N-Vinyltetrahydrocarbazole was prepared essentially by the method of Clemo and Perkin.[22] However, our attempts to fractionate the product under their conditions led to apparent polymerization and simultaneous formation of tetrahydrocarbazole. The N-(2-chloroethyl)-tetrahydrocarbazole was dehydrochlorinated with potassium *t*-butoxide and the product distilled in a short path still at 80° (0.2 mm.); ultraviolet absorption: λ_{max} 302 mμ (ε 8,800), 252 (20,800).

Anal. Calcd. for $C_{14}H_{15}N$: C, 85.2; H, 7.7. Found: C, 84.8; H, 7.6.

N-(2-Carbethoxyvinyl)-indoline (XXVII).—A mixture of 1.96 g. of ethyl propiolate and 2.50 g. of indoline was cooled occasionally over a period of 10 minutes until spontaneous heating had subsided. The mixture was allowed to stand overnight, and the solid which resulted was crystallized from methanol and dried at 80° (0.3 mm.) For 12 hr. to give 3.65 g. (85%) of a product melting at 84–85°; ultraviolet absorption: λ_{max} 330 mμ (ε 34,700), 291 (19,800).

Anal. Calcd. for $C_{13}H_{15}NO_2$: C, 71.9; H, 6.9. Found: C, 72.1; H, 6.9.

N-(1,2-Dicarbomethoxyvinyl)-indoline (XXVIII).—A solution of 1.1 g. of indoline in 50 ml. of methylene chloride was added to a solution of 1.30 g. of dimethyl acetylenedicarboxylate in 50 ml. of methylene chloride. As soon as spontaneous boiling had ceased, the solution was concentrated. The residue, after one recrystallization from methanol and sublimation at 120° (0.2 mm.), afforded 1.91 g. (79%) of white crystals, m.p. 130–131°; ultraviolet absorption: λ_{max} 330 mμ (ε 26,000), 293 (8,550).

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.4; H, 5.8. Found: C, 64.7; H, 6.0.

9-(2-Carbethoxyvinyl)-4a-hydroxy-1,2,3,4,4a,9a-hexahydrocarbazole (XXXa).—To a solution of 1.0 ml. of ethyl propiolate and 0.3 ml. of methanol in 5 ml. of methylene chloride there was added one drop of triethylamine. As soon as spontaneous boiling of the solution ceased, a 1.0-ml. aliquot of this was added to 140 mg. of 4a-hydroxy-1,2,3,4,-4a,9a-hexahydrocarbazole,[23] and the mixture was shaken until a homogeneous solution was obtained. After standing overnight, the solvent had evaporated and a crystalline residue remained. This was recrystallized from ethanol and dried at 80° (0.2 mm.) for 12 hr. to give material melting at 133–134°; ultraviolet absorption: λ_{max} 323 mμ (ε 35,600), 294 (22,000).

Anal. Calcd. for $C_{17}H_{21}NO_3$: C, 71.0; H, 7.4. Found: C, 70.9; H, 7.2.

9-(2,2-Dicarbethoxyvinyl)-4a-hydroxy-1,2,3,4,4a,9a-hexahydrocarbazole (XXXc).—A solution of 100 mg. of 4a-hydroxy-1,2,3,4,4a,9a-hexahydrocarbazole and 0.125 ml. of diethyl ethoxymethylenemalonate in 2 ml. of methylene chloride was allowed to stand for 18 hours and was then concentrated and heated at 100° for 20 minutes. The resulting residue solidified on standing; when this solid was recrystallized from methanol and dried at 80° (0.3 mm.) for 13 hr. there was obtained colorless needles, m.p. 135–136°; ultraviolet absorption: λ_{max} 328 mμ (ε 29,800), 295 (13,600); infrared absorption: λ_{max} 5.91, 5.98 μ.

Anal. Calcd. for $C_{20}H_{25}O_5N$: C, 66.8; H, 7.0. Found: C, 66.9; H, 7.1.

N-Vinyltetrahydrocarbazoles XXXIa, b, c.—The general procedure for dehydrating the 4a-hydroxyhexahydrocarbazoles was to boil the carbinol with an equal weight of picric acid in methanol for 15 minutes. The methanol then was evaporated, and the residue was dissolved in chloroform and poured through a short alumina column to remove the picric acid. Finally, the chloroform was evaporated and the residue was carefully chromatographed on alumina using benzene–chloroform as eluent.

N-(2-Carbethoxyvinyl)-tetrahydrocarbazole (XXXIa) was obtained as a viscous yellow oil by distillation onto a cold finger at 110° (50 μ); ultraviolet absorption: λ_{max} 324 mμ (ε 15,900), 274 (17,500).

Anal. Calcd. for $C_{17}H_{19}NO_2$: C, 75.8; H, 7.1. Found: C, 75.8; H, 7.0.

N-(1,2-Dicarbomethoxy)-tetrahydrocarbazole (XXXIb) was prepared from the crude carbinol XXXb, itself prepared in the same manner as XXXc. The dehydration product crystallized from ethanol and sublimed at 100° (0.2 mm.). It melted at 107–108°; ultraviolet absorption: λ_{max} 340 mμ (ε 6,080), 277 (9,600).

Anal. Calcd. for $C_{18}H_{19}NO_4$: C, 69.0; H, 6.1. Found: C, 68.9; H, 6.0.

N-(2,2-Dicarbethoxyvinyl)-tetrahydrocarbazole (XXXIc) was a yellow oil that was distilled onto a cold finger at 150° (0.1 mm.); ultraviolet absorption: λ_{max} 342 mμ (ε 10,600), 278 (13,300).

Anal. Calcd. for $C_{20}H_{23}NO_4$: C, 70.4; H, 6.8. Found: C, 71.4; H, 6.7.

BERKELEY, CALIF.

[CONTRIBUTION FROM THE REGA INSTITUTE, UNIVERSITY OF LOUVAIN]

The Structure of Factor S of Staphylomycin[1]

BY HUBERT VANDERHAEGHE AND GUIDO PARMENTIER

RECEIVED DECEMBER 17, 1959

Acid hydrolysis of Factor S of Staphylomycin has yielded the products: 3-hydroxypicolinic acid, L-threonine, D-α-aminobutyric acid, L-proline, N-methyl-L-phenylalanine, 4-oxo-L-pipecolic acid and L-phenylglycine. Further work has shown that the sequence of the (amino) acids is in the order given above and that a lactone linkage is present between phenylglycine and the hydroxy group of threonine.

Staphylomycin,[2] an antibiotic produced by a *Streptomyces* related to *S. virginiae*, is very active against Gram-positive bacteria.[3] Its clinical indications are mainly in the treatment of infections of staphylococcal origin.[4]

Paper chromatographic studies have shown the presence of two components active against micrococci.[3] These two components (Factors M I and M II) could be separated on a column of silica gel. Other fractions of the chromatogram, which were essentially inactive against micrococci, increased (up to 2.5 times) the *in vitro* antibacterial activity of Factor M I. From these fractions another product could be obtained in crystalline state. It was named Factor S, because it was more active against *B. subtilis* than Factor M.[5,6]

Factor S is a weak acid (pK'_a 7.7 in dimethylformamide–water, 1:2), soluble in organic solvents and nearly insoluble in water and petroleum ether.[5] The presence of a phenolic group was suggested by the brown-red color obtained with ferric chloride. The presence of this function was also supported

(1) Presented, in part, at the International Congress of Biochemistry, Vienna, September, 1958.

(2) Registered Trade Mark of S.A. R.I.T.

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by the shift of the ultraviolet maximum (305 mμ in neutral or acid solution) in alkaline solution (333 mμ). The fact that the curves obtained in different solvents were practically identical to those observed for Etamycin (Viridogrisein)[7] was a strong indication that Factor S contained the same chromophore (3-hydroxypicolinic acid).

Identification of the Hydrolysis Products of Factor S.—When a total hydrolysate of Factor S (24 hours at 100° in 6 *N* hydrochloric acid) was chromatographed in the system 1-butanol–acetic acid–water followed by water-saturated phenol, or in one direction with 2-butanol–formic acid–water,[8] six ninhydrin-positive spots (I to VI) were found. When a one-directional chromatogram was sprayed with ferric chloride solution, a brown red spot (A) appeared. After a less prolonged hydrolysis, a second brown-red spot (B) was observed. The color reactions with ninhydrin and isatin reagent,[9] and the R_f values had permitted the tentative identification of II as threonine, III as proline and IV as α-aminobutyric acid.[5]

The violet ninhydrin color given by VI was weak and disappeared rapidly. This amino acid gave a red color with *p*-nitrobenzoyl chloride–pyridine, characteristic for N-alkyl amino acids.[10,11] Amino acid V which subsequently has been identified as phenylglycine (α-aminophenylacetic acid), gives a blue color with this reagent. This test is apparently also specific for this amino acid.

