added to a boiling solution of 22 g. of sodium sulfate in 15 ml. of water and 15 ml. of concentrated sulfuric acid. This solution was steam distilled and the distillate made alkaline with sodium hydroxide and extracted with ether. The aqueous fraction was then acidified and extracted with ether. Evaporation of the ether and vacuum distillation of the residue gave 1.2 g. of phenol, b.p. 76–80° (2.5 mm.). The product solidified on cooling and was recrystallized from benzene–ligroin; m.p. 63.5–64.5°.

Anal. Calcd. for $C_7H_5F_3OS$: C, 43.30; H, 2.60. Found: C, 43.15; H, 2.55.

The phenol was converted to the phenoxyacetic acid as usual. After recrystallization from water and vacuum sublimation, it melted at 91.5–92.5°.

Anal. Calcd. for $C_9H_7F_3O_3S$: C, 42.85; H, 2.80. Found: C, 42.56; H, 2.74.

3-Trifluoromethylsulfonylphenoxyacetic Acid.—Our attempts to oxidize 3-trifluoromethylthionitrobenzene to the sulfone according to Yagupolsky and Marenets[36] gave negligible yields. If, however, enough glacial acetic acid was used to increase the solubility of the sulfide in the chromic acid solution, good yields were obtained. In a typical run, 21 g. of 3-trifluoromethylthionitrobenzene was placed in a solution of 16 g. of concentrated sulfuric acid, 25 ml. of water, 10 ml. of glacial acetic acid, and 16 g. of CrO_3. The mixture was refluxed for 50 hr., then poured into ice water and extracted with ether. Evaporation of the ether and vacuum distillation of the residue gave 22.5 g., b.p. 108–112° (1 mm.). After two recrystallizations from ethanol, 16 g. of sulfone of melting point 55–56° was obtained. The 3-trifluoromethylsulfonylnitrobenzene was reduced to the amine and this in turn converted to the phenol. In a solution of 6.3 g. of concentrated sulfuric acid and 22 ml. of water, 6.3 g. of 3-trifluoromethylsulfonylaniline was dissolved. Diazotization was completed with 2.3 g. of sodium nitrite in 7 ml. of water. The diazonium solution was then added dropwise to a boiling solution of 6 g. of sodium sulfate in 45 ml. of water and 45 ml. of concentrated sulfuric acid. The mixture was steam distilled and the distillate extracted with ether. The ether was extracted with sodium hydroxide solution which was then treated with Norit. The alkaline solution was acidified and extracted with ether. Evaporation of the ether and distillation of the residue gave 5.5 g., b.p. 118° (1 mm.). After crystallization from benzene–hexane, the product melted at 72–73°.

Anal. Calcd. for $C_7H_5F_3O_3S$: C, 37.18; H, 2.23. Found: C, 36.90; H, 2.61.

The phenol was converted to the phenoxyacetic acid, which, after recrystallization from water, melted at 101–102°. The yield was very low because of hydrolysis of the $-SO_2CF_3$ function.

Anal. Calcd. for $C_9H_7F_3O_5S$: C, 38.03; H, 2.54. Found: C, 38.33; H, 2.73.

3-Trifluoromethylphenoxyacetic acid was made from a commercial sample of the phenol. After recrystallization from water, it melted at 94.5–95.5°.

Anal. Calcd. for $C_9H_7F_3O_3$: C, 49.10; H, 3.21. Found: C, 48.89; H, 3.46.

3-Trifluoromethoxyphenoxyacetic Acid.—A sample of 3-trifluoromethoxyphenol was kindly supplied by Dr. W. A. Sheppard. This was converted as usual to the phenoxyacetic acid. After recrystallization from water, this substance melted at 89–90°.

Anal. Calcd. for $C_9H_7O_4F_3$: C, 45.77; H, 2.99. Found: C, 46.07; H, 3.27.

4-Trifluoromethoxyacetic acid was prepared by Dr. P. E. Aldrich of du Pont. Its melting point was 87–89°.

3-*n*-Propylphenoxyacetic Acid.—3-*n*-Propylphenol was converted as usual to the phenoxyacetic acid. After recrystallization from water and sublimation, this substance melted at 70.5–72°.

Anal. Calcd. for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27. Found: C, 67.85; H, 7.23.

3-*n*-Butylphenoxyacetic Acid.—3-*n*-Butylphenol was converted as usual to the phenoxyacetic acid. After recrystallization from hexane and subsequent sublimation, the substance melted at 76.5–77.5°.

Anal. Calcd. for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74. Found: C, 68.90; H, 7.78.

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Isolation, Configuration, and Synthesis of Natural *cis*- and *trans*-3-Hydroxyprolines

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3-Hydroxy-L-proline has been isolated from Mediterranean sponge and identified in hydrolysates of collagen of various sources. In addition, a diastereoisomeric 3-hydroxy-L-proline has been found in the antibiotic telomycin. Hydroboration and oxidation of the boron complex of N-carbobenzyloxy-3,4-dehydro-DL-proline methyl ester gave, after saponification and hydrogenolysis, about 70% uniform 3-hydroxy-DL-proline besides 10% *trans*-4-hydroxy-DL-proline. From the absence of 4-allohydroxy-DL-proline and the homogeneity of the synthetic 3-hydroxy-DL-proline, it is concluded that the hydroboration is stereospecific and *trans* with respect to the carboxyl function. The N-tosylmethyl esters of the synthetic *cis*- and *trans*-3-hydroxy-DL-proline and of the natural amino acids from sponge and telomycin, respectively, had identical infrared spectra. *cis*-3-Hydroxy-DL-proline was prepared *via* the 3-ketoproline derivative IV by reduction with sodium borohydride and was found to be identical with 3-hydroxy-L-proline from telomycin with regard to column, paper, and gas chromatographic analysis. This procedure was also used for the preparation of selectively tritiated *cis*-3-hydroxy-DL-proline-3-H^3. Enzymatic studies with D-amino acid oxidase and n.m.r. data confirmed these assignments.

Previous preliminary reports[3,4] from this Laboratory have described the isolation, characterization, and synthesis of the two diastereoisomers of 3-hydroxyproline. The *trans* isomer was isolated from dried Mediterranean sponge and from the antibiotic telomycin. The *cis* isomer was obtained only from telomycin. Independent reports[5,6] from other laboratories have also described the isolation and synthesis of 3-hydroxyprolines. No assignments of configuration have been made.

This paper gives detailed account of the work previously reported and presents additional information on new synthetic approaches.

The key step in the isolation from acid hydrolysates of sponge was the separation of the cyclic amino acids from the primary amino acids by either nitrosation and hydrolysis[7,8] or by treatment of the hydrolysates with 2,4,6-trinitrobenzenesulfonic acid (TNBS)[9] and ion-exchange chromatography[10] on IR-120 resin of the fraction containing the cyclic amino acids. The *trans*- and *cis*-3-hydroxyprolines were separated quantitatively by this method and were easily further purified by recrystallization from aqueous ethanol.

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Isolation of *trans*-3-Hydroxy-L-proline from Mediterranean Sponge. Materials.—The sponge was a dried Mediterranean sponge bought from Yadgi and Co., Inc., Washington, D. C. The resin used for chromatography was Amberlite IR-120, particle size 47–65 μ. The column dimensions were 2 in. × 36 in.; the amount of resin was 1725 ml. Sodium citrate buffer, 0.2 *N*, pH 3.25,[10] was used for elution and was pumped into the column with a peristaltic pump (Sigmamotor, Middleport, N. Y.). The automatic amino acid analyzer used was from Phoenix Precision, Instrument Co., Philadelphia, Pa.

Hydrolysis.—A piece of sponge after it was washed with distilled water and dried weighed 46.1 g.; it was refluxed with 750 ml. of 6 *N* HCl for 24 hr. Variations in the time of hydrolysis, such as 7, 8, 10, and 24 hr, had practically no effect on the yield of 3-hydroxyproline from sponge. The hydrolysate was decolorized with charcoal. The filtrate was evaporated to a sirup and redissolved in 250 ml. of water. This material was used for the preparation of N-nitroso derivatives of the cyclic amino acids as well as for reaction with trinitrobenzenesulfonic acid (TNBS).

N-Nitrosation of the Secondary Amino Acids.[7,8]—Fifty ml. of the above hydrolysate was brought to a volume of 100 ml. by the addition of hydrochloric acid so that the final acidity was 1.0 *N*. To the solution, immersed in a boiling water bath, was added 14 g. of sodium nitrite and the reaction allowed to proceed for 5 min. The flask was then cooled and the solution brought to pH 1.0 by the addition of alkali. The nitrosamino acids were extracted with a total of 125 ml. of ethyl acetate in 5 portions of 25 ml. The combined ethyl acetate extracts containing the N-nitroso derivatives of 3- and 4-hydroxyprolines and proline were dried with anhydrous Na_2SO_4.

