Benzyloxycarbonylglycine dissolved in dimethylformamide was chilled to 0°. Isobutyl chloroformate (0.35 ml., 0.0025 mole) and triethylamine (0.35 ml., 0.0025 mole) were added, and the reaction proceeded for 20 minutes. The hydrobromide oil (1.1 g., 0.0025 mole) produced above was then added, followed by triethylamine (0.35 ml., 0.0025 mole) slowly. The reaction proceeded for 4 hours, then was diluted with ethyl acetate (200 ml.) and extracted with 2 *N* hydrochloric acid, aqueous potassium chloride and aqueous sodium bicarbonate solutions. The organic layer was dried over magnesium sulfate and evaporated under reduced pressure. The solid which formed was recrystallized from warm ethyl acetate twice to give 0.5 g. of product, m.p. 136–139°.

Anal. Calcd. for $C_{25}H_{34}N_4O_{11}$: C, 53.00; H, 6.01; N, 9.89. Found: C, 53.18; H, 6.12; N, 10.00.

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE 39, MASS.]

The Isolation, Characterization and Synthesis of *erythro*-β-Hydroxy-L-leucine, a New Amino Acid from the Antibiotic Telomycin[1]

BY JOHN C. SHEEHAN, K. MAEDA, A. K. SEN AND J. A. STOCK

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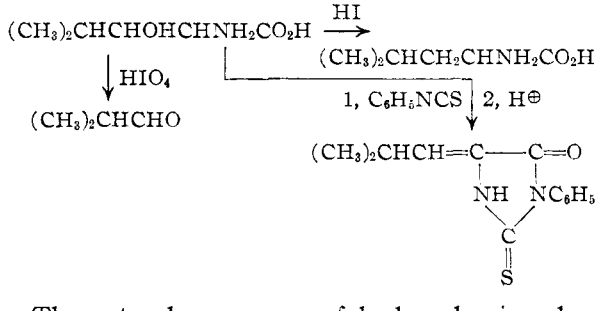
Among the acidic hydrolysis products of the antibiotic Telomycin is an amino acid not previously encountered in nature. Degradation of the pure, isolated amino acid with periodate led to isobutyraldehyde, and reaction with hydriodic acid produced leucine. Optical rotation data, enzymatic evidence and comparison with authentic *threo*- and *erythro*-β-hydroxy-DL-leucine samples established the structure as *erythro*-β-hydroxy-L-leucine. Enzymatic resolution of synthetic N-acetyl-*erythro*-β-hydroxy-L-leucine afforded synthetic *erythro*-β-hydroxy-L-leucine, identical to the amino acid from Telomycin.

The acid hydrolysis of Telomycin, an antibiotic isolated from an unidentified *Streptomyces*,[2] yields a number of ninhydrin-positive components.[3] Several of these have been identified as known amino acids, but among the novel substances present is one with paper chromatographic behavior similar to that of valine. Isolation by preparative paper chromatography and subsequent purification gave a crystalline substance, m.p. 218–222°. Elementary analysis indicated the empirical formula $C_6H_{13}NO_3$, and the reaction with periodate suggested a β-hydroxy-amino acid.[4] Periodate degradation gave isobutyraldehyde, identified as the 2,4-dinitrophenylhydrazone. This result is in accord with the Kuhn–Roth determination which ascribed one C-methyl group per mole, based on the molecular formula $C_6H_{13}NO_3$. The conclusion that the substance was a β-hydroxyleucine was reinforced by the finding that reduction with red phosphorus and hydrogen iodide gave leucine. Quantitative ninhydrin degradation[5] gave a value for the carboxyl group close to that expected for hydroxyleucine. Since the optical rotation of the amino acid showed a positive shift in passing from water to aqueous hydrochloric acid solution, the L-configuration was assigned (Clough–Lutz–Jirgensons rule[6]).

Enzymatic evidence confirmed this assignment (see Experimental). Kenner and his co-workers[7] have recently synthesized the *threo* and *erythro* forms of β-hydroxyleucine and have established that Wieland's one-stage synthesis[8] gives predominantly the *threo* isomer. A comparison by paper chromatography of the β-hydroxyleucine isolated from Telomycin with that synthesized by Wieland's method, and later with samples of the racemic *erythro* and *threo* forms obtained from Professor G. W. Kenner (University of Liverpool, England) and from Professor S. Akabori (Osaka University, Japan), enabled us to assign the natural amino acid to the *erythro* series. That the compound isolated from Telomycin is *erythro*-β-hydroxy-L-leucine was finally confirmed by synthesis, including enzymatic resolution of the intermediate N-acetyl-*erythro*-β-hydroxy-DL-leucine, and by comparison of optical rotations and infrared spectra. The melting points and infrared spectra of the dinitrophenyl derivatives of the natural and synthetic L-amino acids were identical. Reaction of the amino acid with phenyl isothiocyanate led to the 5-isobutylidene derivative of phenylthiohydantoin, the result of a not-unexpected[9] β-hydroxyl elimination.



