groups gave poor results. Thus, the hydrogen bromide–acetic acid procedure[4,18] led to several products which were detected by paper electrophoresis. Saponification followed by catalytic hydrogenation also gave mixtures. Because this pentapeptide was known[2] to be resistant to concentrated hydrochloric acid at 37° the procedure described above was used and was found to give a pure product.

L-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid Diethyl Ester. A.—A 40-mg. sample of carbobenzoxy-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid diethyl ester was dissolved in 3 ml. of ethanol and hydrogenated with palladium–carbon catalyst for 4 hr. The catalyst was removed and the solution concentrated. Addition of water gave 20 mg. of a gelatinous precipitate. Paper electrophoresis in 0.1 *M*, *p*H 5.0 pyridine acetate indicated a single ninhydrin-positive spot (mobility relative to histidine 0.74). However, the Pauly test showed in addition a small amount of starting material (mobility relative to histidine 0.32).

B.—A small sample of the carbobenzoxy pentapeptide ester was treated with an excess of *N* hydrogen bromide in acetic acid at 25° for 90 min.[19] This produced only one detectable compound with the same relative mobility (0.74) as preparation A. These two preparations were used without further purification for microbiological assay and the specific activities, which were nearly identical, were calculated on the basis of the quantitative Pauly reaction.

D-Seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid.—A 41-mg. sample of carbobenzoxy-D-seryl-L-histidine hydrazide was converted to the azide and condensed with L-leucyl-L-valyl-L-glutamic acid diethyl ester as described for the L-serine-containing isomer. The yield was 40 mg. of a gelatinous product. This was hydrolyzed in 1 ml. of concentrated hydrochloric acid at 37° for 130 min. and worked up as described previously for the L-isomer. Almost all of the Pauly-positive and ninhydrin-positive material migrated in paper electrophoresis exactly like the "all L" pentapeptide (mobility relative to histidine 0.18 in 1 *M*, *p*H 5.0 pyridine acetate). However, small amounts of the mono- and diethyl esters also were detected. This material was used without further purification for strepogenin assay.

(18) G. W. Anderson, J. Blodinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952).

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

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[CONTRIBUTION FROM THE MEDICINAL CHEMICAL SECTION, RESEARCH DIVISION, AMERICAN CYANAMID CO.]

1,3,4-Thiadiazole- and Thiadiazolinesulfonamides as Carbonic Anhydrase Inhibitors. Synthesis and Structural Studies

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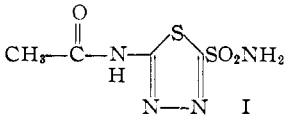
The preparation of 2-(N-methylacetamido)-1,3,4-thiadiazole-5-sulfonamide (II) and of 5-acetylimino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide (XIII) is reported. These compounds, and analogs of II, are potent inhibitors of carbonic anhydrase. The structures of II and related derivatives of 2-acetamido-1,3,4-thiadiazole-5-thiol are established.

The success of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (I)[1,2] as a therapeutically effective inhibitor of the enzyme carbonic anhydrase has



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encouraged further syntheses of structural variants so that the effects of these changes on *in vitro* inhibitory activity and on some pharmacological properties might be studied. Of particular interest were the monobasic acid analogs of the weak dibasic acid I. These differ from I in that the dissociable hydrogen atom on the carboxamide is replaced by an alkyl or aryl group.[3]

The simplest compound of this type, 2-(N-methylacetamido)-1,3,4-thiadiazole-5-sulfonamide (II), was synthesized by first acetylating 2-methylamino-1,3,4-thiadiazole-5-thiol (III)[4] to give 2-(N-methylacetamido)-1,3,4-thiadiazole-5-thiol (IV). Oxidative chlorination[2] of this compound to the sulfonyl chloride followed by amidation with anhydrous liquid ammonia gave the sulfonamide II. Several analogous compounds were prepared by this procedure and are reported in Table I.

(1) Diamox ® Acetazolamide.

(2) R. O. Roblin, Jr., and J. W. Clapp, THIS JOURNAL, **72**, 4890 (1950).

(3) In a previous paper [J. R. Vaughan, Jr., J. A. Eichler and G. W. Anderson, *J. Org. Chem.*, **21**, 700 (1956)] variations on the acyl group of I were reported.

(4) M. Busch and H. Lotz, *J. prakt. Chem.*, [2] **90**, 257 (1914).

The chemical and physical properties of II are especially interesting when compared to I. The acidity and spectra are quite similar, although II is only a monobasic acid. The behavior in alkaline solution, however, is quite different. Compound I is stable at *p*H 11–13 at room temperature for at least 8 hours, but II is deacylated under these conditions to 2-methylamino-1,3,4-thiadiazole-5-sulfonamide (XV).[5] This instability appears to be general; 2-(N-phenylacetamido)-1,3,4-thiadiazole-5-sulfonamide (XXVII) is also rapidly deacylated in dilute alkali.[6]

The resistance of I to alkaline degradation is probably associated with anion formation at the carboxamide, which would inhibit attack at the carbonyl group by hydroxide ion. This mode of stabilization is not available for II or its congeners. However, the validity of this rationalization depends on the assumption that I and II represent the true structures of these compounds. The subsequent investigation was undertaken to confirm these assignments.

Structural Studies.—The preparation of III by alkaline cyclization of 4-methylthiosemicarbazide with carbon disulfide (*cf.* Fig. 1) has not been reported, although Guha[7] prepared the related 2-

(5) Pseudo first-order rate constants for this decomposition were estimated by following the disappearance of the 267 mμ peak in buffers at room temperature: at *p*H 9.2, *k ca.* 2×10^{-5} sec.$^{-1}$; at *p*H 11.0, *k ca.* 2×10^{-3} sec.$^{-1}$.

(6) At *p*H 9.2, *k ca.* 2×10^{-5} sec.$^{-1}$; at *p*H 11.0, *k ca.* 8×10^{-4} sec.$^{-1}$.

(7) P. C. Guha, THIS JOURNAL, **44**, 1510 (1922).