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The Absolute Configuration of α-Hydroxy-β-carboxyisocaproic Acid (3-Isopropylmalic Acid), an Intermediate in Leucine Biosynthesis*

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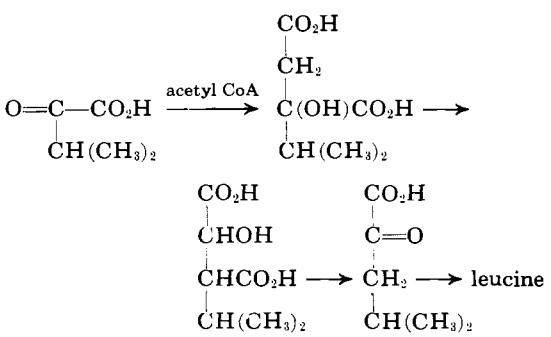
The absolute configuration of α-hydroxy-β-carboxyisocaproic acid isolated from a mutant of *Neurospora crassa* is shown to be *threo*-D_3-α-hydroxy-β-carboxyisocaproic acid. The *threo* configuration was deduced from nuclear magnetic resonance spectra of the *O*-acetyl anhydrides of the two racemates. Optical rotatory dispersion studies of natural α-hydroxy-β-carboxyisocaproic acid ethyl xanthate identified the α asymmetric center as a member of the D-series. Chemical reduction of the natural compound to L(−)-isopropylsuccinate confirmed the configuration of the β-carbon.

Recent work in several laboratories indicates that the following biosynthetic pathway to leucine is operative in several bacteria and fungi (Jungwirth *et al.*, 1961; Gross *et al.*, 1962, 1963; Calvo *et al.*, 1962; Strassman and Ceci, 1963):



This sequence of reactions is analogous to the formation of glutamic acid from oxaloacetic acid in the Krebs cycle. The absolute configuration of the isocitric acid isomer formed in the Krebs cycle has been shown to be *threo*-D_3 (Katsura, 1961).

The determination of the absolute configuration of α-hydroxy-β-carboxyisocaproate, a formidable task by classical organic techniques, was straightforward using nuclear magnetic resonance and optical rotatory dispersion. The configurational assignment was confirmed by chemical degradation.

Results

α-Hydroxy-β-carboxyisocaproate.—The synthesis of the two racemates of α-hydroxy-β-carboxyisocaproate (racemate A, mp 119–119.5°; racemate B, mp 122–122.3°) was described in an earlier report (Calvo *et al.*, 1962).[1]

Optically active α-hydroxy-β-carboxyisocaproate was isolated from *Neurospora crassa* strain D221a by a slight modification of the procedure of Burns *et al.* (1963). Seventy-five liters of culture filtrate was reduced in volume to 1 liter (temperature, *ca* 50°) and, after acidification to *p*H 1, the solution was extracted with three volumes of ether. The ether extract was concentrated to a 200-ml volume and was extracted with 100 ml of 10% $NaHCO_3$. After decolorization three times with 3 g portions of Darco G and acidification to *p*H 1, the solution was extracted three times with 100-ml portions of ether. Evaporation of the ether gave 10 g of a white crystalline residue. The residue was taken up in 5 parts of hot ethyl acetate and 10 parts of chloroform and, after standing overnight, 4.5 g of β-carboxy-β-hydroxyisocaproic acid (mp 164–166°) was removed by filtration. The mother liquors were evaporated to dryness *in vacuo*, the residue was taken up in an excess of ether, and an equal volume of *n*-heptane was added. Slow evaporation of the ether yielded 3 g of crude α-hydroxy-β-carboxyisocaproate. Recrystallization from the same solvent system gave 2 g of product, mp 130–136°. Both *Salmonella typhimurium* strains *leu* 120 and *leu* 128 responded to the isolated α-hydroxy-β-carboxyisocaproate in auxanographic tests (Jungwirth *et al.*, 1961); strain *leu* 120 also responded to β-carboxy-β-hydroxyisocaproic acid.

Racemic 2-Acetoxy-3-isopropylsuccinic Anhydrides.—The two synthetic racemates of α-hydroxy-β-carboxyisocaproate were converted to their *O*-acetyl anhydrides by addition of 4 ml of acetyl chloride to 1 g of racemate. After standing for 2 hours at room temperature, the derivatives were isolated by repeated passage of 50-μl

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[1] In the earlier report, the compound was referred to as 3-isopropylmalate; in this paper the name has been changed to α-hydroxy-β-carboxyisocaproate to conform with the usage of other workers (Gross *et al.*, 1963).