The natural occurrence of hydroxyleucines has been reported by two other groups. Kenner[7,10] has shown that the antibiotic I.C.I. No. 13959 gave, on hydrolysis, *threo*-β-hydroxy-L-leucine. The isolation of a hydroxyleucine, very probably a β-

(1) The presence of a β-hydroxyleucine in Telomycin was reported (J. C. S.) at the Seventeenth International Congress for Pure and Applied Chemistry, held in Munich, Germany, August 30 to September 6, 1959; Meeting Abstracts, p. 108.

(2) M. Misiek, O. B. Fardig, A. Gourevitch, D. L. Johnson, I. R. Hooper and J. Lein, *Antibiotics Ann.*, 852 (1957–1958).

(3) J. C. Sheehan, P. E. Drummond, J. N. Gardner, K. Maeda, D. Mania, A. K. Sen and J. A. Stock, in preparation.

(4) *Cf.* (a) R. Criegee in "Newer Methods of Preparative Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1948, p. 20; (b) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p. 2210.

(5) A. Steyermark in "Organic Analysis," Interscience Publishers, Inc., New York, N. Y., Vol. II, 1954, p. 7.

(6) Reference 4b, p. 83.

(7) S. Dalby, G. W. Kenner and R. C. Sheppard, *J. Chem. Soc.*, 968 (1960).

(8) T. Wieland, H. Cords and E. Keck, *Chem. Ber.*, **87**, 1312 (1954).

(9) H. Fraenkel-Conrat in "Methods of Biochemical Analysis," Interscience Publishers, Inc., New York, N. Y., Vol. II, 1955, p. 386.

(10) G. W. Kenner and R. C. Sheppard, *Nature*, **181**, 48 (1958).

hydroxyleucine, from the ornamental shrub *Deutzia gracilis* has also been described,[11] but its configuration is not known.

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Experimental[12]

Isolation of β-Hydroxyleucine.—A 2.5-g. sample of Telomycin was heated under reflux for 18 hours with 50 ml. of constant-boiling hydrochloric acid in a nitrogen atmosphere and with magnetic stirring. The mixture was taken to dryness under reduced pressure (Dow Corning Silicone antifoam), and the residue was taken up in water and clarified with activated charcoal. The residue from evaporation of the solution was dissolved in 50 ml. of 10% aqueous isopropyl alcohol and applied to 50 sheets of Whatman No. 31 chromatography paper (1 ml. per sheet) as a streak parallel to the shorter edges. The dried papers were developed (4.5 hours) by descending chromatography with butanol–water–acetic acid (4:5:1).

Narrow guide strips, cut from the paper and developed with ninhydrin (0.25% in acetone), indicated the position of the hydroxyleucine (R_f 0.5 – 0.55) and this region was cut from each sheet. The strips were eluted with water until they were ninhydrin-negative, and the combined eluates were taken to dryness *in vacuo*. The residue was transferred in a few ml. of water to a column (*ca.* 13 × 1.3 cm.) of 10 g. of Dowex 50-X4 (H^+), the resin washed with water until free from chloride ($AgNO_3$), and the amino acid eluted from the column with *N* ammonium hydroxide (50 ml.). The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in a few ml. of water, treated with activated charcoal and filtered. The filtrate was taken to dryness and the residue was crystallized from aqueous ethanol, giving 120 mg. of the amino acid as colorless needles, m.p. 218–222° dec., $[\alpha]^{28.5}D$ + 25° (*c* 1.05 in H_2O), $[\alpha]^{28}D$ +35° (*c* 0.41 in *N* hydrochloric acid).

Anal. Calcd. for $C_6H_{13}NO_3$: C, 48.97; H, 8.91; N, 9.52; C-CH_3, (1) 10.20; CO_2, 29.94. Found: C, 49.31; H, 8.84; N, 9.53; Kuhn–Roth C-methyl, 10.50; ninhydrin CO_2,[13] 32.25.