A larger amount of Factor S was hydrolyzed and chromatographed on a column of Dowex 50, using gradually increasing concentrations of hydrochloric acid.[12] No evidence was found for the presence in this hydrolysate of products other than those already mentioned.

All products were separated except III and A. The fraction containing these components was resolved by preparative paper chromatography. Melting point, elemental analysis, optical rotation and ultraviolet spectrum (for V, VI and A) identified II as L-threonine (Thr), III as L-proline (Pro), IV as D-α-aminobutyric acid (AmBut), V as L-phenylglycine (PhGly), VI as N-methyl-L-phenylalanine (N-MePhe) and A as 3-hydroxypicolinic acid (HyPic). Thermal degradation of A to 3-hydroxypyridine, and oxidation of VI with hypochlorite[10,13] to methylamine (isolated as the 2,4-dinitrophenyl derivative) and phenylacetaldehyde (converted to the 2,4-dinitrophenylhydrazone) confirmed their structure. The ferric chloride positive substance B was identified as 3-hydroxypicolinylthreonine.

Some racemization was observed with phenylglycine, as could be expected,[14,15] but was less extensive than in the case of phenylsarcosine.[11]

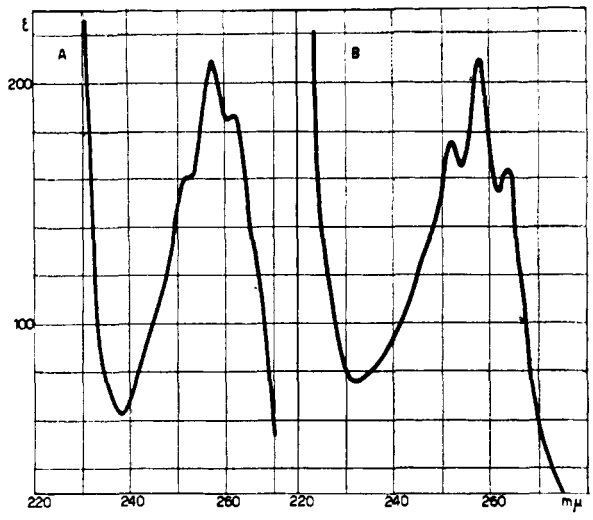

Fig. 1.—Ultraviolet spectra of phenylglycine (curve A) and of N-methylphenylalanine (curve B) in aqueous solution. Phenylalanine presents the same curve as its N-methyl derivative.

A positive shift of the rotation, after acidification, was also observed with N-methyl-L-phenylalanine. This rule, which has been demonstrated to be general for the L-α-amino acids,[16–18] is also valid for the N-methyl-L-α-amino acids.[11,19]

The same phenyl bands are present in the ultraviolet spectra of the aromatic amino acids (PhGly and N-MePhe), but the differences in the extinction values of the maxima permit their identification (Fig. 1).

From the yellow ninhydrin color, it was inferred that the unknown amino acid I ($C_6H_9NO_3$) was an imino acid. As a negative reaction with bromine water eliminated an unsaturated hydroxy-acid, the presence of a carbonyl group was examined. This group was demonstrated by the formation of a dinitrophenylhydrazone. The infrared spectrum (obtained with the hydrochloride) was not very useful for the determination of the structure, because there was overlapping of the carbonyl with carboxyl absorption (5.80 μ with shoulder at 5.76 μ). As the absorption of a five-ring carbonyl occurs at a shorter wave length, both bands could be distinguished in the infrared spectrum of 4-ketoproline hydrochloride (5.65 and 5.75 μ)[20] and hydrobromide (5.65 and 5.81 μ).[21]

Amino acid I was reduced with sodium borohydride, or in the presence of platinum catalyst, to an hydroxyamino acid, which, by treatment with hydrogen iodide and phosphorus, was transformed into pipecolic acid. The oxidation of the hydroxyamino acid with potassium permanganate gave β-alanine, glycine and aspartic acid. Recent studies by Fowden[22] show that these results permit its

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identification as 4-hydroxypipecolic acid. The other possible hydroxypipecolic acids (3- or 5-hydroxy-) could be eliminated, as they easily could be distinguished on a two-dimensional chromatogram with butanol–acetic acid and phenol.[23] Thus, amino acid I has been identified as 4-oxopipecolic acid. As amino acid I gave a negative Fehling reaction, the location of the carbonyl group on C-4 had been considered from the beginning. When the keto group is in the β-position with regard to the imino group, a positive test is obtained.[20,21] This test has not been mentioned in recent studies on 5-oxo-[24] and 3-oxopipecolic acid,[25] but it is likely that it will be positive. Moroever the last mentioned acid, being a β-keto acid, will probably be unstable.

The occurrence of keto-amino acids in nature is rare. It is interesting to note that evidence has been obtained for the presence of 4-oxo-proline in Actinomycin X_2,[26] an antibiotic which also has a cyclopeptide structure.

The reduction of 4-oxopipecolic acid with sodium borohydride or in the presence of platinum catalyst gave a mixture of two hydroxypipecolic acids which could be separated by paper chromatography in the system *t*-amyl alcohol–lutidine.[27] The main component, which is the slower migrating in the just mentioned chromatography system, is considered to be the allo isomer (OH–COOH in *cis* position). It has been shown that the normal (*trans*) isomers of 4-hydroxyproline and 5-hydroxypipecolic acid are the faster moving components on the paper chromatogram,[27] and also on a Dowex 50 column.[24,27,28] We have also been able to obtain pure *allo*-4-hydroxy-L-pipecolic acid by chromatography on a Dowex 50 column. A small amount of L-pipecolic acid, which has been formed during the catalytic reduction in acetic acid, has also been separated. Hydrogenolysis was also observed during the catalytic reduction (in the presence of platinum) of N-carbethoxy-4-oxoproline ester.[20] The proof that all products have the L-configuration is established by the isolation of L-pipecolic acid, and the positive shift of rotation after acidification of 4-oxo- and 4-hydroxypipecolic acid.

It has been shown that the reduction with sodium borohydride of N-carbobenzyloxy-4-oxoproline[21] and -5-oxopipecolic acid,[24] and catalytic reduction (in the presence of platinum in methanol solution) of N-carbethoxy-4-oxoproline ester[20] gives only the allo isomer. The recent observation of Beyerman[29] that sodium borohydride reduction of 5-oxopipecolic acid gives only the normal (*trans*) hydroxyimino acid proves that the bulk of the substituents of the piperidine ring determines the stereochemical course of the reaction. We have observed that sodium borohydride reduction of Factor S (see below) gives about equal amounts of both isomers of 4-hydroxypipecolic acid, and catalytic reduction yields mainly the normal isomer. This is different from the results of the reduction of the free amino acid which yields mainly the allo isomer.

Structure of Factor S.—Factor S was rapidly (after 30 min.) inactivated in 0.1 *N* sodium hydroxide solution. From this solution, Staphylomycin S acid was obtained, which presented two acid functions (pK'_a 5.15 and 8.6 in dimethylformamide–water, 1:1) and gave all the hydrolysis products found with Factor S. As Factor S had only a phenolic acid function,[30] the new acid group must be the carboxyl group of the terminal amino acid which has been liberated by opening the lactone ring. The presence of a six- or higher-membered lactone ring is supported by the 5.74 μ band in the infrared spectrum.[5]

The C-terminal acid was shown to be phenylglycine by hydrazinolysis.[31] This was confirmed by Dakin–West degradation[32,33] which destroys the C-terminal amino acid.

The lactone ring must involve the hydroxyl group of threonine, which is the only hydroxyamino acid present. This was confirmed by the observation that only this amino acid was destroyed when Staphylomycin S acid was treated with chromic acid, whereas no amino acid was affected when Staphylomycin S was submitted to this reaction.

It is interesting to note that $[\alpha]_D$ lactone $-$ $[\alpha]_D$ acid: $-28° - (-45.6°) = +17.6°$ is in the direction predicted by Witkop's application,[24,34] of Hudson's lactone rule to δ- and α-hydroxyamino acids and their lactones. The value obtained should be positive when the carbon atom carrying the hydroxyl group has a D-glyceraldehyde configuration, which is the case for L-threonine.

3-Hydroxypicolinic acid is linked to the amino group of threonine as evidenced by the isolation of 3-hydroxypicolinylthreonine from the acid hydrolysate. The amount of this product was greater when the hydrolysis was less drastic (*e.g.*, 4 hours at 100° in *N* hydrochloric acid). It has already been observed that this bond is particularly resistant.[35] This is also true for antimycic acid, N-(3-aminosalicyloyl)-threonine,[36] and for desamino - actinocinyl - threonine[37] and -bis-(threonine).[38]

From these results the partial formula VII was established, and only the sequence of the amino acids in the central part of the peptide needed to be determined.