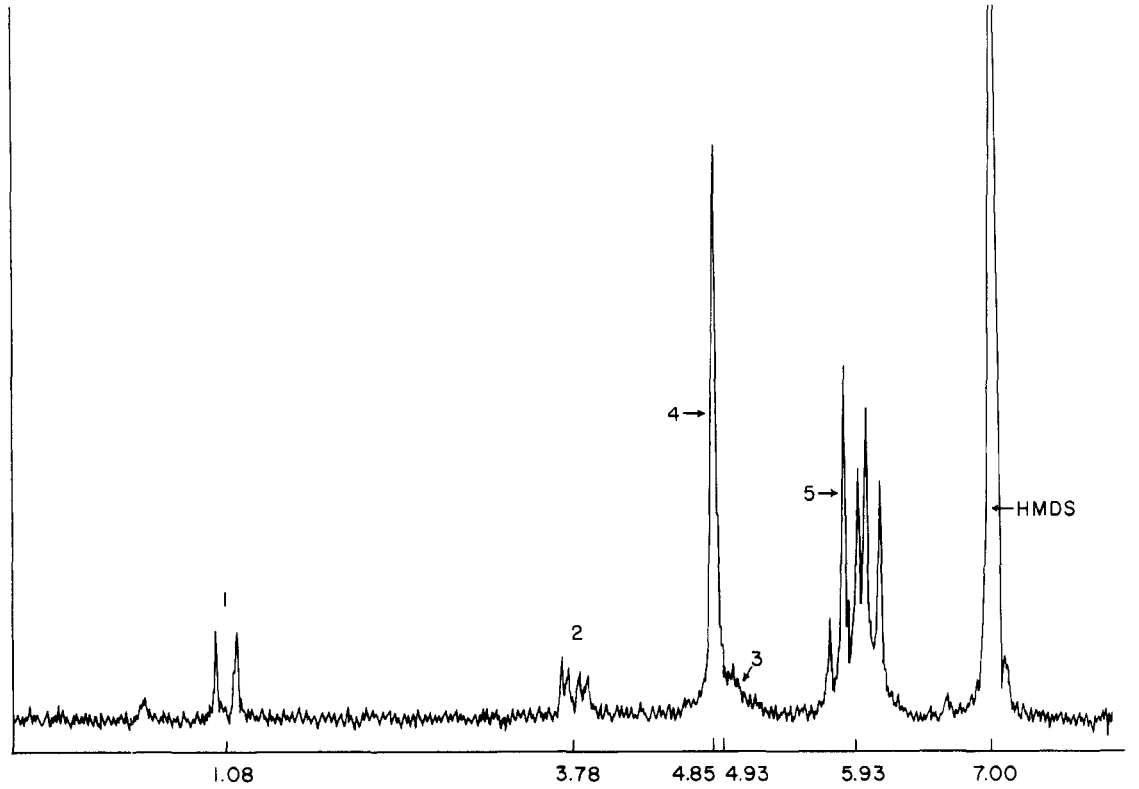


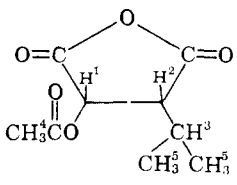
FIG. 1.—NMR spectrum of 2-acetoxy-3-isopropylsuccinic anhydride derived from racemate A of α-hydroxy-β-carboxyisocaproate.

portions of the reaction mixture through a Beckman GC-2 gas chromatograph: column, Beckman No. 74346 (6 ft); temperature, 190°; flow rate (helium), 120 ml/minute. The products were trapped in glass capillary tubes which were inserted in the instrument exhaust and immersed in an ice bath. The retention times were 10.5 and 12.5 minutes, respectively, for the *O*-acetyl derivatives of racemate A and racemate B.

Anal. Calcd for $C_9H_{12}O_5$: C, 54.00; H, 6.04. Found: racemate A: C, 54.31; H, 6.07; racemate B: C, 54.26; H, 5.98.

The infrared spectra of the two derivatives showed peaks at 1880 and 1800 cm^{-1} (anhydride) and at 1765 cm^{-1} (acetate) in the carbonyl region; these same peaks are exhibited by acetoxysuccinic anhydride.

