

Chemical Synthesis of the Isomeric Dimeric Esters of β -Hydroxybutyric Acid*

I. Olsen, J. M. Merrick, and I. J. Goldstein†

ABSTRACT: The dimeric ester of β -hydroxybutyric acid was obtained by condensation of benzyl β -hydroxybutyrate and β -benzyloxybutyric acid followed by hydrogenolysis of the resulting ether-diester. Utilizing this procedure the four possible stereoisomers of the dimer were synthesized.

The major reserve material of many types of bacteria, poly- β -hydroxybutyrate (a polyester of D(-)- β -hydroxybutyric acid), occurs in the form of intracellular granules which easily can be isolated from disrupted cell extracts. Merrick and Doudoroff (1964) have shown that poly- β -hydroxybutyrate granules obtained from *Bacillus megaterium* are degraded by extracts prepared from polymer-depleted cells of *Rhodospirillum rubrum*. Purified preparations of *R. rubrum* extracts contain at least two factors, a heat-stable factor (activator) and a heat-labile factor (depolymerase), both of which must be present for the enzymatic hydrolysis of poly- β -hydroxybutyrate granules to D- β -hydroxybutyric acid (80–85%) and unidentified soluble esters of β -hydroxybutyric acid (15–20%). An esterase present in crude extracts of *R. rubrum* cells completes the hydrolysis of the esterified products to β -hydroxybutyric acid.

An extracellular enzyme isolated from the culture medium of a pseudomonad is also capable of digesting poly- β -hydroxybutyrate, but the mechanism of hydrolysis differs from the enzyme system described above (Merrick *et al.*, 1962). The product consists mainly of the dimeric ester of β -hydroxybutyric acid, 3-O-D-(3-D-hydroxybutyryl)hydroxybutyric acid, although some monomer is also formed. The further metabolism of the dimer by the pseudomonad apparently occurs via its hydrolysis to its monomeric constituent by an intracellular enzyme. The *R. rubrum* esterase is also capable of hydrolyzing the dimer to β -hydroxybutyrate.

Since this dimeric ester may have an important role in the metabolism of poly- β -hydroxybutyrate, its chemical synthesis was undertaken in an effort to establish its chemical nature unequivocally. Further-

When D(-)- β -hydroxybutyric acid is employed as the starting material the dimer formed is identical to the dimeric ester of β -hydroxybutyric acid (3-O-D-[3-D-hydroxybutyryl]hydroxybutyric acid) produced by the enzymatic depolymerization of poly- β -hydroxybutyrate.

more, it was desirable to develop a method of synthesis for all of the various isomers of the dimer so that specificity studies of the dimer esterase could be undertaken. The reactions employed for the synthesis of the dimer are outlined in Figure 1 and were developed initially with DL- β -hydroxybutyric acid. Benzylation of β -hydroxybutyric acid (compound I) with benzyl bromide and powdered potassium hydroxide resulted in the formation of compound II, which after saponification yielded β -benzyloxybutyric acid (compound III). Benzyl β -hydroxybutyrate (compound IV) was obtained by the esterification of compound I with benzyl alcohol employing ethanesulfonic acid as catalyst. The condensation of compounds III and IV was conducted in benzene with ethanesulfonic acid as catalyst and the resulting ether-diester (compound V) was catalytically hydrogenated, yielding the racemic dimeric ester 3-O-(3-hydroxybutyryl)hydroxybutyric acid (compound VI). This procedure was extended to the synthesis of the four possible stereoisomers of the dimer, compounds VII, VIII, IX, and X.

Experimental and Results

Materials. Sodium DL- β -hydroxybutyrate was obtained from Nutritional Biochemicals Corp. Conversion to the free acid was achieved by passage through a column of Dowex 50 (H⁺) (200–400 mesh) or by acidification with 2 N H₂SO₄ followed by continuous extraction into ether for 8 hours. Racemic β -hydroxybutyric acid was resolved into the enantiomers by fractional crystallization of the quinine salts as described by Clarke (1959). The D(-) enantiomer was also obtained by alkaline hydrolysis of poly- β -hydroxybutyrate. Silicic acid for column chromatography was obtained from Mallinckrodt Chemical Works. Thin-layer chromatography was performed on Anasil S-coated (Analabs, Inc.) plates.

Paper Chromatography. Descending paper chromatography was conducted on Whatman No. 1 filter paper. The solvent systems utilized were (A) ether-

* From the Department of Biochemistry, School of Medicine, State University of New York at Buffalo, Buffalo. Received September 29, 1964. This investigation was supported by a research grant (AI-04501) from the National Institutes of Health, U.S. Public Health Service.