Partial hydrolysis of Factor S gave several peptides. The identification of the components

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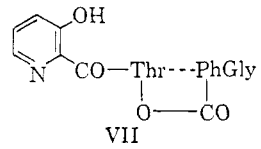


VII

of some of these peptides was difficult because several other ninhydrin-positive spots, probably formed by the decomposition of 4-oxopipecolic acid, were present on the paper chromatogram. Satisfactory results were obtained when this keto amino acid was reduced to hydroxypipecolic acid with sodium borohydride or in the presence of platinum catalyst. A partial hydrolysate, obtained by keeping reduced Factor S in concentrated hydrochloric at 37° for 3 to 5 days, was completely resolved by the system butanol–acetic acid–pyridine–water.[39,40] The fluorescent and ferric chloride-positive peptides were identified as HyPic (Thr, AmBut, Pro) and HyPic (Thr, AmBut, Pro)N-MePhe. The ninhydrin-positive bands were shown to be the two isomers of 4-HyPipec, PhGly, N-MePhe and (Thr,AmBut,Pro). In the hydrolysate of Factor S, hydrogenated in the presence of platinum, a small amount of HyPic was found. In the product reduced with sodium borohydride, another ninhydrin-positive spot, which is probably partially reduced 3-hydroxypicolinic acid, was observed.

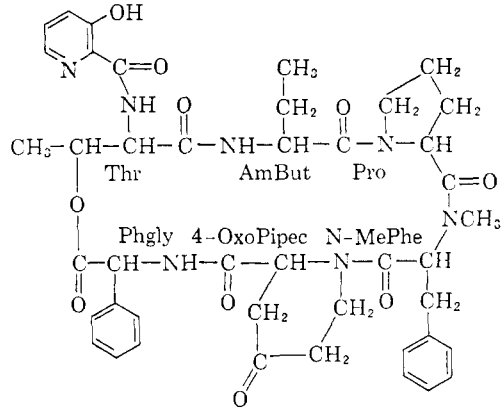
In the partial hydrolysate obtained by alkaline hydrolysis, (4-HyPipec, PhGly) and (N-MePhe, 4-HyPipec) were found.

The place of AmBut and Pro was determined by Edman degradation.[41] For this reaction it was necessary to hydrogenate the 3-hydroxypicolinic acid to the corresponding pipecolic acid (*cf.* ref. 11). In this operation 4-oxopipecolic acid was also reduced. After opening of the lactone ring by saponification, either before or after hydrogenation, Hydrostaphylomycin S acid was treated with phenyl isothiocyanate and the N-terminal amino acid was removed as its phenylthiohydantoin (PTH) derivative. The PTH was identified and the composition of the remaining peptide was examined as described in the Experimental part.

In the first step of the Edman degradation, the phenylthiohydantions (PTH) of 3-hydroxypipecolic acid and of threonine were obtained. It is difficult to interpret this result; especially since this phenomenon was not observed with Etamycin[11] which has a similar structure. One possible explanation is a N,O-peptidyl shift.[42] However, when hydrogenated Staphylomycin S acid was kept overnight in 0.1 *N* sodium hydroxide solution and chromatographed, no free 3-hydroxypipecolic acid was found.

In the second step, not only α-aminobutyric acid but also the C-terminal and the adjacent amino acid, phenylglycine and 4-hydroxypipecolic acid, were removed. Either during the acid cyclization of the first phenylthiocarbonyl peptide, or during the evaporation of the acid solution of the peptide after extraction of the PTH, the last two amino acids had undoubtedly been split off. This secondary reaction could not be avoided, despite the fact that the process was repeated at low temperature. The next step of the degradation established the sequence Pro-N-MePhe. Although the Edman degradation did not proceed satisfactorily, it was possible to establish the sequence Thr-AmBut-Pro-N-MePhe.

As the partial acid and alkaline hydrolysis had already established the sequence N-MePhe-4-oxoPipec-PhGly, the structure of Factor S of Staphylomycin ($C_{43}H_{49}N_7O_{10}$) may be formulated as



The reductions of Factor S are evidence that the keto group of 4-oxo-pipecolic acid is present in the intact molecule, and is not formed during the hydrolysis. This is also supported by the 5.81 μ band in the infrared spectrum,[5] and by the preparation of an oxime. The bands at 6.56 μ and at 5.96 and 6.11 μ may be attributed to the NH and carbonyl groups of the secondary and tertiary amide groups. The last band which is broad contains also the phenyl absorption.

The anti-staphylococcal activity of a mixture containing 30% Factor S + 70% Factor M I is 2.8 times that of Factor M I alone.[6] The activity of a similar mixture of Factor S oxime is 2.2 and of dihydro-S (reduced with sodium borohydride) is 2.0.

Etamycin (Viridogrisein), which is also a 3-hydroxypicolinyl peptide,[11,30] could not be tested in the same proportions because its anti-staphylococcal activity was too high; it was examined at a lower concentration. The activity of a mixture containing 90% of Factor M I and 10% of Etamycin was 2.0 times that of Staphylomycin M I in the cylinder plate assay against *Micrococcus pyogenes* var. *aureus*.[43]

It is also interesting to note that E 129 B,[44] identical with PA 114 B,[45] which presented the same potentiating effect on Staphylomycin M I as Factor S[6] has nearly the same structure. It is also a 3-hydroxypicolinyl peptide containing the same amino acids in the same sequence as in Factor S, but N-methylphenylalanine is replaced by *p*-

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dimethylaminophenyl - N - methylalanine.[46] The synergistic components of the other antibiotics of this group, Streptogramin[47] and Mikamycin,[48] will certainly have a closely related structure.

Other macrocyclic peptide antibiotics, containing an heterocyclic acid, are the actinomycins,[33,49] pyridomycin[50] and echinomycin.[51]

Acknowledgments.—We wish to thank Dr. L. Fowden, University College, London, for his chromatographic studies with our 4-hydroxypipecolic acid. We are indebted to Dr. H. C. Beyerman, Delft, for a sample of 5-hydroxypipecolic acid and to Dr. B. Heinemann, Bristol Laboratories, Syracuse, N. Y., for a sample of Etamycin. The authors wish also to express their appreciation to Dr. M. C. De Wilde-Delvaux for her interpretation of the infrared spectra, and to Mr. L. Verlooy for his excellent technical help.

Experimental[52]

Properties of Factor S.—Factor S was prepared by chromatography on silica gel, and crystallization in methanol and had a m.p. of 240–242°, with sintering at 165–167° (*cf.* ref. 5). When the product was kept in a bath at 170–175° for a few minutes, a substance was obtained which showed only the 240–242° melting point. Its nitrogen analysis and biological activity was unchanged. The other physical constants of the unheated product were: $[\alpha]^{20}D$ −28.0° (*c* 1 in ethanol); λ_{max} in ethanol: 305 mμ (log ε 3.85); in water: 305 mμ (infl.) (log ε 3.13) and 355 mμ (log ε 3.85); in 0.1 *N* hydrochloric acid: 303 mμ (log ε 3.76) and 355 mμ (log ε 3.52); in 0.1 *N* sodium hydroxide: 333 μ (log ε 3.92); in 0.1 *M* phosphate buffer, *p*H 7.0; 350 mμ (log ε 3.82).

Anal. Calcd. for $C_{43}H_{49}N_7O_{10}$: C, 62.67; H, 5.99; N, 11.90; O, 19.42; 2C-CH_3, 3.64; N-CH_3, 1.82; equiv. wt., 823.85. Found: C, 62.69; H, 6.37; N, 11.21; O, 19.45; C-CH_3, 4.36; N-CH_3, 2.37; volatile acid calcd. as CH_3-CO: 0.77; no alkoxy; equiv. wt. (potentiometric titration with 0.1 *N* sodium hydroxide in ethanol): 810 and 821. Average of previous analyses[5]: C, 62.80; H, 6.57; N, 11.47; O, 19.44.[53]

Oxime.—Factor S (205 mg., 0.25 mmole) was treated with hydroxylamine according to the Bryant-Smith method.[5] Calcd. for 1 CO: 2.5 ml. of sodium hydroxide (0.1 *N*). Found: 2.2 ml. The solution was evaporated to dryness, treated with water, the insoluble product was filtered off and recrystallized in ethanol; 105 mg., m.p. 254–255° dec.

Anal. Calcd. for $C_{43}H_{50}N_8O_{10}\cdot H_2O$: C, 60.27; H, 6.11; N, 13.07. Found: C, 59.43; H, 6.23; N, 13.07.