Hydrolysis of the N-Nitrosamino Acids.—The dry ethyl acetate extracts were evaporated to dryness *in vacuo*, redissolved in 6 *N* HCl, and, after addition of 2.4 g. of ammonium sulfamate, heated for 90 min. at 105–110°. Excess acid was removed *in vacuo*. The residue was dissolved in 50 ml. of H_2O adjusted to pH 2.5 to 3.0 and desalted on a column (1.25 in. × 8 in.) of Dowex-50W, X-4, 50–100 mesh, in the acid form. The amino acids were eluted from the column with 7.0 *N* ammonia. The eluate was evaporated to dryness *in vacuo* at 25°. The residue was dissolved in 7 ml. of water and the solution adjusted to pH 2.5 with 0.5 *N* HCl. After filtration the clear solution (about 10 ml.) was used for column chromatography.

Trinitrobenzenesulfonic Acid.—The details of the preparation of trinitrobenzenesulfonic acid (TNBS) were obtained from Prof. Kazuo Satake[9] through Dr. S. Ishii: To a stirred solution of 40 g. (0.16 mole) of picryl chloride in 120 ml. of methanol, maintained at a temperature of 55–60°, was added 18 g. of sodium bisulfite ($NaHSO_3$). After 30 min. a second batch of 18 g. of bisulfite was added and stirring continued for an additional 90 min. The insoluble salts were collected, the filtrate adjusted to pH 5.0 with 0.5 *N* HCl, decolorized with charcoal, and evaporated to dryness *in vacuo*. At this stage the TNBS could be isolated as the acid or as the sodium salt. For the isolation of trinitrobenzenesulfonic acid; the residue from the above filtrate was dissolved in 400 ml. of 3.0 *N* sulfuric acid and extracted with ether in a liquid–liquid extractor. The ether layer was taken down to dryness and the residue crystallized from hot 1.0 *N* HCl (charcoal), giving 10 g. (20% yield) of pale yellow crystals. Recrystallization from 1.0 *N* HCl yielded the colorless monohydrate, m.p. 193–194° (loss of water at 100°).

Sodium Trinitrobenzenesulfonate.—The crude trinitrobenzenesulfonic acid from the bisulfite reaction above was dissolved in 300 ml. of water and filtered. To the filtrate was added an equal volume of saturated sodium chloride solution. The sodium salt which crystallized in the cold overnight was collected, washed with saturated salt solution, and dried in the desiccator over P_2O_5. There was obtained 27.4 g. (54% yield).

Trinitrophenylation of the Primary Amino Acids.—To 25 ml. of sponge hydrolysate was added 13.3 g. of TNBS (sodium salt) equivalent to 1.5 times the total nitrogen contents of the sample. The pH was adjusted to 7.5 to 7.8 over a period of 30–45 min. with 20% aqueous sodium carbonate. The reaction mixture was allowed to stay at room temperature overnight. The next day the trinitrophenylamino acids were removed by filtration. The filtrate was desalted on a column (6 in. × 2 in.) of Dowex-1 acetate. The eluate and water washings were collected and passed through a column of Dowex-50W in the acid form. The secondary amino acids were eluted from the column with 7.0 *N* ammonia and the solution evaporated to dryness *in vacuo* at room temperature. The residue was used for column chromatography.

Column Chromatography.—The material to be chromatographed was dissolved in citrate buffer pH 2.28 and poured on a column of IR-120 as described previously. The pump was adjusted to deliver 180 ml./hr. of citrate buffer pH 3.25 and the fraction collector adjusted to collect 14 ml./tube. Under these conditions 3-hydroxyproline was found in tubes 82–95, 4-hydroxyproline in 105–135, and proline in 206–245. The secondary amino acids were detected in the tubes by putting 10-μl. aliquots/tube to a piece of filter paper which was first dipped into a 0.25% ninhydrin solution in acetone and then steamed for 2 min. The spots for the secondary amino acids when viewed under an ultraviolet light exhibit a characteristic reddish fluorescence. When the chromatographic separation was performed with the total hydrolysate from sponge untreated by nitrous acid or trinitrobenzenesulfonic acid, the large amount of aspartic acid interfered with the separation and identification of 3-hydroxyproline. The tubes containing the 3-hydroxyproline were pooled and desalted on a column of Dowex-50W in the acid form. The amino acid was eluted from the column with 7.0 *N* ammonia and the elution was vaporated *in vacuo*. The combined material from 11 column runs, representing 64 g. of sponge, was dissolved in water, decolorized with charcoal, reduced to a volume of 0.5–1.0 ml., brought to a volume of 10 ml. by the addition of absolute alcohol, and kept in the refrigerator at −10° for several days. The crystals (62 mg.) were collected, washed with cold absolute alcohol, and dried *in vacuo* over P_2O_5. The analytical sample was recrystallized a second time, m.p. > 200° dec.; specific rotation: $[\alpha]^{20}D$ −17.4 ± 1.0° (*c* 1.0 in H_2O), $[\alpha]^{20}D$ +13.3 ± 2.0° (*c* 0.5 in 1 *N* HCl).

Anal. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.82; H, 6.74; N, 10.46.

With ninhydrin reagent on paper the amino acid gives a yellow color which turns to brown when the paper is exposed to steam. When viewed under an ultraviolet light the spot exhibits a brick-red fluorescence which is characteristic of the cyclic secondary amino acids. 3-Hydroxyproline gives a bluish color with isatin reagent. It reacts with 1,2-naphthoquinone-4-sodium sulfonate[11] to give a reddish orange color characteristic of the cyclic secondary amino acids.

On the automatic amino acid analyzer[10] the peak due to natural (*trans*) 3-hydroxyproline appears after 95 ml. of effluent (Fig. 4) at 30–50°. The ninhydrin color has a maximum absorption at 440 mμ, similar to 4-hydroxyproline and proline. On a 2-dimensional paper chromatogram[12] it occupies a position directly on top of natural 4-hydroxyproline (ref. 12, Fig. 5).

Preparation of Trichloroacetic Acid (TCA)-Soluble Collagen from Sponge.—Dried Mediterranean sponge (1.8 g.) was extracted with 0.3 *M* trichloroacetic acid in a water bath at 90–100° for 30 min.[13] The TCA extract was put inside a cellophane bag and dialyzed exhaustively in the cold against distilled water for 2 days with several changes of distilled water. The dialyzed material was lyophilized; total N = 15.0%.

The sedimentation pattern of the TCA extract of sponge indicated that the material is monodispersed in the ultracentrifuge in 0.15 *M* citrate buffer at pH 3.65. The analysis of the hydrolysate gave values differing from those of the total sponge hydrolysate that had been pretreated with trinitrobenzenesulfonic acid (Table I).

TABLE I

CYCLIC AMINO ACID ANALYSES OF MATERIALS FROM VARIOUS SOURCES (RESIDUES/10^5 G.)

Cyclic amino acids	Bovine collagen	Rat skin collagen	Sponge collagen (TCA-soluble)	TNBS treated hydrolysate from total sponge	Cell wall of anthrax bacillus[b]
trans-3-Hydroxy-L-proline	2	2	11	6[a]	11
4-Hydroxy-L-proline	63	68	78	47	
L-Proline	97	119	49	39	2

[a] In addition to six residues of *trans*-3-hydroxy-L-proline about one residue of a different hydroxyproline (presumably 4-allohydroxy-D-proline resulting from acid-catalyzed epimerization of 4-hydroxy-L-proline) was found. [b] The cell wall of anthrax bacillus was obtained from Dr. L. Mester, Institut de Chime des Substances Naturelles, Gif-Sur-Yvette, France.

Isolation of *cis*-3-Hydroxy-L-proline from the Antibiotic Telomycin.—One gram of telomycin[14] was hydrolyzed with 100 ml. of 6.0 *N* HCl at 105–110° for 24 hr. The hydrolysate was decolorized with charcoal and concentrated to a sirup repeatedly *in vacuo*. It was dissolved in 50 ml. of water, neutralized by the addition of 5.0 *N* NaOH, and treated with a solution of 2.3 g. of TNBS (sodium salt) in 10 ml. of water. The reaction mixture was

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maintained at pH 7.5–7.8 by the addition of 20% sodium carbonate solution. When (after 1–2 hr.) the pH no longer changed, the reaction mixture was allowed to stand in the dark at room temperature overnight. The yellow precipitate of the trinitrophenylamino acids was removed by filtration and washed with water. The combined filtrate and washings were adjusted to a pH of 3.0 with 1.0 *N* HCl and desalted on a column (2 in. × 6 in.) of Dowex-1, X-4 resin in the acetate form. The effluent and washings, about 370–450 ml., were adjusted to pH 3.0 and desalted on a column (1.25 in. × 9 in.) of Dowex-50W, X-4 resin in the hydrogen form. The cyclic secondary amino acids were eluted with 7.0 *N* ammonia and the solution evaporated *in vacuo* at room temperature.