Nuclear Magnetic Resonance Studies.—Figures 1 and 2 show the NMR spectra of the *O*-acetyl anhydrides of the two racemates. The spectra were taken with a Varian Associates spectrometer operating at 60 mc/second. The samples were run in $CDCl_3$ (*c*, *ca* 14%) with hexamethyldisiloxane (HMDS; Chamberlain, 1959) as an internal reference. Chemical shifts are expressed in ppm from benzene. Peaks 1–5 in both spectra correspond to the hydrogens indicated by superscripts in structures I and II.



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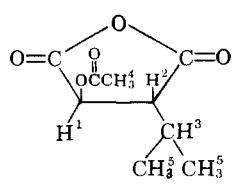

II

The areas under peaks 1–5 are in the ratio 1:1:1:3:6. The signal from H^1 is split into a doublet by H^2, while H^2 itself yields a signal showing a quartet structure, being split by H^1 and H^3. The high degree of splitting of peak 3 identifies it as the isopropyl tertiary hydrogen. It is clear from the complex splitting of peak 5 that the isopropyl methyl hydrogens are not equivalent.

α-Hydroxy-β-carboxyisocaproic Acid Ethyl Xanthate.—A slight modification of the procedure of Holmberg (1925) was used for the preparation of α-hydroxy-β-carboxyisocaproic acid ethyl xanthate. Lithium hydroxide was used in place of NaOH and the xanthate derivative was further purified by passage through a 4 g silicic acid column prepared by the method of Kinnory *et al.* (1955). The desired product ran close to the chloroform eluent front as a yellow band. The material from the column was extracted into Na_2CO_3 and, after acidification, into ether. One-half of α-hydroxy-β-carboxyisocaproate yielded 52 mg (6.5%) of a viscous amber oil which did not solidify after standing several weeks. Repeated attempts to obtain the derivative in crystalline form were unsuccessful.

Anal. Calcd for $C_{10}H_{16}O_5S_2$: C, 42.84; H, 5.75; S, 22.88. Found: C, 43.09; H, 6.01; S, 22.69.

The product was further characterized by a comparison of its ultraviolet and infrared spectra with those of L-malic acid ethyl xanthate. The ultraviolet spectra of the two derivatives were almost superimposable: ϵ_{375} = 31.2 cm^2 $mmole^{-1}$, ϵ_{355} = 50.6, ϵ_{330} = 30.2, ϵ_{316} = 92.5. The infrared spectra of the two derivatives were similar, both showing strong absorption bands at 1220–1205, 1200–1180, and 1086–1075 cm^{-1}.

Optical Rotatory Dispersion Studies.—Optical rotatory dispersion measurements were made with a Rudolph recording spectropolarimeter: $[\alpha]_{450}$ −200°, $[\alpha]_{372}$ −910°, $[\alpha]_{336}$ +375°, $[\alpha]_{320}$ +110° (*c*, 0.2 in methanol at 25°). As can be seen in Figure 3, the rotatory dispersion curve of α-hydroxy-β-carboxyisocaproic acid ethyl xanthate exhibits a negative Cotton effect.

Reduction of α-Hydroxy-β-carboxyisocaproic Acid to Isopropylsuccinic Acid.—The procedure was analogous to that of Abderhalden and Heyns (1934). α-Hydroxy-