Total Hydrolysis.—Factor S was hydrolyzed in 6 *N* hydrochloric acid (10 mg./ml.) in a sealed tube for various periods of time. The maximum yield, as judged by the colorimetric assay with ninhydrin,[54] was obtained after 24 hours. This solution was dried in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide, and submitted to paper chromatography. Two dimensional chromatograms revealed 6 ninhydrin positive spots, with R_f values (in 1-butanol–acetic acid–water 4:1:5, and in water-saturated phenol): I (0.12, 0.50), II (0.12, 0.46), III (0.20, 0.85), IV (0.29, 0.64), V (0.41, 0.79), VI (0.57, 0.96). Subsequently, it was found that all ninhydrin-positive substances could be separated with the system 2-butanol–88% formic acid–water, 150:30:20[8]: I (0.09), II (0.15), III (0.25), IV (0.33), V (0.49), VI (0.64). When a chromatogram (developed with 1-butanol–acetic acid–water, 4:1:5) was sprayed with 1% ferric chloride solution, a brown red spot A (R_f 0.46) and sometimes a weaker second spot B (R_f 0.84) appeared.

The ninhydrin spots were violet except I and III which were yellow. When a chromatogram was sprayed with isatin reagent,[19] and kept in the dark, a blue spot appeared quickly on the place of III and slowly a lavender spot on IV. The place on the chromatogram and the color reactions suggest that III is proline and IV is α-aminobutyric acid. (*cf.* ref. 55). The R_f values and the test of Schwartz[56] for hydroxyamino acids, identify II as threonine. The color of VI with ninhydrin was not intense, and disappeared quickly. The ninhydrin color of V was also abnormal: intense brown which turned to violet. When a chromatogram was sprayed with *p*-nitrobenzoyl chloride–pyridine reagent,[10] VI gave a red color and V gave a blue color. This test indicates that VI is an N-alkylamino acid; this color test is apparently also specific for V, which has been identified as phenylglycine.[57]

Isolation of the Hydrolysis Products.—The acid hydrolysate, described below, was adsorbed on a column (diameter 34 mm., height 1 m.) containing 1 kg. of Dowex 50 × 8,[60] and eluted with *N* hydrochloric acid[12]; 10-ml. fractions were taken at a rate of 50–60 ml./hour. After 250 tubes were collected, 4 *N* hydrochloric was fed to a 3-l. mixing chamber.[61,62] The Dowex 50 × 8, 200–400 mesh, resin was purified as described by Moore and Stein[63] and reduced to H-form with 1 *N* hydrochloric acid. The column was washed with the same acid until 100 ml. of eluate left, upon evaporation, no residue of salt. From every tenth tube (every fifth in the region of a peak) 0.5 ml. was pipetted off, diluted with 1.5 ml. of water, neutralized with 2 *N* sodium hydroxide (*p*H meter), and the ninhydrin reagent[64] added. The ferric chloride-positive substances were detected by examining the extinction at 300 mμ. The ammonia–amine peak was located by adding Nessler reagent to aliquots of the tubes in the region of the aminobutyric acid peak. Because we had found that the N-methylamino acids gave a color with ninhydrin only at a concentration of 0.5 mg./ml., it was unlikely that we could find the N-alkylamino acid (VI) with this test. For this reason, all ninhydrin and ultraviolet negative tubes (ten grouped together) were evaporated *in vacuo* to dryness. In a second chromatogram, the amino acids V and VI were easily located by using the ultraviolet absorption at 257 mμ.

The tubes of a peak were pooled and evaporated to dryness *in vacuo*. With amino acids I, V and VI, the residues were dried *in vacuo* over phosphorus pentoxide and sodium hy-

(46) F. W. Eastwood, B. K. Snell and A. R. Todd, *J. Chem. Soc.*, 2286 (1960), and personal communication.

(47) J. Charney, W. P. Fischer, C. Curran, R. A. Machlowitz and A. A. Tytell, *Antibiotics & Chemotherapy*, **3**, 1283 (1953).

(48) (a) M. Arai, K. Okabe, J. Yonehara and H. Umezawa, *J. Antibiotics (Japan) Ser. A*, **11**, 21 (1958); (b) K. Watanabe, H. Yonehara, N. Tanaka and H. Umezawa, *ibid.*, **12**, 112 (1959).

(49) H. Brockmann and H. Muxfeldt, *Angew. Chem.*, **68**, 67 (1956).

(50) K. Maeda, *J. Antibiotic (Japan), Ser. A*, **10**, 94 (1957).

(51) W. Keller-Schierlein, M. Lj. Mihailovic and V. Prelog, *Helv. Chim. Acta*, **42**, 305 (1959).

(52) All melting points are uncorrected. The microanalyses were performed by Dr. A. Bernhardt, Mülheim, Germany.

(53) The identification of the hydrolysis products has caused a revision of our earlier formula: $C_{38-39}H_{47-48}N_6O_9$ to $C_{43}H_{49}N_7O_{10}$. Our previous (790) and present (810–821) titration data are more reliable than our molecular weight determined by isothermal distillation (740–750).[5]

(54) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).

(55) A. Saifer and I. Oreskes, *Anal. Chem.*, **28**, 501 (1956).

(56) D. P. Schwartz, *ibid.*, **30**, 1855 (1958).

(57) Waser[58] and Edlbacher[59] have shown that this test, carried out in a test-tube and in the presence of sodium carbonate or sodium hydroxide, gives a violet-blue color with α-amino acids. Plattner and Nager[10] found that this reagent, applied on a paper chromatogram, gave a red color with N-methyl-α-amino acids (except sarcosine), and a violet-blue color with α-amino acids when the chromatogram was sprayed afterward with sodium hydroxide solution. Sheehan, *et al.*,[11] has shown that this reaction was positive with a great number of N-methyl-α-amino acids, and also with N-ethylleucine. Most amino acids give no color,[10,11] but we found that phenylalanine, tyrosine and histidine give a yellow, tryptophan and glycine an orange and phenylglycine a blue spot. Pipecolic acid, 4-hydroxy- and 5-hydroxypipecolic acid give a rose, 3-hydroxypipecolic an orange color, but this reaction is less sensitive (100–150 μg. per spot) than with N-alkyl-α-amino acids. Nipecotic acid, proline and hydroxyproline are negative.

(58) E. Waser and E. Brauchli, *Helv. Chim. Acta*, **7**, 757 (1924).

(59) S. Edlbacher and F. Litvan, *Z. physiol. Chem.*, **265**, 241 (1940).

(60) Dowex 50 × 8 was used instead of Dowex 50 × 4 because a small scale experiment had shown that this resin gave sufficient separation. The column was also maintained at room temperature.

(61) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).

(62) H. Schwab, W. Rieman and P. A. Vaugham, *Anal. Chem.*, **29** 1357 (1957).

(63) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

droxide, and some experiments were performed on the amino acid hydrochlorides. Free amino acids were obtained with an indicator-treated Dowex 2 × 8 (200–400 mesh) column[64] (diameter 1 cm., height 10 cm.). After adding the amino acid solution, the column was washed with water and a small yellow-orange buffer zone was formed by adding 10 ml. of 0.5 *N* acetic acid. The amino acid and acetic acid zones were displaced with 0.1 *N* hydrochloric acid. The effluent, containing the amino acid and a small part of the acetic acid zone, were evaporated under reduced pressure, and recrystallized in the appropriate solvent.

Factor S (1.2 g., 1.45 mmole) was hydrolyzed at 100° in 100 ml. of 6 *N* hydrochloric acid, under nitrogen in a pressure bottle during 24 hours. After cooling, nitrogen was led through the solution, and washed through two wash bottles containing 2 *N* sodium hydroxide. A saturated barium hydroxide solution was added to the sodium hydroxide solution. As no precipitate was obtained, we may conclude that no carbon dioxide was formed during the hydrolysis.

Two-thirds of the hydrolysate was distilled off. The acidity of the distillate, which had been cooled with ice, was reduced (from *p*H −1 to 1) by adding a concentrated sodium hydroxide solution. This solution was distilled again. No volatile organic acids were found by paper chromatography with the system ethylamine–butanol.[65] Methanol, which could have been present as a very stable solvate of factor S, was not detected.[66] This distillate did not give a precipitate with 2,4-dinitrophenylhydrazine, indicating the absence of aldehydes, ketones and keto acids. This test was also negative when performed on an ether extract of the hydrolysate.

The partially concentrated hydrolysate was evaporated to dryness under reduced pressure, dissolved in a small volume of water and put on the Dowex 50 column.