† This work was done during the tenure of an Established Investigatorship of the American Heart Association.

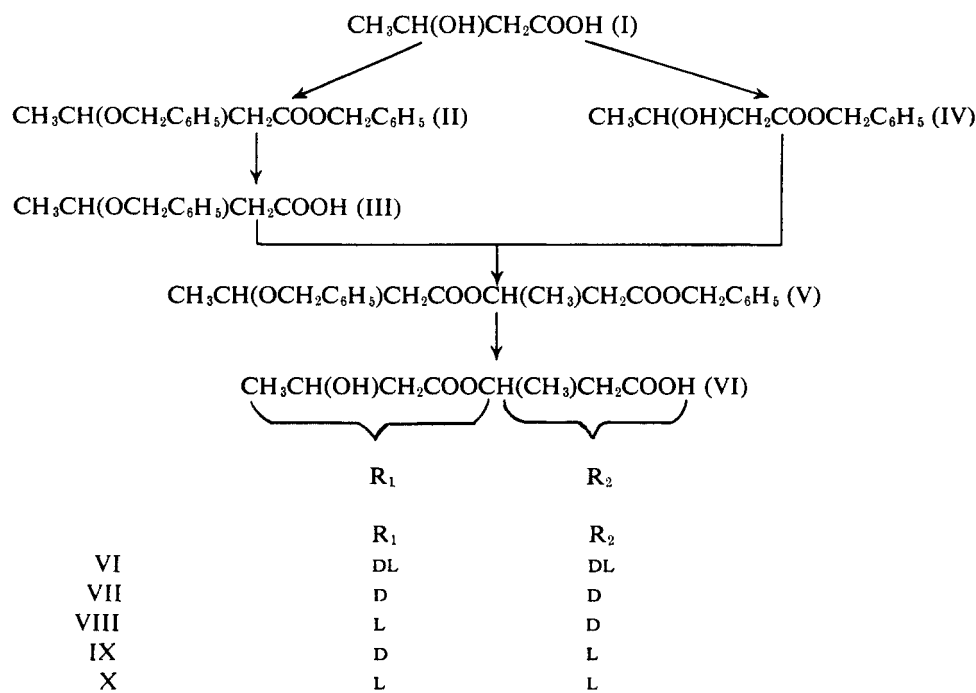


FIGURE 1: Sequence of reactions involved in the synthesis of the dimeric ester of β -hydroxybutyric acid.

benzene-formic acid (50:50:12.5, v/v); (B) 1-butanol saturated with 1.5 N aqueous ammonia; and (C) 1-butanol-ethylamine, prepared as described by Manganielli and Brofazi (1957).

Silicic Acid Column Chromatography. Silicic acid (100 mesh) was washed with chloroform and dried. The fine particles were removed by suspending the silicic acid in water, decanting the supernatant fluid after 30 minutes, and repeating the procedure several times. Following activation at 95° overnight, the silicic acid was ground thoroughly with distilled water in a ratio of 65 ml water to 100 g silicic acid. It was then added to the column in a slurry of chloroform (saturated with water).

Synthesis of Benzyl β -Hydroxybutyrate (Compound IV). Compound I (25 mmoles, 2.6 g) was refluxed for 16 hours with redistilled benzyl alcohol (27 g, 250 mmoles) in 125 ml of anhydrous benzene containing 25 mg of ethanesulfonic acid. Benzene was removed from the reaction mixture by vacuum distillation (removal of solvent in all subsequent operations was accomplished by vacuum distillation), and the resulting solution was dissolved in ether and extracted with 5% NaHCO_3 to remove any remaining free acid. The ether was evaporated and the residue was dried over anhydrous sodium sulfate. Benzyl alcohol was removed by distillation of the residue at 50°/0.15 mm, leaving compound IV, as a viscous oil, bp 95–97°/0.15 mm, n_D^{25} 1.5084; yield, 85%. Thin-layer chromatography of the product using the solvent system benzene-ether-ligroin-carbon disulfide (50:25:25:10, v/v) indicated only one spot (detected by exposure of the plate to iodine vapor). Saponification of the product gave com-

pound I as the only acid present as shown by paper chromatography.

The *p*-nitrobenzoyl derivative of compound IV was prepared in the usual manner utilizing pyridine as the solvent (Vogel, 1954). The product, initially isolated as an oil, was crystallized from ethanol-water. The white, needlelike crystals melted at 52–53°.

*Anal.*¹ Calcd. for $\text{C}_{18}\text{H}_{17}\text{O}_6\text{N}$ (343): C, 62.93; H, 4.99; N, 4.08. Found: C, 63.00; H, 4.99; N, 4.05.