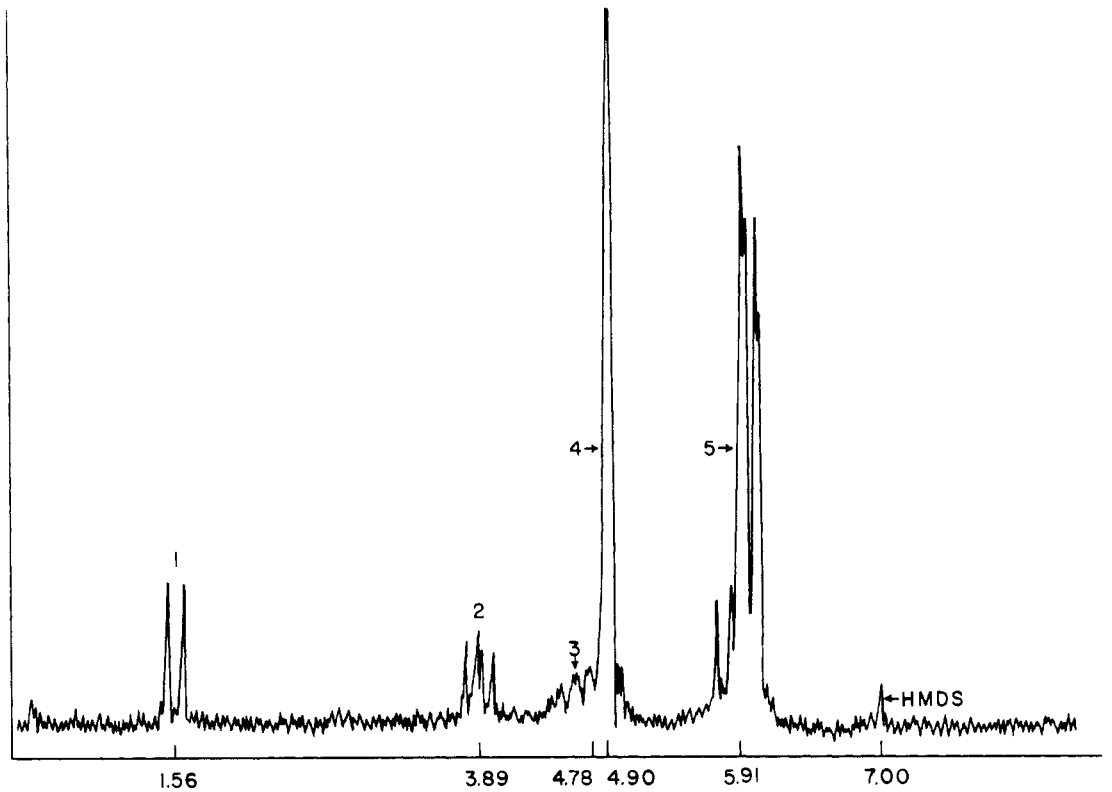

FIG. 2.—NMR spectrum of 2-acetoxy-3-isopropylsuccinic anhydride derived from racemate B of α-hydroxy-β-carboxyisocaproate.

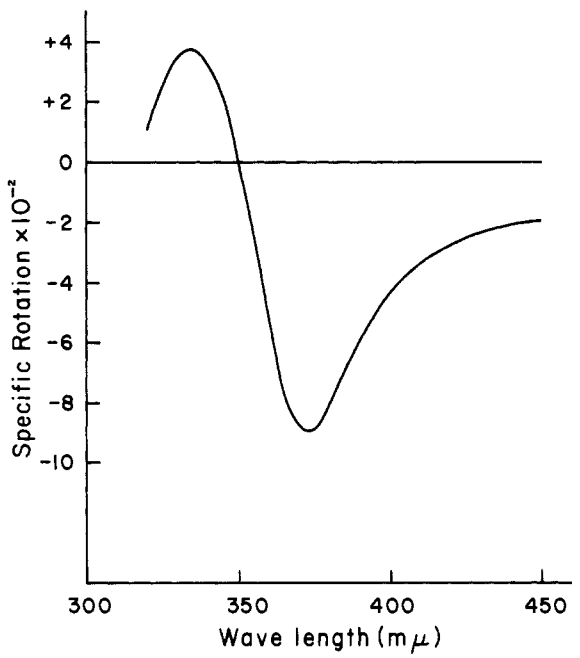

FIG. 3.—Optical rotatory dispersion curve of natural α-hydroxy-β-carboxyisocaproic acid ethyl xanthate.

β-carboxyisocaproic acid (0.08 g), 0.025 g of red phosphorus, and 1 ml of hydriodic acid (specific gravity 1.5) were heated in a sealed tube at 150° for 7 hours. The reaction mixture was cooled and the red phosphorus filtered off. The clear filtrate was evaporated to a small volume on a steam bath and evaporated further several times after repeated addition of water until the solution was colorless. The oily residue (0.07 g) solidified on standing overnight in a desiccator and was sublimed at 70° *in vacuo*. The sublimate (0.04 g, mp 85–86°) was further purified by crystallizing twice from ether-ligroin to a constant melting point of 87–88°; neutralization equivalent, 79.6 (theoretical, 80.0). The final product was levorotatory, $[\alpha]_D^{25} = -22.4°$ (1.56% in water). Freudenberg and Lwowski (1954) report $[\alpha]_D^{20} = +23.7°$ (10% in water) for D(+)-isopropylsuccinic acid.

Anal. Calcd for $C_7H_{12}O_4$: C, 52.52; H, 7.55. Found: C, 52.29; H, 7.35.

The infrared absorption spectrum of the product lacked the absorption maximum at 3500 cm^{-1} present in the spectrum of α-hydroxy-β-carboxyisocaproic acid.