The TNBS-treated hydrolysate from 2 g. of telomycin in a volume of 5.0 ml. of water was chromatographed on a column of IR-120 following the same technique as that used for the isolation from sponge. The fractions containing the *trans*-3-hydroxyproline were located in tubes 78–111, while the *cis* isomer was contained in tubes 159–190. The respective fractions of the two isomers were pooled, passed through a column of Dowex-50W [H^+], eluted with ammonia, and evaporated to dryness. Recrystallization of the solids from aqueous ethanol (containing 3% water) yielded, respectively, 62 mg. of *trans*-3-hydroxy-L-proline and 70 mg. of *cis*-3-hydroxy-L-proline. Further recrystallization of the two isomers from aqueous ethanol yielded analytically pure samples.

trans-3-Hydroxy-L-proline: $[\alpha]^{20}D$ −15.3 ± 1.0° (*c* 1.00 in H_2O), $[\alpha]^{20}D$ +17.4 ± 2.0° (*c* 0.50 in 1.0 *N* HCl).

Anal. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.88; H, 6.99; N, 10.60.

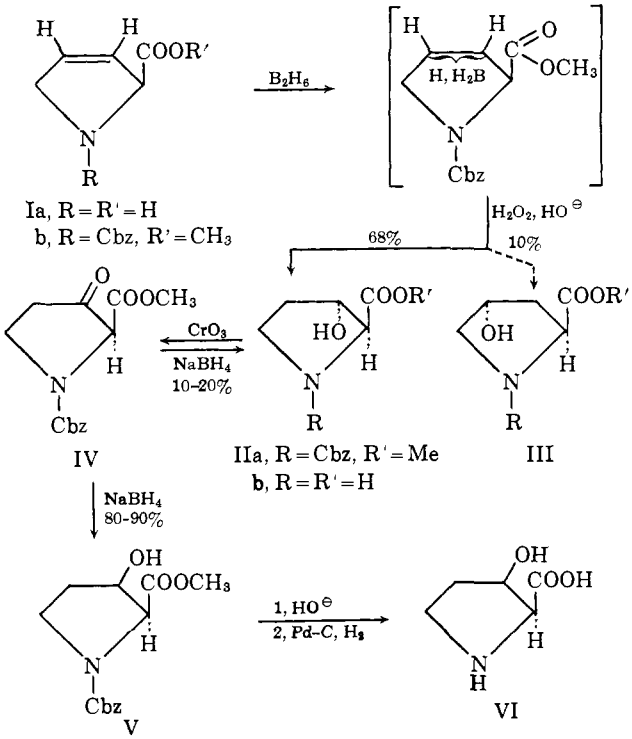
The physical and chemical properties and the color reactions were identical with those given by *trans*-3-hydroxy-L-proline from sponge.

cis-3-Hydroxy-L-proline: $[\alpha]^{20}D$ −91.5 ± 1.5° (*c* 0.61 in H_2O). When the same solution was analyzed by a Rudolph recording spectropolarimeter it gave $[\alpha]^{20}D$ −90.2° (in water), $[\alpha]^{20}D$ −54.3 ± 2.0° (*c* 0.50 in 1 *N* HCl).

Anal. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.68; H, 6.68; N, 10.64.

The *cis* isomer gave the same color reactions with ninhydrin, isatin, and 1,2-naphthoquinone-4 sodium sulfonate as the *trans* isomer.

On the automatic amino acid analyzer the peak due to the *cis* isomer overlapped that of threonine. The yellow color with ninhydrin absorbs maximally at 440 mμ.



The synthesis of one diastereoisomer of 3-hydroxy-DL-proline was accomplished by stereospecific hydroboration[15] of the methyl ester of N-carbobenzyloxy-3,4-dehydro-DL-proline (I)[16] following the procedure of Brown and Zweifel.[17] The resulting alkylborane was oxidized with alkaline hydrogen peroxide, and the product was hydrogenolized to a mixture of isomeric 3- and 4-hydroxyprolines and separated by column chromatography. On the automatic amino acid analyzer[18] the reaction mixture was resolved into 68% 3-hydroxyproline, 10% 4-hydroxyproline, and a trace of 4-allohydroxyproline. The other diastereoisomer of 3-hydroxyproline was not detected on the amino acid analyzer which separates the diastereoisomeric 3- and 4-hydroxyprolines accurately.

An alternate procedure was discovered when conditions for the oxidation of the alkylborane were used which did not lead to saponification of the ester. In that case, the mixture of the N-carbobenzyloxy-*trans*-3- and -4-hydroxy-DL-proline methyl esters was resolved on a silicic acid column. This procedure offers preparative advantages.

The production of only the *trans* diastereoisomers in this synthetic procedure extends Brown's observations on the stereospecificity of the hydroboration reaction to the heterocyclic series. The carbomethoxy group in I not only directs the approach of the boron to the less hindered *trans* side but also preponderantly to the 3-position. Apparently the carbomethoxy group exercises both a steric and inductive control leading to a preferred polarization of the 3,4-double bond. It has been pointed out previously that this 3,4-double bond in dehydroproline and derivatives is stable to reagents known to isomerize double bonds.[19] There is again no indication of noticeable double bond isomerization under the conditions of hydroboration. The product expected from hydroboration (or hydrolysis) of such a rearranged olefin, *viz.*, 2,3-dehydroproline, would be δ-amino-α-ketovaleric acid.

The *trans* assignment to the major product from the hydroboration is further strengthened by the sequence of reactions leading to *cis*-3-hydroxy-DL-proline which proceeded in analogy to the preparation and reduction of 4-keto-DL-proline.[20] The methyl ester of *trans*-N-carbobenzyloxy-3-hydroxy-DL-proline (IIIa) in acetone on oxidation with 8.0 *N* chromium trioxide solution in 50% sulfuric acid gave the 3-oxo compound IV, which was obtained as a chromatographically pure oil after fractionation on a silica gel column. By the criteria of gas, as well as thin-layer chromatography, compound IV proved to be homogeneous. The infrared spectrum of IV contained a band at 5.63 μ characteristic of five-membered ring ketones.

Reduction of the ketone IV with a large excess of sodium borohydride in dimethoxyethane *in the absence of water* yielded a mixture (about 4:1) of *cis* (V) and *trans* (IIa) isomers as shown by gas chromatography. Separation was possible at the stage of the N-carbobenzyloxy esters IIa and V by fractionation on a silicic acid column and elution with benzene–ethyl acetate (4:1). In 50% *aqueous* dimethoxyethane the amount of *trans* isomer decreased to less than 10% as assayed by both gas chromatography and separation on silicic acid.

Inspection of space-filling models suggests that only two relatively unhindered conformations, VII and VIII, exist for the ester function which retain *trans* geometry about the ester C–O bond.[21] In one of these

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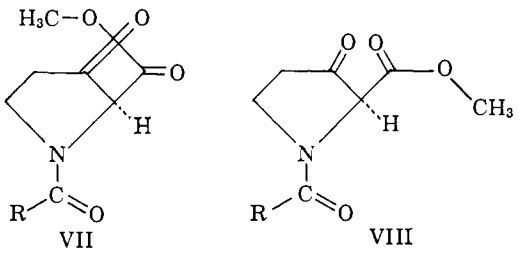


VII VIII

conformations (VII), the borohydride ion is repelled electrostatically by the negative charge of the ester carbonyl; in the other (VIII), the bulk of the methyl group shields the ketonic carbonyl from attack *cis* to the ester. In aqueous media[22] the size of the boron hydride ion is probably increased by conversion to $[HOBH_3]^{\ominus}$,[23] hence the *cis* product predominates more than in dimethoxyethane where the reducing species is $[BH_4]^{\ominus}$. In addition, the presence of a basic or nonbasic nitrogen affects the steric course of reduction of ketoprolines and ketopipecolic acid derivatives[24] in a manner not fully rationalized. The difference in the reduction of 4-ketoproline[20] and 3-ketoproline raises also the question of enolization.