Synthesis of β -Benzyloxybutyric Acid (Compound III). To a vigorously stirred suspension of powdered 90% KOH (12.5 g) in ether (125 ml) was added a solution of compound I (2.6 g, 25 mmoles) in ether (15 ml) over a period of 1 hour. The mixture was stirred under reflux for 12 hours, after which time benzyl bromide (34.2 g, 200 mmoles) was added dropwise, and the refluxing was continued for an additional 24 hours. The reaction product (compound II) was saponified by the addition of water (50 ml), followed by vigorous stirring for 3 hours. After removal of the aqueous phase, the organic layer was extracted with water (3×50 ml). The combined aqueous extracts were acidified to pH 2 with cold 2 N H_2SO_4 and extracted continuously with ether for 8 hours. The ether extract was evaporated and the remaining syrup was dissolved in chloroform. Paper chromatography revealed that the product (compound III) was contaminated with a small amount of compound I but contained no crotonic acid. Further purification of compound III was achieved by column

¹ The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

chromatography on silicic acid. The chloroform solution was applied to a column prepared from 200 g of silicic acid, and compound III was eluted with chloroform (saturated with water). After removal of the chloroform, compound III was obtained as a syrup in 50% yield, n_D^{24} 1.0517, and was shown to be homogenous in solvent systems A, B, and C (Table I).

TABLE I: R_F Values.

Acid	Solvent System		
	A	B	C
I	0.57	0.15	0.25
VI ^a	0.74	0.25	0.46
Crotonic acid		0.28	0.43
III		0.56	0.68

^a Enzymatically prepared or chemically synthesized dimer gave identical R_F values.

The *p*-bromoanilide was prepared in the usual fashion by means of the acid chloride (Vogel, 1954), giving white needles after recrystallization from ethanol water, mp 83–85°.

Anal. Calcd. for $C_{17}H_{18}O_2NBr$ (348): C, 58.62; H, 5.17; N, 4.02; Br, 22.97. Found: C, 58.54; H, 5.21; N, 4.06; Br, 22.96.

Synthesis of Racemic Dimeric Ester, 3-O-(3-Hydroxybutyryl)hydroxybutyric Acid (Compound VI). To a solution of compounds III (1.28 g, 6.6 mmoles) and IV (1.28 g, 6.6 mmoles) in anhydrous benzene (15 ml) was added ethanesulfonic acid (50 mg). The reaction mixture was heated under reflux for 16 hours. The solvent was removed and the residue was dissolved in ether. Extraction with 5% $NaHCO_3$ served to remove the free acid. The ether was removed and the resulting syrup was dissolved in absolute ethanol and hydrogenated for 8 hours in the presence of 10% palladium-charcoal catalyst (300 mg). The reaction mixture was filtered, the solvent removed, and the residue dissolved in ether and extracted with 5% $NaHCO_3$. The aqueous layer was acidified with 2 N H_2SO_4 and the acids were extracted continuously with ether for 8 hours. After evaporation of the ether solution, paper chromatography in solvent systems A, B, and C revealed the presence of compound VI as the principal product using the enzymatically prepared dimer as a standard. Impurities included compound I as well as several unidentified faster-moving acids. The syrup was dissolved in chloroform and applied to a column prepared from 150 g of silicic acid. The contaminating acids were eluted by washing the column with chloroform (saturated with water). Elution of compound VI was accomplished with 2.5% 1-butanol in chloroform. Increasing the 1-butanol concentration to 15% resulted in the elution of compound I. After the eluate containing the dimer was evaporated, the syrup was dissolved in

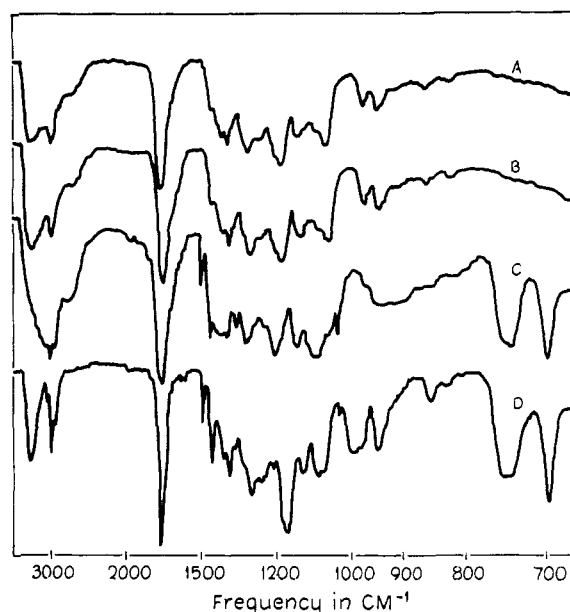


FIGURE 2: Infrared absorption spectra. A, Dimer (enzymatically prepared); B, dimer (chemically prepared, compound VI); C, compound III; D, compound IV.