(1) Fractions 171 to 189 gave 240 mg. (1.33 mmoles) of the hydrochloride of **amino acid I**. These crystals were dissolved in absolute ethanol and the precipitate, obtained by adding ether, was triturated with acetone, and gave 147 mg. of a white product, m.p. 193–194° dec. This product decomposed partially when a conversion to the free amino acid with a Dowex 2 column was attempted; $[\alpha]^{20}D$ −12.8 ± 1° (*c* 0.9 in water), +2 ± 1° (*c* 1.0 hydrochloride dissolved in water), +12.5 ± 1° (*c* 1.0 in 5 *N* hydrochloric acid).[67]

Anal. Calcd. for $C_6H_9NO_3 \cdot HCl$: C, 40.13; H, 5.61; N, 7.80; Cl, 19.75; O, 26.75. Found: C, 40.63; H, 5.84; N, 7.80; Cl, 19.14; O (by diff.), 26.60; C-CH_3, 1.15.

The infrared spectrum (KBr pellet) presented the bands at 2.98 and 3.44 μ with secondary maxima at 3.6, 4.0 and 4.2 μ, typical for the amine hydrochlorides. A strong band was present at 5.80 μ with inflection at 5.76 μ.

This amino acid shows only end absorption in the ultraviolet, does not decolorize bromine water, gives a negative Fehling reaction, even on heating, and behaves as a neutral amino acid during paper electrophoresis at *p*H 6.

The dinitrophenylhydrazone was prepared by heating for 15 minutes 90 mg. (0.5 mmole) of amino acid I with 110 mg. (0.55 mmole) of 2,4-dinitrophenylhydrazine in 5 ml. of ethanol containing a drop of concentrated hydrochloric acid; 115 mg. of yellow crystals, m.p. 250–261° dec., was obtained.

Anal. Calcd. for $C_{12}H_{13}N_5O_6 \cdot HCl$: C, 40.06; H, 3.92; N, 19.48. Found: C, 40.26; H, 3.76; N, 19.46.

(2) Fractions 206 to 234 gave 137 mg (1.15 mmoles) of the hydrochloride of **amino acid II**. After conversion to the free amino acid with a Dowex 2 column, and crystallization in ethanol–acetone, 90 mg. of white crystals was obtained, m. p. 253–254° dec., $[\alpha]^{22}D$ −27.5 ± 0.5° (*c* 1.0 in water). The value given for L-**threonine** is −28.3°.[17,68] This amino acid gives a yellow precipitate with periodic acid–Nessler reagent.[69]

Anal. Calcd. for $C_4H_9NO_3$: C, 40.33; H, 7.62; N, 11.76. Found: C, 40.32; H, 7.68; N, 11.60.

(3) Fractions 377 to 408 yielded 166 mg. (1.10 mmoles) of the hydrochloride of **amino acid IV**. The product was adsorbed on a column of Dowex 2. The washings were distilled, and the distillate was titrated with 0.1 *N* hydrochloric acid; 0.4 ml. of this acid was used for neutralization, which corresponds to 0.04 mmole of amine. When other amino acid hydrochlorides are adsorbed on the column and the washings distilled in the same way, no consumption of acid occurred.

The amino acid was eluted from the column, and crystallized from water–ethanol; 104 mg., m.p. 278–280° dec.; $[\alpha]^{22}D$ −7.5 ± 0.5° (*c* 2.0 in water), −19.5 ± 0.5° (*c* 1.5 in 6 *N* hydrochloric acid). The values recorded in the literature for L-α-aminobutyric acid are: m.p. 270–280° dec.; $[\alpha]D$ 8.4 ± 0.5° (*c* 4.0 in water), 18.65° (*c* 4.8 in 6 *N* hydrochloric acid);[70] + 9.3° (water) and +20.6° (in 5 *N* hydrochloric acid).[17]

Anal. Calcd. for $C_4H_9NO_2$: C, 46.60; H, 8.80; N, 13.59. Found: C, 46.53; H, 8.83; N, 13.58.

A sample of this D-α-**aminobutyric acid** was also transformed into the DNP derivative by the method of Sanger.[71] The m.p. was 129–131°; the recorded[72] m.p. is 133°.

(4) **Amino acid III** was present in fractions 461 to 495, and the ferric chloride positive **substance A** in the tubes 461 to 525. All these fractions were combined and evaporated to dryness under reduced pressure; 370 mg. of product was dissolved in 3 ml. of water, and partially neutralized (to *p*H 3.5) with 2 *N* sodium hydroxide. This solution was applied as a streak on heavy paper (50 × 50 cm.), (Schleicher and Schull No. 2230) according to the technique of Brownell, *et al.*[73] The chromatogram was developed overnight (the two paper wicks were kept) with 1-butanol–acetic acid–water, 4:1: 5. The two components were located, by spraying one of the Whatman No. 1 paper sheets which had been in contact with the heavy paper, with isatin reagent, the other with ferric chloride solution. The proline band was eluted with 0.02 *M* acetic acid, and the solution was evaporated. After crystallization in 1-butanol–ether, 122 mg. of product was obtained, which gave upon recrystallization in absolute ethanol–ether 52 mg. of crystals, m.p. 216–218°, $[\alpha]^{20}D$ −80.0 ± 0.5° (*c* 1.0 in water). The value recorded[74] for L-**proline** is −85°.

Anal. Calcd. for $C_5H_9NO_2$: C, 52.16; H, 7.88; N, 12.17. Found: C, 52.31; H, 8.03; N, 12.07.

The product of the other band was eluted with the same solvent. After evaporation to dryness under reduced pressure, it was crystallized in ethanol; 80 mg. of crystals, m.p. 210–211° dec. The ultraviolet spectrum in ethanol, 0.02 *N* hydrochloric acid and 0.02 *N* sodium hydroxide was identical to that described for **3-hydroxypicolinic acid**.[75] This acid, prepared by the method of Urbanski,[76] had a m.p. 210–212° dec. and gave a red color with ferric chloride solution and a yellow color with ferrous sulfate.

Anal. Calcd. for $C_6H_5NO_3$: C, 51.80; H, 3.62; N, 10.07. Found: C, 52.10; H, 3.55; N, 9.98.

3-Hydroxypicolinic acid (50 mg.) was decarboxylated by heating in an oil-bath at 200–210°, until gas evolution had ceased (6–7 min.). The residue was sublimed *in vacuo* at 110–115° (0.5 mm.). Eighteen milligrams of a white product with m.p. 124–126° was obtained. The m.p. was unchanged upon admixture of an authentic sample of 3-hydroxypyridine.

Partial decomposition of 3-hydroxypicolinic acid could easily be detected by paper chromatography with the system 1-butanol–acetic acid–water 4:1:5; R_f of 3-hydroxypicolinic acid 0.45 and of 3-hydroxypyridine 0.63. Chromatography in the same system, together with the color reaction with ferric chloride, was useful for the identification of

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(65) R. M. Manganelli and F. R. Brofazi, *Anal. Chem.*, **29**, 1441 (1957).

(66) F. Feigl, "Spot Tests," Vol. II, Elsevier, Amsterdam, 1954, p. 244.

(67) The value of $[\alpha]D$ was determined with the hydrochloride, but is calculated on the free amino acid. The value of $[\alpha]D$ of the free amino acid was obtained by adding an equivalent amount of sodium hydroxide solution.

(68) V. E. Price, J. B. Gilbert and J. P. Greenstein, *J. Biol. Chem.*, **179**, 1169 (1949).

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(70) K. Vogler, *Helv. Chim. Acta*, **30**, 1766 (1947).

(71) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(72) K. R. Rao and H. A. Sober, THIS JOURNAL, **76**, 1328 (1954).

(73) H. H. Brownell, J. G. Hamilton and A. A. Casselman, *Anal. Chem.*, **29**, 550 (1957).

(74) M. S. Dunn and L. B. Rockland, *Advances in Protein Chem.*, **33**, 295 (1947).

(75) L. R. Fibel and P. E. Spoerri, THIS JOURNAL, **70**, 3908 (1948).

(76) T. Urbanski, *J. Chem. Soc.*, 1104 (1946), states that 3-hydroxypicolinic acid gives a red color with ferrous sulfate; this reagent probably contained ferric ion.

the hydroxypyridines: 2-OH (0.66, orange), 3-OH (0.63, brown-red), 4-OH (0.48, yellow). They were cleanly separated with the system *t*-amyl alcohol–2,6-lutidine (R_f 0.69, 0.90, 0.46). Fluorescence in the ultraviolet light is also very useful in the location of all these products.

(5) Fractions 433 to 454 contained 41 mg. of the ferric chloride-positive **substance B**. Its ultraviolet spectrum also presented a maximum at 305 mμ. It was further purified by paper chromatography as described for the fractions 461 to 525. The product was hydrolyzed in 6 *N* hydrochloric acid at 110° for 24 hr. and gave 3-hydroxypicolinic acid and threonine. A negative ninhydrin and a positive ferric chloride reaction are evidence that B should be formulated as **3-hydroxypicolinylthreonine.**

(6) Fractions 620 to 670 did show some absorption at 300 mμ, and gave upon evaporation 12 mg. of a product that presented a blue color with ferric chloride. It was unchanged upon further hydrolysis. Prolonged hydrolysis of phenylglycine yields a similar product.