Periodate Titration.—One mole of amino acid ($C_6H_{13}NO_3$) consumed 1.01 moles of periodate (standardized against sodium arsenite).

Periodate Degradation.—A mixture of 3 ml. of 0.02 *M* periodic acid solution and 5 ml. of *M* sodium bicarbonate solution was added to a solution of 7.4 mg. (0.050 mmole) of the hydroxyleucine (from Telomycin) in 5 ml. of water. After 5 minutes 2.5 ml. of 0.02 *M* sodium arsenite solution was added, followed by 1.0 ml. of *N* sulfuric acid. The mixture was then distilled until about 7 ml. of distillate had been collected in a receiver containing 12 mg. (0.061 mmole) of 2,4-dinitrophenylhydrazine in 0.8 ml. of 2 *N* hydrochloric acid. The product (which separated immediately) was collected and recrystallized from ethanol, giving 10.5 mg. (0.042 mmole) of hydrazone, m.p. 186–186.5°, unchanged on admixture with authentic isobutyraldehyde 2,4-dinitrophenylhydrazone. The infrared spectra were identical.

Phosphorus and Hydriodic Acid Reduction of Hydroxyleucine.—A mixture of 3 mg. of hydroxyleucine (from Telomycin), 18 mg. of red phosphorus and 0.1 ml. of freshly distilled constant-boiling hydriodic acid was heated in a sealed tube at 150–155° for 20 hours. The mixture was diluted with 2 ml. of water and filtered through Celite. The filtrate and washings were taken to dryness *in vacuo*, the residue was dissolved in 3 ml. of water, and the solution was treated with Amberlite resin IR-4B (OH^-) to bring the *p*H of the solution to 6. The residue was indistinguishable from leucine by paper chromatography in two systems, (a) 1-butanol–water–acetic acid (4:5:1) and (b) *t*-amyl alcohol saturated with water and containing a little 8-hydroxyquinoline. System b distinguishes among leucine, norleucine and isoleucine.[14]

5-Isobutylidene-3-phenyl-2-thiohydantoin.—Reaction of hydroxyleucine (from Telomycin) with phenyl isothiocyanate on a 0.1 mmolar scale by Edman's procedure[15] gave a 65% yield of the dehydrated phenylthiohydantoin of hydroxyleucine, m.p. 219–220° (from aqueous ethanol).

Anal. Calcd. for $C_{13}H_{14}N_2OS$: C, 63.38; H, 5.73. Found: C, 63.54; H, 5.83.

Paper Chromatography of *erythro*- and *threo*-β-Hydroxyleucines.—Two solvent systems, known[7] to differentiate readily between the *erythro* and *threo* forms, were used: (a) *n*-butyl alcohol saturated with 10% aqueous diethylamine[16a] (R_f's: *erythro*, 0.41; *threo*, 0.48); (b) *n*-butyl alcohol–water–acetone–concentrated ammonium hydroxide (8:6:1:1)[16b] (R_f's: *erythro*, 0.30; *threo*, 0.44).

Synthetic *erythro*-β-Hydroxy-DL-leucine.—A solution of 11.15 g. (0.076 mole) of N-benzoyl-(*erythro* and *threo*)-β-hydroxy-DL-leucine ethyl ester[7] in 100 ml. of 6 *N* hydrochloric acid was heated under reflux for 20 hours. The cooled solution was filtered, extracted with ether (100 ml. × 2) to remove benzoic acid, and evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 *N* acetic acid and transferred to a column (20 × 2.5 cm.) of Dowex 1-X4 anion-exchange resin, previously washed with 0.5 *N* acetic acid. The combined ninhydrin-positive fractions (25 to 135 ml. of 0.5 *N* acetic acid eluate) were concentrated to a volume of 50 ml., diluted with 60 ml. of ethanol, and stored in a refrigerator. After 16 hours crystals were collected, affording 1.7 g. (29%) of *erythro*-amino acid, shown by paper chromatography to contain only a trace of the *threo* form (solvent system a, previous paragraph).

Anal. Calcd. for $C_6H_{13}NO_3$: C, 48.96; H, 8.90; N, 9.52. Found: C, 49.23; H, 8.70; N, 9.76.

Concentration of the filtrate to 20 ml. and addition of 20 ml. of ethanol gave a further 2.45 g. (45%) of crystalline product consisting of an approximately 1:1 mixture of the *threo*- and *erythro*-amino acids.