In our first experiments the mixture of V and IIa was converted to the free amino acids by saponification and hydrogenolysis and analyzed by ion-exchange technique. Ratios approaching 1:1 were then observed.[4] However, in this case the complete oxidation of the starting material was in doubt. In addition the effect of base in the process of saponification is not known. The *cis* isomer is saponified in about one-third the time of the *trans* isomer and may not be stable to the prolonged action of base.

When the reduction of the 3-ketoproline derivative IV was carried out with sodium borohydride-H^3 followed by alkaline hydrolysis, hydrogenolytic removal of the carbobenzyloxy residue, and column separation, *cis*- and *trans*-3-hydroxy-DL-proline-3-H^3 were obtained which were utilized for incorporation studies into the peptide moiety of actinomycin analogs.[25]

It was ascertained by gas and column chromatography that no interconversion took place between the *cis* and *trans* isomers under conditions of hydrolysis, *viz.*, on prolonged treatment (24 hr.) with mineral acid or alkali. Destruction by acid under these conditions does not exceed 10%.

The synthetic and natural *cis*-3-hydroxyprolines proved to be inseparable by paper chromatography in five solvent systems (Table II), by ion-exchange chromatography, and by high voltage paper electrophoresis. The infrared solution spectra of N-tosyl-*cis*- and -*trans*-3-hydroxy-DL- (synthetic) and -L-prolines (from sponge and telomycin) were identical.

Enzymatic Oxidation of 3-Hydroxyprolines.—The absolute configuration of the amino acids was determined by measuring the oxygen consumption of the natural and synthetic *trans*- and *cis*-3-hydroxyproline in enzymatic dehydrogenation with D-amino acid oxidase. Figure 1 shows that the synthetic substrates and D-alanine consumed almost the theoretical amount of oxygen (based on the D-form), whereas the natural *cis*- as well as *trans*-3-hydroxyprolines and L-alanine consumed no oxygen at all.

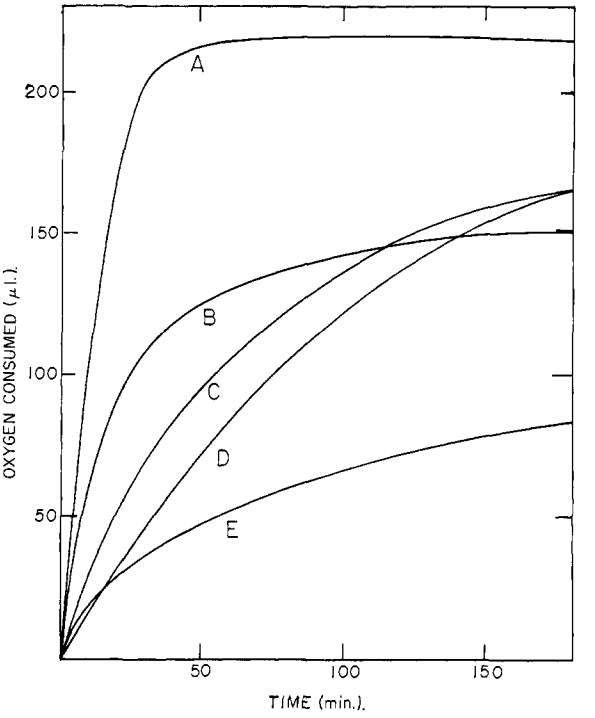


Fig. 1.—Oxidation of various secondary cyclic amino acids by D-amino acid oxidase in relation to D-alanine: A, D-alanine; B, 4-allo-hydroxy-D-proline; C, *cis*-3-hydroxy-DL-proline; D, *trans*-3-hydroxy-DL-proline; E, 3,4-dehydro-DL-proline.

TABLE II

R_f VALUES OF THE NATURAL AND SYNTHETIC *cis*- AND *trans*-3-HYDROXYPROLINES

(Paper chromatography, descending technique)

	R_f			
	trans		*cis*	
Solvent system	Natural[a] (L)	Synthetic (DL)	Natural (L)	Synthetic (DL)
Methanol–pyridine–H_2O (20:1:5)	0.39	0.39	0.35	0.35
1-Butanol–pyridine–H_2O (1:1:1)	.36	.36	.35	.35
Ethanol–formic acid–H_2O (12:3:5)	.57	.57	.62	.61
t-Amyl alcohol–2,4-lutidine–H_2O (178:178:110)	.66[b]	.65[b]	.43[b]	.43[b]
t-Butyl alcohol–formic acid–H_2O (70:15:15)	.35	.36	.29	.27

[a] Both compounds from sponge and telomycin. [b] Instead of the solvent front the R_f of valine is taken as a reference for the mobility of the 3-hydroxyprolines in this solvent system of high resolving power.

It is clear, therefore, that the natural amino acids belong to the L-series, a fact which is further supported by the rotational data (Table III) which obey the rule of Lutz and Jirgensons.[26]

Oxidation of *trans*-3-hydroxy-DL-proline by D-amino acid oxidase produces a dehydro compound, presumably IX, whose catalytic hydrogenation yields mainly *cis*-3-hydroxy-L-proline, accompanied by proline (hydrogenolysis of the "allylic" hydroxyl). Oxidation of *trans*-3-hydroxy-DL-proline may also be effected by the action of hydrogen peroxide and Cu^{++} ions, producing DL-IX, which, upon hydrogenation, forms *cis*-3-

carbobenzyloxymethyl ester derivatives of *cis*- and *trans*-hydroxyproline, 3-ketoproline and 3,4-dehydroproline show a characteristic doubling of the O-methyl group in the n.m.r. spectra. The two components are of about equal intensity and cannot be due to splitting because the separation is solvent dependent (8–9 c./sec. in deuteriochloroform, 4–5 c./sec. in acetonitrile at 60 Mc./sec., 40° probe temperature). Temperature dependence studies are in progress to prove the existence of a conformational equilibrium due to restricted rotation either at the ester carbonyl and/or the amide carbonyl.

(22) *Cf.* H. O. House, H. Babad, R. B. Toothill, and A. W. Noltes, *J. Org. Chem.*, **27**, 4141 (1962).

(23) H. C. Brown and K. Ichikawa, *Tetrahedron*, **1**, 214, 221 (1957).

(24) A. V. Robertson, E. Katz, and B. Witkop, *J. Org. Chem.*, **27**, 2676 (1962).

(25) *Cf.* E. Katz and H. Weissbach, *J. Biol. Chem.*, **238**, 666 (1963).

(26) O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); *cf.* M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **77**, 716 (1955).

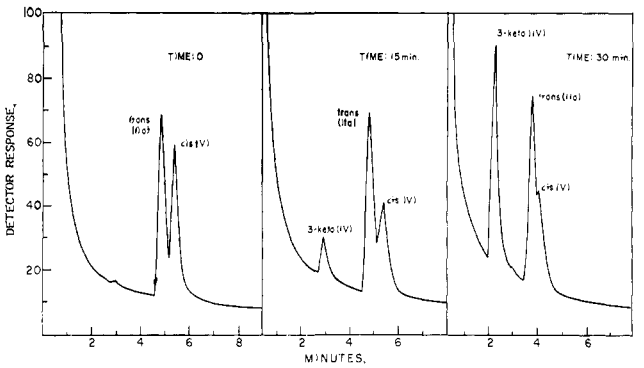
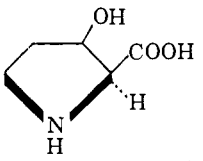
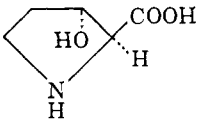


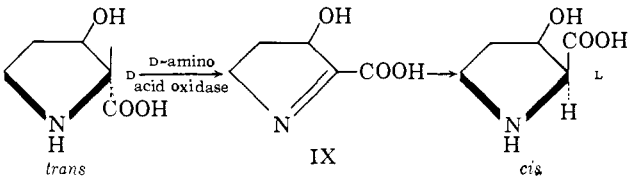
Fig. 2.—Preponderant disappearance of N-carbobenzyloxy-*cis*-3-hydroxyproline methyl ester relative to the *trans* compound on oxidation with chromic acid.

hydroxy-DL-proline. Sodium borohydride carries out this reduction more selectively and without the formation of proline. When *cis*-3-hydroxy-DL-proline was oxidized with D-amino acid oxidase the resulting mixture was easily separable on a column of IR-120 ion-exchange resin into *cis*-3-hydroxy-L-proline and s-3-hydroxy-$\Delta^{1,2}$-pyrroline-2-carboxylic acid. Reduction with sodium borohydride gave *cis*-3-hydroxy-D-proline.