(7) Fractions 790 to 905 gave 240 mg. (1.25 mmoles) of the hydrochloride of **amino acid V**, with m.p. 245° dec. and sintering at 215°; 50 mg. of this product was dissolved in 10 ml. of ethanol, and titrated (using a *p*H meter) with 0.1 *N* ethanolic sodium hydroxide. The two inflection points of the curve gave an equivalent weight of 149. All fractions were desalted with a Dowex 2 column, and gave 140 mg. of white plates, m.p. 249–251° dec. The product which had a low solubility in water was recrystallized in that solvent; m.p. 262–263° dec., $[\alpha]^{20}D$ 104 ± 1° (*c* 0.5 in water) and 147 ± 1° (*c* 1.0 in 6 *N* hydrochloric acid). The values recorded for **L-phenylglycine** (α-aminophenyl acetic acid) are +114° (in water) and +168° (in 5 *N* hydrochloric acid).[77] For the m.p. see ref. 78. The ultraviolet spectrum is given in Fig. 1.

The preparation of the free amino acid with the Dowex 2 column was difficult because the product precipitated on the resin. Neutralization with lithium hydroxide and recrystallization in water–ethanol gave better results.

Anal. Calcd. for $C_8H_9NO_2$ (151,16): C, 63.59; H, 6.00; N, 9.27. Found: C, 63.65; H, 6.08; N, 9.27.

(8) Fractions 1020 to 1170 yielded 283 mg. (1.30 mmoles) of the hydrochloride of **amino acid VI**. After crystallization in acetone, the m.p. was 168–170° dec. Titration of 85 mg. of the hydrochloride with 0.1 *N* ethanolic sodium hydroxide solution gave an equivalent weight of 183. All solutions and mother liquors were evaporated and passed through a Dowex 2 column. The eluate was concentrated, and upon addition of ethanol, 125 mg. of white crystals, m.p. 248–250° dec., was obtained. Recrystallization in the same solvent mixture raised the m.p. to 258–260° (dec. and sublimation), $[\alpha]^{20}D$ 0° (*c* 0.5 in water), +21 ± 2° (*c* 3.0 in *N* hydrochloric acid), +27 ± 2° (*c* 0.9 in 5 *N* hydrochloric acid). Fischer and Lipschitz[79] give + 17.7 (*c* 6.3 in *N* hydrochloric acid). We found for **N-methyl-L-phenylalanine**, prepared by the method of these authors,[79] $[\alpha]^{20}D$ 0° (*c* 0.5 in water), +21.8 ± 0.3° (*c* 0.9 and 6.0 in *N* hydrochloric acid), +25.5 ± 0.3° (*c* 0.9 in 5 *N* hydrochloric acid). The ultraviolet spectrum is given in Fig. 1.

Anal. Calcd. for $C_{10}H_{13}NO_2$ (179.21): C, 67.02; H, 7.31; N, 7.82. Found: C, 67.14; H, 7.49; N, 7.65.

Degradation of N-Methyl-L-phenylalanine.—Amino acid VI (110 mg., 0.6 mmole) was treated with equimolecular quantities of sodium hydroxide and sodium hypochlorite as described by Plattner and Nager[19]; 0.17 mmole of amine was obtained which after reaction with fluorodinitrobenzene yielded 30 mg. of yellow crystals, m.p. 174–176°, undepressed upon admixture of 2,4-dinitro-N-methylaniline. The aldehyde, which had the typical odor of phenylacetaldehyde, gave 42 mg. of 2,4-dinitrophenylhydrazone, m.p. 108–110°, which was raised to 119–121° (*cf.* ref. 80) by recrystallization in ethanol. There was no depression upon admixture of an authentic sample.[81]

Reduction of Amino acid I (4-Oxo-L-pipecolic Acid) (1).–Amino acid I hydrochloride (135 mg., 0.75 mmole) was neutralized with an equivalent amount of 0.2 *N* sodium hydroxide solution and 100 mg. of sodium borohydride dissolved in 7 ml. methanol was added. After 1.5 hours at room temperature, the solution was heated on the steam-bath for 5 minutes, cooled, and acidified. The solution was desalted with a Dowex 2 column, and the eluate was concentrated *in vacuo*. The residue was crystallized in an ethanol–acetone mixture; 80 mg. of white crystals, m.p. 257–258° dec.

Anal. Calcd. for $C_6H_{11}NO_2$: C, 49.65; H, 7.64; N, 9.64; O, 33.07. Found: C, 49.65; H, 7.23; N, 9.65; O, 33.36; no $C-CH_3$.

When this **4-hydroxypipecolic acid** was chromatographed in the system *t*-amyl alcohol–2,6-lutidine–water, 178:178:114[27] (descending, 4 days), we observed one main component with R_{ala}[82] 0.87 and a minor one 1.27; we found as R_{ala} for the two isomers of 3-hydroxypipecolic acid, (dec. 282–284° after browning at 240°, prepared by the method of Fowden[22]), 0.95 and 1.67, and for 5-hydroxy-D-pipecolic acid,[29] 1.31 (*cf.* ref. 27). The three hydroxypipecolic acids could be separated on a two-dimensional chromatogram with 1-butanol–acetic acid–water, 4:1.5, and water-saturated phenol. Our amino acid was also chromatographed by Dr. L. Fowden in the four solvents described in ref.[22] and no separation from 4-hydroxypipecolic acid could be detected. 3-Hydroxypipecolic acid gives a brown-black precipitate with periodic acid–Nessler reagent.[69] This reaction is negative with 4-hydroxypipecolic acid.

(2) When the neutralized ethanol solution (3 ml.) of amino acid I (9 mg.) was shaken overnight with hydrogen (2 atm.) in the presence of platinum catalyst, the same mixture of 4-hydroxypipecolic acids with the same relative amounts of isomers was obtained.

(3) Amino acid I hydrochloride (340 mg.) was dissolved in 25 ml. of acetic acid, and hydrogenated for 5 hours in the presence of 150 mg. of platinum catalyst (Adams) at 50° and 4 atm. This mixture contained, as found by paper chromatography, mainly the slower migrating component of 4-hydroxypipecolic acid, some of the other isomer and pipecolic acid. This solution was evaporated, and the residue was eluted on the Dowex 50 × 8 column with gradually increasing concentrations of hydrochloric acid as described above. Tubes 293 to 313 contained only the main component. After evaporation, desalting with Dowex 2 and crystallization in ethanol, 85 mg. of **allo-4-hydroxy-L-pipecolic acid**, m.p. 263–264° dec., was obtained; $[\alpha]^{20}D$ −14.5 ±1° (*c* 1.1 in water), +15.3 ±1° (*c* 0.6 in 5 *N* hydrochloric acid). Tubes 555 to 565 gave 12 mg. of residue. The free amino acid was obtained by passing through the Dowex 2 column; 3.5 mg., dec. 256–258°, $[\alpha]^{20}D$ −25 ±5° (*c* 0.1 in water). The values recorded for L-pipecolic acid are −25.2°[83] and −24.6°.[84]

Reduction and Oxidation of 4-Hydroxypipecolic Acid.—(1) 4-Hydroxypipecolic acid (10 mg.) was heated with 1 ml. of hydrogen iodide (d. 1.96) and 30 mg. of red phosphorus in a sealed tube at 135–140° for 6 hours. The solution was evaporated to dryness, neutralized with Dowex 2, and chromatographed in the system 2-butanol–88% formic acid–water, 150:30:20. It contained pipecolic acid and starting material. (2) 4-Hydroxypipecolic acid (10 mg.) was dissolved in 0.2 ml. of sulfuric acid 1:10, 1 ml. of 1% potassium permanganate solution in sulfuric acid 1:10 was added, and the solution was heated at 60° for 10 minutes. The reaction mixture was diluted with 1 ml. of water, neutralized with barium carbonate, centrifuged, and the supernatant was used for two-dimensional chromatograms; β-alanine (++), glycine (+), aspartic acid (+) and starting material were found. 3-Hydroxypipecolic acid gave, besides starting material, γ-aminobutyric acid (++) and β-alanine (+). These results are not identical to those of Fowden,[22] but are in substantial agreement. Oxidation of 5-hydroxy-

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(78) "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 84.

(79) E. Fischer and W. Lipschitz, *Ber.*, **48**, 360 (1935).

(80) R. Shriner and R. Fuson, "Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1948, p. 229.