N-Acetyl-*erythro*-β-hydroxy-DL-leucine.—Acetic anhydride (175 mg., 1.72 mmoles) was added during 30 minutes and with vigorous stirring to an ice-cooled solution of 168 mg. (1.14 mmoles) of *erythro*-β-hydroxy-DL-leucine and 183 mg. (1.73 mmoles) of sodium carbonate in 1.5 ml. of water. Sodium carbonate was added to bring the *p*H to 7.5, and stirring was continued for an additional 30 minutes at room temperature. The solution was stirred for 5 minutes with about 5 ml. of Dowex 50W-X8 cation exchange resin, and the mixture was set aside in a refrigerator for 2 hours, then filtered. Lyophilization of the filtrate afforded 223 mg. of crude product which was crystallized from ethyl acetate, giving 167 mg. (77%) of the acetyl compound, m.p. 174°.

Anal. Calcd. for $C_8H_{15}NO_4$: C, 50.77; H, 7.99. Found: C, 51.20; H, 8.07.

Resolution of N-Acetyl-*erythro*-β-hydroxy-DL-leucine. Isolation of *erythro*-β-Hydroxy-L-leucine.—The procedure is patterned after that reported for the resolution of N-acetyl-O-benzyl-DL-threonine.[17] A suspension of 1 g. of Takadiastase in 10 ml. of water was stirred at 0° for 2 hours and filtered through Celite. The filtrate was stored at 0° until required. A 1.6-ml. portion of the enzyme solution was added to a solution of 155 mg. (0.8 mmole) of N-acetyl-*erythro*-β-hydroxy-DL-leucine in 0.26 ml. of 2 *N* sodium hydroxide solution (*p*H 7.2). After storage for 24 hours at 37°, the solution was transferred to a column (20 × 1 cm.) of Dowex 50W-X8 (H^+) cation exchange resin and the column was eluted with 2 *N* ammonium hydroxide. The combined ninhydrin-positive fractions (35–85 ml. of eluate) were evaporated to dryness under reduced pressure at 40°. Solution of the residue (73 mg.) in 1 ml. of water and addi-

(11) J. Jadot and J. Casimir, *Biochim. Biophys. Acta*, **48**, 400 (1961).

(12) Melting points are corrected. The microanalyses are by Dr. S. M. Nagy and his associates unless otherwise noted.

(13) Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y.

(14) E. D. P. Thompson and A. R. Thompson in "Progress in the Chemistry of Organic Natural Products," Vol. XII, Springer-Verlag, Vienna, 1955, p. 285.

(15) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(16) (a) T. L. Hardy and D. O. Holland, *Chemistry and Industry*, 855 (1952); (b) K. N. F. Shaw and S. W. Fox, *J. Am. Chem. Soc.*, **75**, 3421 (1953).

(17) Y. Murase, K. Okawa and S. Akabori, *Bull. Chem. Soc. Japan*, **33**, 123 (1960).

tion of 5 ml. of ethanol gave a crystalline precipitate which contained *erythro*-β-hydroxyleucine as the sole ninhydrin-positive component. Paper electrophoresis showed the filtrate to contain a number of ninhydrin-positive substances. The *erythro*-amino acid was purified by treating the aqueous solution (10 ml.) with 0.3 g. of activated charcoal, lyophilizing the filtrate, and recrystallizing the residue from water–ethanol; 28 mg. (47%) of *erythro*-β-hydroxy-L-leucine was thus obtained as colorless needles, m.p. 219–222° dec., $[\alpha]^{30}D$ + 22° (*c* 1.0 in water). The infrared spectrum (KBr) was identical with that of the hydroxyleucine obtained from the acid hydrolysate of Telomycin.

Anal. Calcd. for $C_6H_{13}NO_3$: C, 48.96; H, 8.90; N, 9.52. Found: C, 49.46; H, 8.62; N, 9.55.

2,4-Dinitrophenyl-*erythro*-β-hydroxy-L-leucine.—Dinitrophenylation[17] of both the isolated and synthetic amino acids gave DNP derivatives which were crystallized from aqueous ethanol and had identical m.p.'s (173–174°) and infrared spectra (KBr).

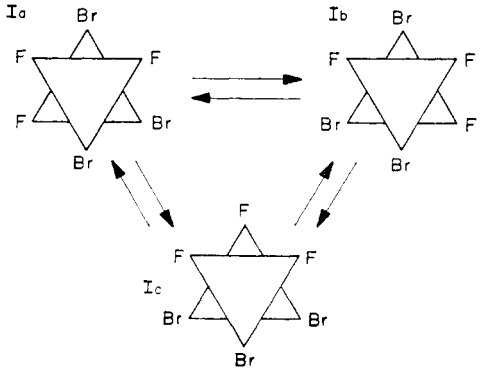
Anal. Calcd. for $C_{12}H_{15}N_3O_7$: C, 46.01; H, 4.82; N, 13.41. Found (for Telomycin product): C, 46.55; H, 5.28; N, 13.70; (for synthetic product): C, 45.83; H, 4.60.