TABLE III

APPLICATION OF THE LUTZ–JIRGENSONS RULE TO *cis*- AND *trans*-3-HYDROXY-L-PROLINE

Formula	$[\alpha]^{20}D$ in H_2O	$[\alpha]^{20}D$ in 1.0 *N* HCl	Δ
 *cis*(from telomycin)	−91.5 ± 1.5°	−54.3 ± 2°	+37.2°
 *trans*(from sponge)	−17.4 ± 1°	+13.3 ± 2°	+30.7°

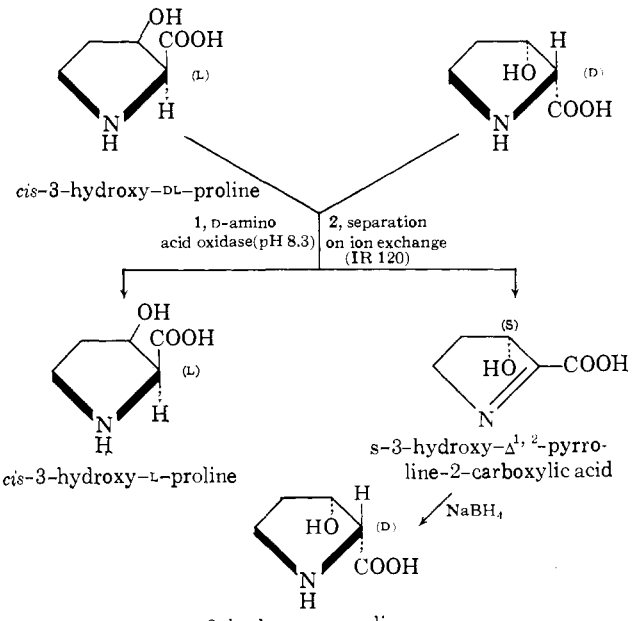


*This method provides a new way for the preparation of cis-3-hydroxy-*D- *and* -L-*proline in addition to the conventional enzymatic resolution by leucine amino peptidase (hog kidney acylase).*

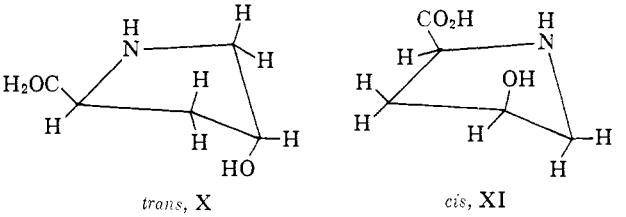
Conformational Considerations.—Relative rates of saponification of the *trans*-3-hydroxyproline ester, according to preliminary results obtained by gas chromatograpic analysis (see above), were higher than for the *cis*-ester.[27] Similarly, when a mixture of the *cis* and *trans* isomers V and IIa was exposed to brief chromic acid oxidation, the analysis of the resulting mixture by gas chromatography (Fig. 2) as a function of time showed the preponderant disappearance of the *cis* isomer V, in which nonclassical (Pitzer) strain between OH and COOR leads to steric acceleration.[28]

(27) *Cf.* T. C. Bruice and T. H. Fife, *J. Am. Chem. Soc.*, **84**, 1973 (1962).

(28) J. Schreiber and A. Eschenmoser, *Helv. Chim. Acta*, **28**, 1529 (1955).



The rate of oxidation of the two 4-hydroxyprolines X and XI[29] is less affected by the relative position of the



carboxyl group. The results on oxidation of a mixture of the two hydroxyprolines reflect this situation (Fig. 3). Although *cis*- or allohydroxyproline disappears somewhat faster, there is not the same striking difference between oxidation of the *cis* and *trans* isomer as with the two 3-hydroxyprolines.

Incomplete permanganate oxidation gave glycine and β-alanine in amounts not significantly different for *cis*- and *trans*-3-hydroxy-L-proline (Table IV). It was not possible to establish clearly the expected relationship between *trans*-3-hydroxy-L-proline and *erythro*-β-hydroxy-L-glutamic acid (XIII), on the one hand, and *cis*-3-hydroxy-L-proline and *threo*-β-hydroxy-L-glutamic acid (XIII), on the other hand, although only XIII was observed (in traces) in the oxidation of *cis*-3-hydroxy-L-proline.

TABLE IV

OXIDATION OF *cis*- AND *trans*-HYDROXY-L-PROLINES WITH AQUEOUS PERMANGANATE

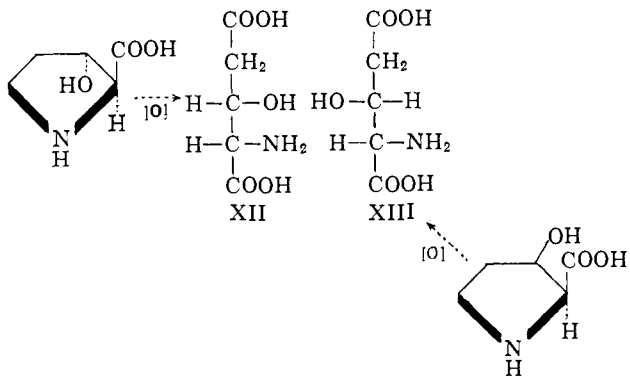
3-Hydroxy-L-proline	Glycine, %	β-Alanine, %	β-Hydroxy-L-glutamic acid[b]
cis-	3.9–5.2	21–35[a]	Traces of *threo* (and *erythro*)
trans-	1.1–3.2	10–18	Trace of *erythro*

[a] The results reflect variations in procedure. The lower figures result from oxidations at room temperature, the higher one at 40–60° for 1–4 hr.; in each experiment 8.6 μmoles of compound in 0.5 ml. of 1.0 *N* H_2SO_4 was mixed with 0.1 ml. of 0.1 *N* $KMnO_4$ in 1.0 *N* H_2SO_4. After the discharge of color the reaction mixture was desalted on Dowex-50W (H^+) and examined on an automatic amino acid analyzer. [b] *Cf.* T. Kaneko and R. Yoshida, Japanese Patent 2174 (1961); *Chem. Abstr.*, **56**, 8837 (1962). We are greatly indebted to Prof. Kaneko for his kind donation of reference samples.

In addition to the conventional enzymatic resolution by leucine amino peptidase (hog kidney acylase).

A more thorough investigation of this kind, *e.g.*, the exact relative rates of chromic acid oxidation of 3-

(29) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, **5**, 513 (1962).



and 4-hydroxyproline derivatives by the methods of Westheimer[30] and Kwart,[31] may permit certain conclusions with regard to the relative strain between functional groups, *i.e.*, steric acceleration and, indirectly, their conformations in the five-membered ring system.

The n.m.r. spectra of *cis*- and *trans*-3-hydroxyproline in D_2O show a clear difference with regard to the splitting of the peak due to the C-2 proton. Decoupling at appropriate frequencies confirmed the assignment of peaks to individual protons. The C-2 proton peak in the *cis* compound was a doublet indicating $J_{2,3}$ = 4 c.p.s., while that of the *trans* compound was an unresolved but broader peak, indicating $J_{2,3} < 1$ c.p.s. From the general relationship between coupling constant and dihedral angles these findings offer support, in a qualitative manner, for the assignments of *cis* and *trans* structures independent of the stereochemistry of hydroboration. In a quantitative sense the numerical values for the coupling constants are much lower than expected on the assumption of a planar five-membered ring and applicability of the Karplus equation. There are two possible conclusions: either the Karplus equation is not readily applicable to this 5-membered ring system, or the pyrrolidine ring in 3- and 4-hydroxyprolines is considerably puckered,[29] a conclusion which has also been reached for cyclobutane derivatives.[32]

The infrared spectra (0.005 *M* in CCl_4) of *trans*-N-carbobenzyloxy-3-hydroxyproline methyl ester (strong band at 3610 cm.$^{-1}$ low half-intensity band width 22 cm.$^{-1}$, slight shoulder at 3580 cm.$^{-1}$) and of *cis*-N-carbobenzyloxy-3-hydroxyproline methyl ester (similar but much broader band at 3603 cm.$^{-1}$, $\Delta\gamma^1/_2$ = 58 cm.$^{-1}$, shoulder at ~3500) indicate at best weak bonding of the hydroxyls to a π-electron center but do not provide further evidence for *cis* and *trans* assignments.

Experimental

Benzyloxycarbonyl-3,4-dehydro-DL-proline Methyl Ester (Ib). Benzyloxycarbonyl-3,4-dehydro-DL-proline[16] (25.0 g., 0.9 mole) was dissolved in ether (50 ml.) and treated gradually with an excess of diazomethane in ether during 30 min. Esterification proceeded rapidly and the excess of reagent was destroyed by dropwise addition of acetic acid. The reaction mixture was evaporated, leaving the methyl ester as a colorless oil (26.0 g., 99%) which has not yet been obtained crystalline. A sample was pumped at 80° (1 mm.) overnight and analyzed without further purification. Vapor phase chromatography showed that the oil was at least 99% pure. The nuclear magnetic resonance spectrum contained only the expected peaks whose area agreed with the ester formulation Ia.[33]

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 63.49; H, 5.97; N, 5.88.