5% $NaHCO_3$ and the residual butanol was removed by ether extraction. The bicarbonate solution was acidified with 2 N H_2SO_4 and the dimer was isolated by continuous extraction into ether. Evaporation of the ether gave a water-soluble oil in 35% yield. The dimer was shown to be homogenous by paper chromatography in solvent systems A, B, and C, and gave R_F values identical to the dimer isolated from the enzymatic depolymerization of poly- β -hydroxybutyrate (Table I). Saponification of the ester followed by paper chromatography indicated that compound I was the only acid present. Further evidence which suggests that the chemically and enzymatically prepared dimer are identical was obtained from the infrared absorption spectra (Figure 2) and from the refractive indices (Table II).

Anal. Calcd. for $C_8H_{14}O_5$ (190): C, 50.53; H, 7.37. Found: C, 50.58; H, 7.40.

Chemical Synthesis of the Dimers VII, VIII, IX, and X (See Figure 1). Benzyl β -hydroxybutyrate and β -benzyloxybutyric acid were prepared from both the L(+) and D(–) isomers of compound I as described. The four stereoisomers of compound VI were synthesized as previously described. Table II gives the specific optical rotations of each of the isomers as well as that of the enzymatically prepared dimer.

The reaction sequence described allows for the chemical synthesis of all of the dimeric esters of β -hydroxybutyric acid. The chemically prepared dimer (compound VII) is identical in all respects with the dimer obtained from the enzymatic depolymerization of poly- β -hydroxybutyrate (R_F , infrared absorption

TABLE II: Specific Optical Rotations and Refractive Indices of the Dimers.

Dimer	Specific Optical Rotation		Re- fractive Index, n_D^{24}
	$[\alpha]_D^{25}$	Concentration in Water	
Enzymatically prepared VII	-20.1	0.84	1.4518
Chemically pre- pared			
(a) VII	-19.7	1.40	
(b) X	+21.0	1.59	
(c) VIII	+ 8.4	2.71	
(d) IX	- 5.9	2.49	
(e) VI			1.4495

spectrum, refractive index, and optical rotation). See Figure 2 and Table II. These results unequivocally establish the nature of the enzymatically prepared dimer. Furthermore, the synthetic dimer (compound VII) could be utilized as a substrate for the *R. rubrum* esterase and was completely hydrolyzed by the dimer esterase of the pseudomonad (M. Doudoroff, personal communication).

The major problem encountered in the synthesis of compound VI was the introduction of a suitable blocking group for the labile hydroxyl group. In the foregoing synthesis, the benzyl group was effectively utilized as the blocking agent. It offers several advantages since the desired benzyl derivatives are easily prepared by standard procedures and readily removed under mild conditions of hydrogenolysis. This group

has, in fact, been used to great advantage in the protection of the hydroxyl group in carbohydrates due to its relative inertness during chemical manipulation (McCloskey, 1957). The benzyl group has been used in the present synthesis to mask the hydroxyl group of one reactant (compound III) and the carboxyl function of a second (compound IV). Condensation of these two substances was achieved in the presence of ethane-sulfonic acid. Unmasking of the condensation product (compound V) was accomplished by hydrogenolysis in the presence of a palladium-charcoal catalyst to yield the desired dimer (compound VI). By utilizing the procedures described in this communication, it may be possible to chemically synthesize the trimeric esters as well as higher homologs of β -hydroxybutyrate esters. It may also be of general use in the synthesis of di- or trimeric esters of other 3-hydroxyalkanoic acids.

Acknowledgment

The authors gratefully acknowledge the interest and advice of Dr. Howard J. Schaeffer throughout the course of this work.

References

- Clarke, H. (1959), *J. Org. Chem.* 24, 1610.
- McCloskey, C. M. (1957), *Advan. Carbohydrate Chem.* 12, 137.
- Manganelli, R., and Brofazi, F. (1957), *Anal. Chem.* 29, 1441.
- Merrick, J. M., Delafield, F., and Doudoroff, M. (1962), *Federation Proc.* 21, 228.
- Merrick, J. M., and Doudoroff, M. (1964), *J. Bacteriol.* 88, 60.
- Vogel, A. (1954), *A Textbook of Practical Organic Chemistry*, 2nd ed., London, Longmans, pp. 263, 357.