DISCUSSION

The absolute configuration of the two asymmetric centers in natural α-hydroxy-β-carboxyisocaproic acid is established from the experimental results reported above.

Anomalous dispersion curves of *N*-dithiocarbalkoxy derivatives of nine L-amino acids show positive Cotton effects while the reverse is true of members of the D-series (Sjöberg *et al.*, 1959). These same authors report that rotatory dispersion curves of xanthate derivatives of L-malic acid, L-lactic acid, and L-mandelic acid exhibit positive Cotton effect curves. The presence of a second adjacent asymmetric center does not appear to influence the anomalous dispersion pattern as evidenced by the nearly identical positive Cotton effect in the curves of L-isoleucine and L-*allo*isoleucine. The negative Cotton effect curve of natural α-hydroxy-β-carboxyisocaproic acid ethyl xanthate indicates that, with reference to the α asymmetric center, this compound is a member of the D-series.

An assignment of the acetoxy anhydrides of racemates A and B to structures I and II, respectively, may be made on the basis of the NMR spectra. Karplus (1959) has calculated the coupling constants of two interacting protons as a function of their dihedral angle. The theory predicts a higher coupling constant

for hydrogens with a dihedral angle of 0° than for those with a dihedral angle of 120°. The pertinent coupling constants, J_{12}, may be calculated from peak 1 of Figures 1 and 2. On the basis of theory, then, structure I corresponds to the spectrum in Figure 1 (J_{12} = 9.38 cps) and structure II to Figure 2 (J_{12} = 7.32 cps). The correlation with theory cannot be considered unequivocal evidence of the correctness of the configurational assignments because of the relatively small differences in the coupling constants and because the theory treats only one of the factors which may affect the observed coupling constants (Karplus, 1963). More reliable deductions can be made from the comparison of observed coupling constants in a series of closely related structures.

Bright (1961) examined the NMR spectra of a series of substituted succinic anhydrides closely related in structure to compounds I and II. The coupling constant, J_{12}, for the acetoxysuccinic anhydride derived from L-*erythro*-β-deuteriomalate (Gawron and Fondy, 1959; Anet, 1960) was 9.6 ± 0.2. In the NMR spectra of the anhydrides of *N*-acetylaspartic acid and *O*-acetylmalic acid, coupling constants of 9.0 and 6.8 were observed for the former, as compared to 8.9 ± 0.2 and 6.6 for the latter, the larger value in each case being assignable to the interaction of the adjacent hydrogens having the smaller dihedral angle. The anhydrides of the two diastereoisomers of *N*-acetyl-β-methylaspartic acid showed coupling constants, J_{12}, of 10.2 and 8.1. Thus, the experimental data on six different compounds of quite similar structure yielded coupling constants which fell into two distinct groups, with average values of 9.5 and 7.2 cps, the former group containing a compound of known configuration. The present experimental data fit nicely into this pattern and support the assignment of structure I for the anhydride derived from racemate A (which includes the isomer of biological origin).

Confirmation of the configuration about the β-carbon is provided by the chemical conversion of natural α-hydroxy-β-carboxyisocaproic acid to L(−) isopropylsuccinic acid, a compound of known configuration.

The unambiguous name, *threo*-D_s-α-hydroxy-β-carboxyisocaproic acid, is proposed for the isomer of biological origin. This nomenclature is in harmony with that suggested by Vickery (1962) for the isomers of isocitric acid. It is of interest that the natural isomer of α-hydroxy-β-carboxyisocaproate has the same absolute configuration as the isomer of isocitric acid which is formed in the Krebs cycle (Katsura, 1961).

Extracts of *N. crassa* and *S. typhimurium* catalyze the conversion of *threo*-D_s-α-hydroxy-β-carboxyisocaproate to dimethylcitraconate (Gross *et al.*, 1963). From the known configuration of the reactant and product, it is concluded that a *trans* elimination of water is involved in the enzymatic reaction. This finding is consistent with the "*trans*" nature of those enzyme-catalyzed elimination reactions studied previously, namely, fumarase and aspartase (Gawron *et al.*, 1961), aconitase (Gawron *et al.*, 1958), and β-methylaspartase (Bright, 1961).

Note Added in Proof

A full paper describing NMR studies of substituted succinic anhydrides has just appeared (Bright, H. J., Lundin, R. E., and Ingraham, L. L. (1964), *Biochemistry 3*, 1224). The data and conclusions agree essentially with those cited above.

Acknowledgments

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