(30) F. H. Westheimer and N. Nicolaides, *J. Am. Chem. Soc.*, **71**, 25 (1949).

(31) H. Kwart and P. S. Francis, *ibid.*, **81**, 2116 (1959).

(32) J. M. Conia, J.-L. Ripoll, L. A. Tushaus, C. L. Neumann, and N. L. Allinger, *ibid.*, **84**, 4982 (1962).

(33) The interesting splitting present in the n.m.r. spectrum will be discussed separately (L. F. Johnson, A. V. Robertson, J. N. Shoolery, and B. Witkop, in preparation).

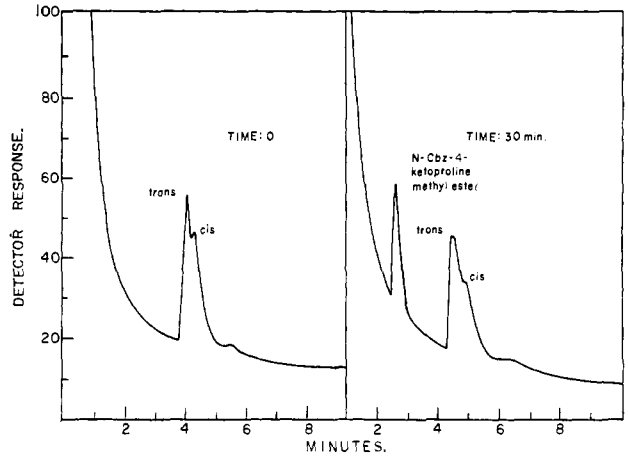


Fig. 3.—Almost identical rate of disappearance of N-carbobenzyloxy-*cis*-(allo)-4-hydroxy- and N-carbobenzyloxy-*trans*-4-hydroxyproline methyl esters on oxidation to N-carbobenzyloxy-4-ketoproline methyl ester with chromic acid.

A. Hydroboration with Saponification of the Ester Ib. Benzyloxycarbonyl-3,4-dehydro-DL-proline methyl ester (Ib, 10.5 g., 40 mmoles) was dissolved in dry tetrahydrofuran (50 ml.) and treated with diborane (15 mmoles) at 0°. The diborane was generated by the dropwise addition (1 hr.) of a solution of sodium borohydride (0.84 g.) in diglyme (36 ml.) to a magnetically stirred solution of boron trifluoride etherate (5.6 ml.) in diglyme (7 ml.).[17] A slow stream of dry nitrogen was used to carry the diborane into the olefin solution. When the borohydride addition was complete, the diborane generator was heated at 70–80° for 1 hr. and then disconnected. The olefin reaction mixture was left at room temperature for another hour. Excess of diborane was decomposed cautiously with water and the alkylborane was oxidized by the addition of 3.0 *N* sodium hydroxide (6.0 ml.) followed by 30% hydrogen peroxide (5 ml.) drop by drop (10 min.). During the peroxide addition, the temperature of the magnetically stirred reaction mixture was kept below 40° by using a bath of water at room temperature. Then 14 ml. of 3.0 *N* sodium hydroxide was added to complete the hydrolysis of the methyl ester. Initially two layers formed, but a homogeneous solution was obtained by stirring rapidly for an hour and the alkaline solution was left for another hour. The reaction mixture was diluted with water (200 ml.) and extracted with ether (3 × 100 ml.) to remove neutral material. The aqueous phase was acidified at 0° with concentrated hydrochloric acid (40 ml.) and the oil which precipitated was extracted into ether (3 × 50 ml.). The combined ether extracts were dried over magnesium sulfate and evaporated, leaving the crude mixture of benzyloxycarbonylhydroxyprolines as an oil (6.8 g.). The oil was dissolved in a mixture of ethanol (200 ml.), water (60 ml.), and acetic acid (40 ml.) and the protecting group was hydrogenolyzed with hydrogen at room temperature and atmospheric pressure in the presence of 10% palladium-on-charcoal (2 g.). Gas uptake was initially very rapid, but the reaction rate then slowed and the hydrogenation was left overnight for completion. The catalyst was filtered off and the filtrate was evaporated. The residue of crude hydroxyprolines, 3.2 g., could not be readily crystallized and was purified by filtration on 100 ml. of Dowex 50W (H^+). The impurities were removed from the column by washing with water until the eluate was neutral. The amino acids were then eluted with 2 *N* ammonium hydroxide (250 ml.). Evaporation of this fraction *in vacuo* gave an off-white solid residue (2.3 g.). The purified amino acid mixture was recrystallized by dissolving it in the minimum volume of boiling ethanol and adding acetone (*ca.* 50 ml.) at the boiling point; yield of white crystals, 1.9 g. The composition of this mixture was determined on an automatic amino acid analyzer to be: *trans*-3-hydroxy-DL-proline, 68%; 4-hydroxy-DL-proline, 10%; allo-4-hydroxy-DL-proline, <1%; ninhydrin-negative material, 21%.

***trans*-3-Hydroxy-DL-proline by Direct Recrystallization.**—Slow recrystallization of the above product from ethanol–water gave 49% of a colorless crystalline product which contained 94.4% *trans*-3-hydroxy-DL-proline and 5.6% 4-hydroxy-DL-proline. A second recrystallization gave 47% of 100% pure *trans*-3-hydroxy-DL-proline, identical with the material prepared by column chromatography (see below).

Anal. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 46.01; H, 6.98; N, 10.62.

The same analytically pure homogeneous *trans*-3-hydroxy-DL-proline was also obtained by preparative chromatography on a 2 in. × 36 in. column of Amberlite IR-120, 47–65 μ, and the use of

citrate buffer pH 3.25 for elution as described under the isolation procedure from sponge.

N-Tosyl-*trans*-3-hydroxy-DL- (and L)-proline Methyl Ester.—To a stirred solution of *trans*-3-hydroxy-DL-proline (15 mg.) in 4 ml. of 0.2 *N* KOH was added pulverized *p*-toluenesulfonyl chloride (30 mg.) and the mixture was stirred at 0° for 2 hr. The mixture was extracted with ether and the ether extract was discarded. The aqueous solution was then acidified, extracted with ether, and the ethereal solution was washed with water, dried, and evaporated to give N-tosyl-*trans*-3-hydroxy-DL-proline (27 mg.). After methylation with diazomethane and recrystallization from ether, N-tosyl-*trans*-3-hydroxy-DL-proline methyl ester was obtained as colorless cubes, m.p. 99–100°, yield 21 mg.

Anal. Calcd. for $C_{13}H_{17}NO_5S$: C, 52.17; H, 5.73; N, 4.68. Found: C, 52.11; H, 5.54; N, 4.70.

Likewise, *trans*-3-hydroxy-L-proline, obtained from telomycin, was converted to N-tosyl-*trans*-3-hydroxy-L-proline methyl ester, which crystallized as colorless platelets, m.p. 101–102°.

The infrared spectra of synthetic and natural N-tosyl-*trans*-3-hydroxyproline methyl ester in chloroform solution were superposable.

B. Hydroboration of N-Carbobenzyloxy-3,4-dehydro-DL-proline Methyl Ester (Ib) without Saponification.—To a stirred solution of N-carbobenzyloxy-3,4-dehydro-DL-proline methyl ester (Ib, 1.0 g.) in 50 ml. of dimethoxyethane (DME) at 0° was added an excess of diborane (generated from 300 mg. of 82% $LiBH_4$ and 4 ml. of 20% BF_3 in DME) over a period of 5 min. After 20 min., an aliquot of the reaction mixture was examined by gas chromatography (SE-30, 1% on GCP; 190°)[34] which indicated the complete disappearance of the starting material. Careful addition of water decomposed the excess diborane. The borane complex was oxidized by addition of 20 g. of K_2CO_3 and 5 ml. of 30% H_2O_2 and allowed to stand at room temperature for 2 days with occasional agitation. Inorganic salt was removed by filtration and the cake was washed with dimethoxyethane. The combined filtrate and washings were evaporated *in vacuo*, redissolved in ether, and the ethereal solution was washed, dried, and evaporated, giving 0.9 g. of an oily residue. Gas chromatographic examination of the oil, after acetylation with acetic anhydride in pyridine, showed the presence of N-carbobenzyloxy-*trans*-3-hydroxy-DL-proline methyl ester and of N-carbobenzyloxy-4-hydroxy-DL-proline methyl ester in a ratio of 4:1.