(81) When an hydrolysate of Factor S was chromatographed on an analytical Dowex 50 × 8 column, as described by Moore and Stein,[62] the amino acids threonine, proline and α-aminobutyric acid were found at their expected place, 4-oxopipecolic acid at the place of hydroxyproline, and phenylglycine between valine and methionine (peak at tube 260); N-methylphenylalanine was not detected.

(82) R_f of alanine, considered as the unity.

(83) F. E. King, T. J. King and A. J. Warwick, *J. Chem. Soc.*, 3590 (1950).

(84) R. M. Zacharius, J. T. Thompson and F. C. Steward, THIS JOURNAL, **76**, 2908 (1954).

pipecolic acid yields mainly glutamic acid and some aspartic acid.[85]

Separation of Allo-hydroxyproline from Hydroxyproline. As a sample of allo-hydroxyproline was needed for paper chromatographic studies, the separation of the isomers obtained by alkaline isomerization was attempted; 400 mg. of L-hydroxyproline was heated at 180–190° for 24 hours in 40 ml. of a saturated barium hydroxide solution. The solution was filtered to remove the precipitate of silica, neutralized with sulfuric acid and put onto the Dowex 50 × 8 column described above. The components were also eluted with gradually increasing concentrations of hydrochloric acid. Paper chromatography with the system *t*-amyl alcohol–lutidine[27] did show that fractions 286–295 contained HyPro; 296–305, HyPro plus a trace of alloHyPro; 306–315, about equal amounts of the two isomers; 316–327, alloHyPro plus a trace of HyPro (54 mg. after desalting with Dowex 2); 327–339, alloHyPro (4 mg). Elution with hydrochloric acid is less favorable for the separation of these isomers than the buffer solutions used by Piez.[28]

Staphylomycin S Acid. 1. Preparation.—Factor S (164 mg.) was dissolved in 5 ml. of 0.1 *N* sodium hydroxide, and the solution was kept at room temperature for 2 hours. After 0.5 hour, 99% of the biological activity was destroyed. The solution was neutralized with 0.7 ml. of *N* hydrochloric acid, and the white precipitate was filtered off; 110 mg., m.p. 139–143°. After recrystallization in methyl propyl ketone–ether, the m.p. is 141–143°, $[\alpha]^{20}D$ −45.6 ±1° (*c* 1.0 in ethanol); λ_{max} in ethanol: 304 mμ (log ε 3.92); in water: 305 mμ (log ε 3.65) and 348 mμ (log ε 3.70); in 0.1 *N* hydrochloric acid: 304 mμ (log ε 4.00); in 0.1 *N* sodium hydroxide: 334 mμ (log ε 3.94).

Anal. Calcd. for $C_{43}H_{51}N_7O_{11}\cdot H_2O$: C, 60.06; H, 6.21; N, 11.40; equiv. wt., 859.85. Found: C, 60.51; H, 6.54; N, 10.96; equiv. wt., 870 (potentiometric titration with 0.1 *N* sodium hydroxide in dimethylformamide–water, 1:1) and pK_a' 5.15 and 8.6; 860 (in ethanol) and pK_a' 5.6 and 9.2.

When chromatographed in butanol–acetic acid, the R_f value (0.9) of Staphylomycin S acid was the same as that of Staphylomycin S. Both products could be detected by fluorescence in ultraviolet light and with ferric chloride, but not with ninhydrin. After total hydrolysis, all components found for Factor S could be detected on the paper chromatogram.

2. Chromic Acid Oxidation.—Staphylomycin S acid (10 mg.) was treated with chromium trioxide solution as described for Etamycin.[11] After hydrolysis and chromatography in 2-butanol–formic acid–water, 150:30:20, all amino acids were found except threonine which was present only in trace amounts. When Factor S was submitted to the same reaction, all amino acids were present in the hydrolysate.

3. C-Terminal. (a) **Hydrazinolysis.**[31]—A solution of 3 mg. of Staphylomycin S acid in 0.6 ml. of anhydrous hydrazine (distilled from barium oxide) was heated in a sealed tube at 100° for 8 hours. After evaporation of the hydrazine in a desiccator over sulfuric acid, the residue was dissolved in 3 ml. of water, and shaken with 0.3 ml. of benzaldehyde. After centrifugation, the water layer was removed and the benzaldehyde was washed with 1 ml. of water. The two aqueous layers were combined and shaken again with 0.3 ml. of benzaldehyde. The aqueous layer was chromatographed with the system 2-butanol–formic acid.[8] Only phenylglycine could be detected.

(b) **Dakin–West Degradation.**[32,33]—Staphylomycin S acid (10 mg.) was heated with 0.5 ml. of pyridine and 1.25 ml. of acetic anhydride in a sealed tube at 130° for 6 hours. The solvents were evaporated under reduced pressure, the residue was taken up in water and evaporated again, and finally hydrolyzed in 1.5 ml. of 6 *N* hydrochloric acid at 100° for 24 hours. The acid was removed in a vacuum desiccator containing phosphorus pentoxide and sodium hydroxide, and the residue was chromatographed. All amino acids were found except phenylglycine which was present in trace amounts. The aspect of the chromatogram was the same when the residue, before paper chromatography, was dissolved in 5 ml. of 0.5 *N* ammonia, and air was blown through this solution during 20 minutes (in order to oxidize the aminoketone to a pyrazine).

We also obtained the same result when acetylation and decarboxylation were effected at lower temperature (*cf.* ref. 86). Staphylomycin S acid (8 mg.) was dissolved in 3 ml. of acetic anhydride and 2 ml. of pyridine, and heated at 80° for 6 hours. The mixture was stirred with a stream of nitrogen, the volume being kept constant by addition of acetic anhydride–pyridine. The reaction mixture was worked up as described above. Aeration was necessary here.

4. Hydrogenation.—Staphylomycin S acid was dissolved in 40 ml. of acetic acid, and the solution was shaken overnight under hydrogen (3 atm.) in the presence of 150 mg. of platinum (Adams) catalyst. The catalyst was filtered off, and the solvent was removed by freeze-drying. Paper chromatography in 2-butanol–formic acid[8] revealed only one component. The spot was not fluorescent in the ultraviolet but was detected with a modified Rydon and Smith reagent.[87] The peptide could not be found with the usual ninhydrin reagent. A rose color was obtained with ninhydrin in acetic acid[88] at a concentration of 5 mg./ml. Factor S gave no color at the same concentration.[89] A sample was hydrolyzed in 6 *N* hydrochloric acid (24 hours at 100°) and two-dimensional paper chromatography in 1-butanol–acetic acid–water, 4:1:5, and water-saturated phenol revealed the amino acids: threonine, α-aminobutyric acid, proline, phenylglycine, N-methylphenylalanine, 3- and 4-hydroxypipecolic acid and a small amount of pipecolic acid; no ferric chloride-positive substance was present. This product was used for Edman degradation.

Unchanged starting material was recovered when the hydrogenation was attempted in ethanol solution.

Reduction of Factor S. (1).—A solution of 30 mg. of Factor S in 3 ml. of acetic acid was hydrogenated over 15 mg. of platinum oxide (Adams catalyst) at 50° and 50 p.s.i. for 5 hours. This product was hydrolyzed (24 hr. at 100° in 6 *N* HCl), and chromatographed in 1-butanol–acetic acid–water, 4:1:5, and water-saturated phenol. Not only 3- and 4-hydroxypipecolic but also some pipecolic acid was formed, and phenylglycine was partially transformed into cyclohexylglycine.

(2).—A solution of 300 mg. of Factor S in 100 ml. of ethanol was hydrogenated overnight in the presence of 150 mg. of platinum oxide at 50 p.s.i. When a sample of this solution was chromatographed on paper with 2-butanol–formic acid,[8] two spots (R_f 0.71 and 0.91) were detected with a modified Rydon and Smith reagent.[87] The second spot was fluorescent with ultraviolet light and ferric chloride positive. The two substances were separated on a column (diam. 1.5 cm.) of silica gel (10 g.). In the product with R_f 0.91 (eluted with 5–10% acetone–chloroform) only 4-oxopipecolic acid was reduced; in the second product (eluted with acetone after elimination of the mixture of the two products with 20–50% acetone–chloroform) 3-hydroxypicolinic acid and 4-oxopipecolic acid were transformed into 3- and 4- hydroxypipecolic acid. The first product was used for partial hydrolysis studies and the second one for Edman degradation.