COMMUNICATIONS TO THE EDITOR

OPPOSITE RELATIVE SIGNS OF GEMINAL AND VICINAL FLUORINE-FLUORINE N.M.R. COUPLING CONSTANTS IN A SATURATED FLUOROCARBON BY DOUBLE RESONANCE

Sir:

We wish to report the determination of the relative signs of the geminal and vicinal F^{19}–F^{19} n.m.r coupling constants in a saturated fluorocarbon by use of the audio side band phase detection decoupling technique.[1,2] The utility of this decoupling technique for F^{19}–F^{19} decoupling has been demonstrated recently.[3] In this communication, we describe the results of double irradiation experiments on 1,1,2-trifluoro-1,2,2-tribromoethane (I). The fluorine n.m.r. spectrum of I was studied previously by Nair and Roberts,[4] who have also discussed the temperature dependence of the spectrum of I. They demonstrated that molecules of this type are undergoing rapid rotation about the C–C bond at room temperature but that at low temperature it is possible to "freeze" molecules into a mixture of their various possible rotational configurations. The three possible rotational configurations of I are Ia, Ib and Ic. Of these three



configurations Ic would be expected to be the least stable on steric grounds because the 1-bromine atom is flanked by both of the 2-bromine atoms. Ia and Ib are mirror images and obviously have the same energy.

We have studied the low temperature spectrum of I (50% in CS_2) at 56.4 Mc. and found that at −110° the spectrum is that of an ABX type with J_{AB} = |165| cps., J_{AX} (or $J_{A'X}$) = 16.2 cps. and J_{BX} (or $J_{B'X}$) = 18.6 cps. (Fig. 1a). The chemical shift between the center of gravity of the AB region and the X region is about 705 cps.

It would appear that at this temperature I has been "frozen" primarily into a mixture of the magnetically equivalent rotational configurations Ia and Ib. However, there are additional lines in the spectrum indicating the presence of Ic. There is a sharp doublet about 388 cps. upfield from the center of gravity of the AB region and a triplet about 250 cps. upfield from the center of the X region of Ia and Ib. The intensity perturbations and spacings of these sets of lines are consistent with those of an A_2X system with a coupling of 18.8 cps. and a chemical shift of 570 cps. Relative area measurements suggest that Ic is only present to the extent of 5–7%.

At −110° we carried out double irradiation experiments at the audio frequencies of 670 and 756 cps. with a $\gamma H_1/2\pi$ of 25 cps. The results are shown in Fig. 1 where each transition has been numbered. By assigning the neighboring nuclear spin states to each transition in Fig. 1a, it is possible to predict what changes in the spectrum will take place on double irradiation between certain sets of the lines for the various possible relative sign assignments for the three coupling constants. This procedure is described in detail elsewhere.[5,6,7]

When the AB region is observed with a decoupling frequency of 670 cps. lines 10 and 12 are receiving the strong irradiation while lines 5 and 6 are being observed. It is predicted that if J_{AX} and J_{AB} have the same relative signs then no change in lines 5 and 6 should be observed, but if J_{AX} and J_{AB} have different signs then lines 5 and 6 should collapse (as observed in Fig. 1b). When the X region is observed at the same decoupling frequency lines 5 and 6 are being strongly irradiated. If J_{AX} and J_{AB} have the same relative signs then lines 9 and 11 should collapse, but if J_{AX} and J_{AB}

(1) R. Kaiser, *Rev. Sci. Instr.*, **31**, 963 (1960).
(2) R. Freeman, *Mol. Phys.*, **3**, 435 (1960).
(3) D. D. Elleman and S. L. Manatt, *J. Chem. Phys.*, in press.
(4) P. M. Nair and J. D. Roberts, *J. Am. Chem. Soc.*, **79**, 4565 (1957).
(5) R. Freeman and D. H. Whiffen, *Mole. Phys.*, **4**, 321 (1961).
(6) S. L. Manatt and D. D. Elleman, *J. Am. Chem. Soc.*, **84**, May (1962).
(7) D. D. Elleman and S. L. Manatt, *J. Chem. Phys.*, in press.