A. Isolation of N-Carbobenzyloxy-*trans*-3-hydroxy-DL-proline Methyl Ester (IIa).—Elution of the mixture from a column of silicic acid (mesh 100–200) with benzene–ethyl acetate (4:1) gave as the more rapidly moving component 440 mg. of N-carbobenzyloxy-*trans*-3-hydroxy-DL-proline methyl ester, which crystallized in colorless prisms, m.p. 81–82°, undepressed on admixture with a synthetic sample prepared under B.

Anal. Calcd. for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 60.49; H, 6.20; N, 5.01.

N-Carbobenzyloxy-4-hydroxy-DL-proline Methyl Ester (IIIa).—The slower-moving component (93 mg.) was then eluted from the column with the same solvent system. The identity with an authentic sample was confirmed by direct comparison on thin-layer chromatography (Brinkmann Silica Gel G, benzene–ethyl acetate [4:1]) and gas chromatography.

B. N-Carbobenzyloxy-*trans*-3-hydroxy-DL-proline Methyl Ester (IIa) from *trans*-3-Hydroxy-DL-proline.—A mixture of 100 mg. of *trans*-3-hydroxy-DL-proline in 5 ml. of 1.0 *N* KOH and 0.4 ml. of benzyl chloroformate in 4 ml. of benzene was stirred for 45 min. at 0°. The mixture was diluted with 50 ml. of water, extracted with ether, and the ether layer discarded. The aqueous phase, acidified with 1.0 *N* HCl to pH 2, was re-extracted with ether. The ether extract was washed with salt solution, dried, and treated with diazomethane in ether. Evaporation of the solvent yielded N-carbobenzyloxy-*trans*-3-hydroxy-DL-proline methyl ester as an oil (182 mg.) which became crystalline on standing. Recrystallization from isopropyl ether gave cubic crystals, m.p. 80–81°. This product is identical with that obtained by the hydroboration of N-carbobenzyloxy-3,4-dehydro-DL-proline methyl ester.

Conversion of N-Carbobenzyloxy-*trans*-3-hydroxy-DL-proline Methyl Ester to *trans*-3-Hydroxy-DL-proline.—A solution of 1.42 g. (5 mmoles) of N-carbobenzyloxy-*trans*-3-hydroxy-DL-proline methyl ester (IIa) in 10 ml. of dimethoxyethane was treated with 10 ml. of 1.0 *N* KOH and allowed to stand at room temperature for 1 hr. under nitrogen. After removal of the organic solvent at room temperature, the aqueous mixture was extracted with ether and the ether extract discarded. The solution was acidified, re-extracted with ether, and the ether extract was washed, dried over anhydrous Na_2SO_4, and evaporated to dryness giving an oily acid (1.31 g.) which was dissolved in 50 ml. of methanol–water (1:1) and hydrogenated over palladium-on-charcoal catalyst. Removal of the catalyst and evaporation of the solvent *in vacuo* gave a crystalline product which on further recrystallization from ethanol–water (80:70) yielded 510 mg. (80%) of colorless crystalline *trans*-3-hydroxy-DL-proline, analytically and chromatographically pure.

N-Carbobenzyloxy-3-keto-DL-proline Methyl Ester (IV).—A solution of 600 mg. (1.5 mmoles) of N-carbobenzyloxy-*trans*-3-hydroxy-DL-proline methyl ester (IIa) in 180 ml. of acetone was oxidized with 1.5 ml. of 8.0 *N* CrO_3 in 50% H_2SO_4 and allowed to stand at room temperature for 65 min. A small quantity of ethanol was added to decompose excess oxidant. Evaporation of the solution under reduced pressure gave an oily substance which was extracted with 200 ml. of ether. The ether solution was washed with salt water, dried over anhydrous Na_2SO_4, and evaporated to give the ketone (590 mg., yield 93%). The ketone IV, after chromatographic purification on Mallinckrodt silicic acid, failed to crystallize. However, thin-layer chromatography and gas chromatography indicated the sample to be homogeneous and the infrared spectrum of the compound revealed a strong peak at 5.63 μ, characteristic of a ketone carbonyl in a five-membered ring.

Reduction of N-Carbobenzyloxy-3-keto-DL-proline Methyl Ester (IV) with $NaBH_4$.—To a solution of 2.0 g. (7.2 mmoles) of N-carbobenzyloxy-3-keto-DL-proline methyl ester (IV) in 100 ml. of dimethoxyethane was added 100 mg. of sodium borohydride in 1.0 ml. of water, and the reduction was allowed to proceed at room temperature for 15 min. Acetic acid was added to decompose the excess reagent and the solution evaporated *in vacuo*. The residue was extracted with ether and the ether solution was washed with water, dried, and evaporated to give an oily residue (2.0 g.). Gas chromatographic analysis of this oil, after acetylation with acetic anhydride in pyridine, revealed the presence of 80% of N-carbobenzyloxy-*cis*-3-hydroxy-DL-proline methyl ester (V) and less than 20% of the *trans* isomer IIa.

Separation of the Esters IIa and V on Silicic Acid.—The oily mixture (2.0 g.) was dissolved in 20 ml. of benzene–ethyl acetate (4:1) and chromatographed in four equal portions on a column (18.5 × 6 cm.) of Mallinckrodt silicic acid (mesh: 100–200) with the same solvent mixture. Fractions of 22 ml./tube were collected on a fraction collector. The *cis*-ester V (1.46 g.) appeared in fractions 62–85, the *trans*-ester (150 mg.) in fractions 94–120. The total yield of the two isomeric esters was 81%. The conversion of the pure esters IIa and V to *trans*- and *cis*-3-hydroxy-DL-prolines is described in the following.

Preparation of *cis*- and *trans*-3-Hydroxy-DL-proline.—The mixture of *cis*- and *trans*-3-hydroxy-N-carbobenzyloxy-DL-proline methyl ester from another $NaBH_4$ reduction of 0.4 g. of unpurified keto ester IV was dissolved in 50 ml. of dimethoxyethane, treated with 2.5 ml. of 1.0 *N* KOH, and allowed to stand at room temperature for 2 hr. Water was carefully added and the mixture was extracted with ether and the ether extract discarded. After acidification, the aqueous solution was extracted with ether and the ether solution was washed with water, dried, and evaporated to give a mixture of *cis*- and *trans*-3-hydroxy-N-carbobenzyloxy-DL-prolines (350 mg.), which was dissolved in methanol–water (2:1) and hydrogenated over palladium to give a mixture of 147 mg. of *cis*- and *trans*-3-hydroxy-DL-proline. By the amino acid analyzer this sample contained 504 μmoles of *trans* and 288 μmoles of *cis*, or a total of 0.79 mmole of amino acid mixture (60% yield from starting material).

The entire material was dissolved in citrate buffer pH 2.2 and chromatographed on a column of IR-120 (see column chromatography), yielding crystalline *trans*-3-hydroxy-DL-proline (35.7 mg.) and *cis*-3-hydroxy-DL-proline (24.5 mg.); yield of both isomers, 23%. The *cis*-3-hydroxy-DL-proline was analytically pure.

Anal. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.83; H, 6.94; N, 10.66.

Titration Data.—The pK values of *cis*- and *trans*-3-hydroxy-DL-proline, as determined by titration of 5-mg. samples of the amino acids in 2.0 ml. of water with both 1.0 *N* HCl and 1.0 *N* NaOH in a Radiometer titrator with an Auto-Burette, were indistinguishable: $pK_{COOH} = 1.92 \pm 0.05$, $pK_{NH} = 9.73 \pm 0.05$. For comparison the pK values for proline and 3,4-dehydro-DL-proline were determined independently through the courtesy of Dr. Simon, ETH, Zurich.

Substance	pK_{MeS} HCl	pK_{MeS} $(CH_3)_4N^{\oplus}$	pK_{H_2O} HCl	pK_{H_2O} $(CH_3)_4N^{\oplus}$
L-Proline	<3.2	10.58	<3.0	>10.5
DL-3,4-Dehydroproline	<2.9	9.61	<3.0	9.57

Synthesis of *cis*- and *trans*-3-Hydroxyproline-3-H^3.—Stereospecifically labeled *cis*- and *trans*-3-hydroxy-DL-proline-3-H^3 were synthesized by reduction of crude N-carbobenzyloxy-3-keto-DL-proline methyl ester (IV) with $NaBH_4^3$ (obtained from New England Nuclear Corp., Boston, Mass.), followed by alkaline hydrolysis, catalytic hydrogenation, and chromatographic separation. The specific activities of crystalline *cis*- and *trans*-3-hydroxy-DL-prolines were 0.61 μc./μmole and 0.134 μc./μmole.