(3).—To a solution of 50 mg. of Factor S in 3 ml. of ethanol was added 25 mg. of sodium borohydride dissolved in 2 ml. of water. The *p*H of the solution was adjusted to 8.2–8.5 by adding 5% acetic acid solution. After keeping for 1 hour at room temperature, the mixture was acidified to *p*H 4 by adding 6 *N* hydrochloric acid. The solution was concentrated *in vacuo*, at low temperature, to half-volume, and the residual aqueous suspension was extracted with chloroform. This solution after washing and drying was evaporated. This product was used for partial hydrolysis. The complete hydrolysis revealed besides the unchanged acids, *viz.*, threonine, α-aminobutyric acid, proline, phenylglycine, N-methylphenylalanine, 3-hydroxypicolinic acid, also 4-hydroxypipecolic acid and a ninhydrin positive substance (Nh in the partial hydrolysate), probably formed by partial reduction of 3-hydroxypicolinic acid.

The activity in the anti-Staphylococcus assay of mixture containing 30% of this reduced Factor S and 70% of Factor M I is 2.0 times that of Factor M I. When Factor S is used, the activity increases 2.8 times.[6] When the *p*H is

(85) A. Virtanen and S. Kari, *Acta Chem. Scand.*, **8**, 1290 (1954).

(86) R. G. Shepherd, *et al.*, THIS JOURNAL, **78**, 5067 (1956).

(87) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).

(88) K. A. Piez, F. Irreverre and H. L. Wolff, *J. Biol. Chem.*, **223**, 687 (1956).

(89) The positive ninhydrin reaction, obtained before with Factor S,[5] was probably due to an impurity.

not controlled during the sodium borohydride reduction, the product is inactive probably because the lactone ring is hydrolyzed in the alkaline solution. It is possible that even at *p*H 8.2–8.5, some of the lactone is opened.

Partial Hydrolysis in Acid Solution.—No definite information could be obtained by heating Factor S in dilute acid. The partial decomposition of 4-oxopipecolic acid was probably the cause of these difficulties in the interpretation. For this reason partially reduced Factor S was used.

Dihydro S (15 mg.), obtained by reduction in the presence of platinum, was kept in concentrated hydrochloric acid at 37° for 3 days. The solution was evaporated in a desiccator over solid sodium hydroxide and phosphorus pentoxide. The hydrolysate was applied as a streak on a sheet of Whatman 3 MM paper, and developed (descending) with pyridine–acetic acid–water–butanol, 10:10:50:40.[39,40] The fluorescent bands near the solvent front (F_3, 0.94; F_2, 0.87; F_1, 0.79) were cut off. On the guide strip cut from the remaining paper were found one weakly fluorescent (F_0, 0.50) and five ninhydrin-positive bands (Na, 0.14; Nc, 0.18; Nd, 0.22; Ne, 0.27; Nf, 0.39; Ng, 0.58). To this paper was sewn a sheet of 3 MM paper of the same size as the surface removed. The chromatogram was developed again with the same solvent mixture, in order to improve the separation of the ninhydrin positive bands. All bands were cut out and eluted with 0.02 *N* acetic acid. The eluate was evaporated to dryness, dissolved in 0.5 ml. of 6 *N* hydrochloric acid and hydrolyzed at 100° during 24 hr. The hydrolysate was evaporated to dryness and examined by one- and two-dimensional paper chromatography. Color reactions (ferric chloride, *p*-nitrobenzoyl chloride–pyridine) were also used for the identification. Na and Nc were identified as the isomers of 4-hydroxypipecolic acid. There was always a weak band (Nb) at the upper edge of Na, but the amount was too small for identification. Nd and Ne gave Thr, AmBut, Pro. The amount of Nd was very small. If N-MePhe, which gives a much less sensitive ninhydrin reaction, had been present, it would not have been detected. Nf was PhGly and Ng N-MePhe; F_0 was 3 HyPicolinic acid, F_1 gave 3 HyPic, Thr, AmBut, Pro; and F_2 and F_3, 3 HyPic, Thr, AmBut, Pro, N-MePhe.

The same picture was obtained with partial hydrolysates (3, 4 and 5 days at 37° in concd. HCl) of Factor S, reduced with sodium borohydride, except that no 3-HyPic (F_0) was found. But another ninhydrin-positive band (Nh 0.69) was detected. This is thought to be partially reduced 3-hydroxypicolinic acid.

Partial Hydrolysis in Alkaline Solution.—Factor S (25 mg.), reduced with sodium borohydride, was dissolved in 0.5 ml. of 0.1 *N* sodium hydroxide and kept at 50° for 2 to 5 days. The solution was neutralized with 0.1 *N* hydrochloric acid, and the precipitate of starting material was centrifuged off. The supernatant was chromatographed on Whatman 3 MM paper, as described for the partial acid hydrolysis. Besides a broad fluorescent band (R_f 0.9) which gave all amino acids upon total hydrolysis, two ninhydrin-positive bands were detected. One (R_f 0.35) gave 4-HyPipec and PhGly, the other (R_f 0.7) N-MePhe and 4-HyPipec upon acid hydrolysis.

Edman Degradation.[90]—Hydro S (100 mg.) was treated with phenyl isothiocyanate at *p*H 8.9 after treatment with sodium hydroxide to open the lactone ring as described for Etamycin.[11] The same reaction was also performed, but without sodium hydroxide treatment, with hydrogenated Staphylomycin S acid. The acid peptide solution, after extraction of the phenylthiohydantoin, was evaporated at low temperature in a desiccator or by freeze-drying.

The progress of the degradation was determined by four methods: A, paper chromatography of the PTH's in pyridine–heptane, 3:7[91]; B, paper chromatography of the amino acids obtained by hydrolysis (24 hr.) of the PTH's in 6 *N* hydrochloric acid at 150°; C, or in hydroiodic acid (d. 1.7) at 140° for 2 hr.[92]; D, chromatography of the amino acids obtained by total hydrolysis of the remaining peptide.

Step 1, method A: The spot showed the characteristic pink center of PTH-threonine. As PTH-3-hydroxypipecolic acid has the same R_f value,[11] its presence could not be demonstrated. Method B gave threonine (+), glycine (++), alanine (+), α-aminobutyric acid (++) and 3-hydroxypipecolic acid (+). Method D showed that 3-hydroxypipecolic acid and threonine had been removed.

Step 2: Several spots were present (method A). Method B gave α-aminobutyric acid and phenylglycine. Method C gave the same amino acids and another spot, probably iodopipecolic acid. The hydrolysis of the peptide gave only proline and N-methyl-phenylalanine (method D).

Step 3: Method C gave proline and method D, N-methyl-phenylalanine.

(90) A thorough discussion of the method has been given by H. Fraenkel-Conrat in D. Glick, "Methods of Biochemical Analysis," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1955, p. 383

(91) Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953).

(92) D. F. Elliott and W. S. Peart, *Biochem. J.*, **65**, 246 (1957).

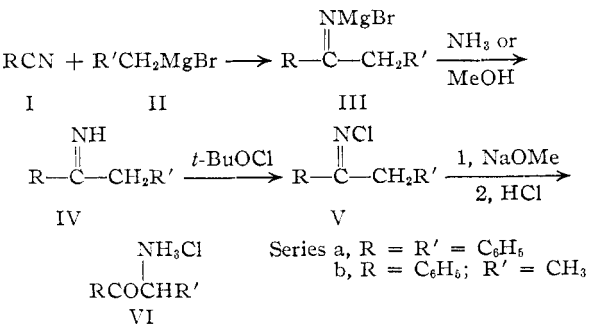
LOUVAIN, BELGIUM

COMMUNICATIONS TO THE EDITOR

REACTIONS OF AMINES. VI. SYNTHESIS OF α-AMINO KETONES AND ACIDS FROM IMINO DERIVATIVES[1,2]

Sir:

In earlier communications it has been shown that α-amino ketones may be prepared in good yields by the base catalyzed rearrangement of N,N-dichloro-*sec*-alkylamines.[1,3,4] We now report the modification of this synthesis as indicated in the charts to permit the preparation by sequence I → VI of α-amino ketones or by sequence VII → XIII of α-amino acids (or their esters) in 20–60% over-all yield from the corresponding nitriles.



With respect to the α-amino ketone sequence, Campbell[5] already has described the preparation of benzyl phenyl N-chloroketimine (Va) by a very similar sequence of reactions, although in his attempts to cause the further reaction of Va with base, he unfortunately chose to employ aqueous bases and thereby failed to complete the sequence.

(1) Paper V, THIS JOURNAL, **82**, 459 (1960).

(2) This work was supported in part by grants G-3689 and G-11339 of the National Science Foundation.

(3) H. E. Baumgarten and F. A. Bower, THIS JOURNAL, **76**, 4561 (1954).

(4) G. H. Alt and W. S. Knowles (*J. Org. Chem.*, in press) have reported the isolation of the intermediate N-chloroketimine in the base-catalyzed rearrangement of N,N-dichlorocyclohexylamine and the subsequent rearrangement of the former to α-aminocyclohexanone.

(5) K. N. Campbell, THIS JOURNAL, **59**, 2058 (1937).