(34) For details on gas chromatography see the following paper: K. Morita, F. Irreverre, F. Sakiyama, and B. Witkop, *J. Am. Chem. Soc.*, **85**, 2832 (1963).

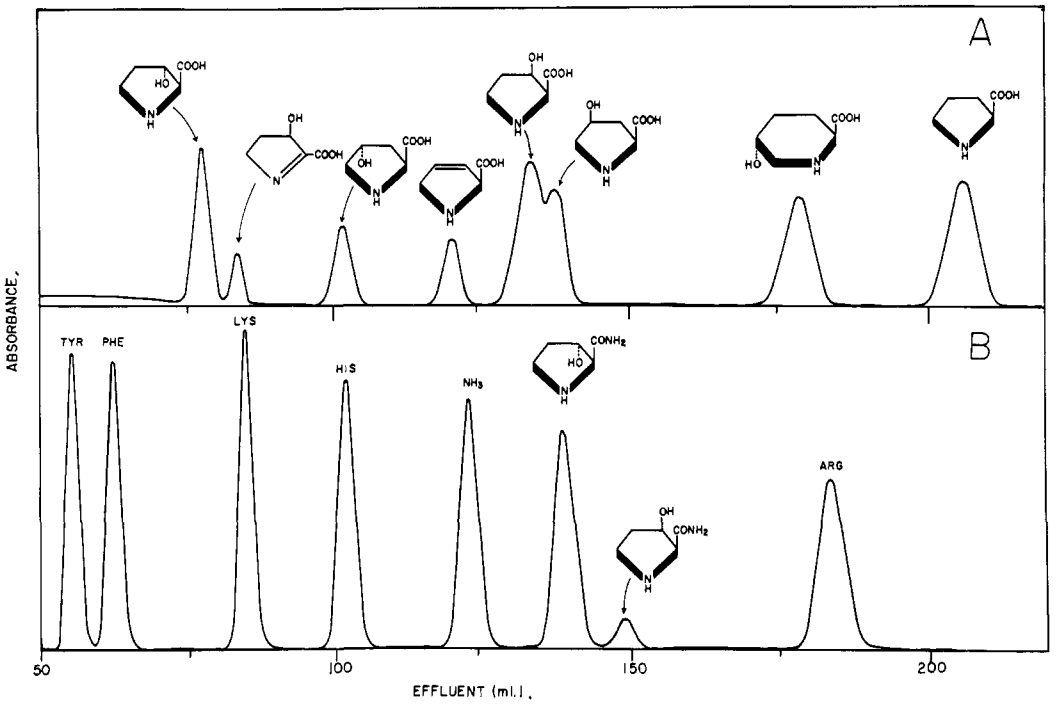


Fig. 4.—Position of the cyclic secondary amino acids on the automatic amino acid analyzer: A, The pyrrolidine and piperidine carboxylic and derivatives were eluted from a 150-cm. column of Amberlite IR-120 at 30 and 50°, with 0.2 *N* sodium citrate, pH 3.25 and 4.25. The ninhydrin values of the following amino acids were determined at λ 440 mμ: *trans*-3-hydroxy-L-proline (1 mμ), 3-hydroxy-Δ'-pyrroline-2-carboxylic acid (0.77 mμ), *trans*-4-hydroxy-L-proline (1 mμ), 3,4-dehydro-DL-proline (4 mμ), *cis*-3-hydroxy-L-proline (1 mμ), *cis*-4-hydroxy-D-proline (1 mμ), 5-hydroxy-L-pipecolic acid (2 mμ). DL-Pipecolic acid (4 mμ) was read at 570 mμ. B, The basic amino acids (0.25 μmole of the markers, ~4 μmoles of the *trans*- and *cis*-3-hydroxyproline amide) were eluted from a 50-cm. column of Amberlite IR-120 at 50°, with 0.7 *N* sodium citrate (pH 5.28); *cf.* D. R. Kominz, *J. Chromatog.*, **9**, 253 (1962). Ninhydrin colors were read at λ 570 mμ.

The reduction conditions and results of this experiment and the preceding one are compared in Table V.

TABLE V
REDUCTION OF CARBOBENZYLOXY-3-KETOPROLINE METHYL ESTER WITH SODIUM BOROHYDRIDE

N-Carbobenzyloxy-3-ketoproline methyl ester	—Solvent, ml.— DME	H_2O	$NaBH_4$, mg.	Reaction time, min.	Molar ratio[a] *trans*	*cis*
2 g.	100	1	100	15	2	3
800 mg.	10	10	20 + 2 ($NaBH_4$)	45	1	12

[a] The molar ratios were determined by chromatographic separation on silicic acid.

The low value of the activity for the *trans* isomer is considered to be due to the dilution of the compound with unlabeled material present in the starting ketone IV as the N-carbobenzyloxymethyl ester as a result of incomplete oxidation.

Enzymatic Oxidation of *cis*- and *trans*-3-Hydroxyprolines.—Enzymatic oxidation of synthetic and natural *trans*- and *cis*-3-hydroxyprolines in relation to D-alanine, 3,4-dehydro-DL-proline, 4-allohydroxy-D-proline, was followed manometrically by the usual Warburg technique under the following reaction conditions:

D-Amino acid oxidase (pig kidney, Worthington)	15.0 mg.
Sodium pyrophosphate buffer (0.1 *M*, pH 8.2)	2.0 ml.
Catalase	0.1 ml. (30 units)
Flavin adenine dinucleotide (10^{-4} *M*)	1 drop (0.04 ml.)
Substrate: D-amino acids	20 μmoles
DL-amino acids	40 μmoles
Atmosphere	Air
Temperature	37°

The values for oxygen consumption are plotted in Fig. 1. The D-amino acids used and the synthetic *trans*- and *cis*-3-hydroxy-DL-proline consumed oxygen, while the natural *cis*- and *trans*-3-hydroxyprolines were inert.

Examination of the enzymatic reaction by the amino acid analyzer showed a new peak (yellow ninhydrin color, λ_{max} 440 mμ) adjacent to *trans*-3-hydroxyproline. Both *trans*- and *cis*-hydroxy-DL-proline gave identically located products indistinguishable from the product of nonenzymatic oxidation of *trans*-3-hydroxyproline with hydrogen peroxide and cupric ions.[35] After 3 hr. the *cis*- and *trans*-3-hydroxy-D-proline were oxidized to the extent of 90.5 and 73%, respectively.

Oxidation of *cis*-3-Hydroxy-DL-proline by D-Amino Acid Oxidase.[36]—The incubation mixture contained 20 mg. of *cis*-3-hydroxy-DL-proline, 137 mg. of D-amino acid oxidase (Worthington), 300 units of catalase, and 5 ml. of 0.025 *M* phosphate buffer pH 8.3. The mixture was incubated at 37° for 24 hr. with oxygen bubbling through the solution. The protein was removed with 2.5 ml. of 40% trichloroacetic acid and the excess TCA extracted with ether. Examination of the deproteinized solution by the automatic amino acid analyzer showed 100% oxidation of the D-form. The mixture, presumably $\Delta^{1,2}$-pyrroline-3-hydroxy-2-carboxylic acid and *cis*-3-hydroxy-L-proline was chromatographed on a column of IR-120 by the same technique used for the isolation of the natural *cis* and *trans* substances. The fractions containing the dehydro compound were pooled and desalted on a column of Dowex-50 W in the hydrogen cycle. The desalted product showed only one peak by the amino acid analyzer.

The dehydro compound in a volume of 5.0 ml. of water (pH 8.5) was reduced with 8.6 mg. of $NaBH_4$ for 30 min. at room temperature. The reaction mixture, after acidification with *N* HCl to pH 1–2, was evaporated to dryness *in vacuo*. The material was extracted twice with methanol (5 ml.) and the methanolic solution evaporated to dryness. It was then desalted on Dowex-50 W in the hydrogen cycle. The reduced product cochromatographed with *cis*-3-hydroxyproline.

Composite Column Chromatogram.—Figure 4 presents a composite picture of the sequence of elution of the various new amino acids reported in this paper as the peaks appear on the automatic recorder of an amino acid analyzer.

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(35) *Cf.* L. Macholán and J. Vencálková, *Ber.*, **96**, 237 (1963).

(36) *Cf.* J. R. Parikh, J. P. Greenstein, M. W. Winitz, and S. M. Birnbaum, *J. Am. Chem. Soc.*, **80**, 953 (1958).