The Synthesis of L-*glycero*-Tetrulose 1-Phosphate (L-Erythrulose 1-Phosphate)*

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L-*glycero*-Tetrulose 1-phosphate (L-erythrulose 1-phosphate) has been prepared by a series of reactions that parallel those used previously for the syntheses of the D-isomer (Chü and Ballou, 1961. (−)-Inositol, prepared from quebrachitol by demethylation with HI, was acetonated in acetone and zinc chloride to give 1,2:5,6-di-*O*-isopropylidene-(−)-inositol. After oxidation with sodium periodate and reduction of the dialdehyde with sodium borohydride, the inositol derivative yielded 2,3:4,5-di-*O*-isopropylidene-L-mannitol. Benzoylation then gave 1,6-di-*O*-benzoyl-2,3:4,5-di-*O*-isopropylidene-L-mannitol. This compound was partially hydrolyzed and isomerized in an acidic aqueous acetone mixture to 1,6-dibenzoyl-3,4-isopropylidene-L-mannitol, which, without isolation, was benzoylated to give 3,4-*O*-isopropylidene-1,2,5,6-tetra-*O*-benzoyl-L-mannitol. The acetone group was removed by acid hydrolysis and 1,2,5,6-tetra-*O*-benzoyl-L-mannitol was obtained. The tetrabenzoyl-L-mannitol was cleaved by oxidation with lead tetraoxide in glacial acetic acid, and the 2,3-dibenzoyl-L-glyceraldehyde was oxidized to 2,3-dibenzoyl-L-glyceronic acid with peroxypropionic acid. The acid was converted to 2,3-dibenzoyl-L-glyceronyl chloride through the action of thionyl chloride, and then was condensed with diazomethane at −50° to give 1-deoxy-1-diazo-3,4-di-*O*-benzoyl-L-*glycero*-tetrulose. Hydrolysis of the diazo-ketone yielded 3,4-di-*O*-benzoyl-L-*glycero*-tetrulose, which, on acetalation in dry methanol and trimethylorthoformate with sulfuric acid as the catalyst, was converted to 3,4-di-*O*-benzoyl-L-*glycero*-tetrulose dimethyl acetal. Phosphorylation of this acetal with diphenylphosphorchloridate in pyridine gave a sirup. By reductive removal of the phenyl groups and saponification of the acyl substituents, L-*glycero*-tetrulose 1-phosphate dimethyl acetal was obtained and was isolated as the crystalline *bis*-cyclohexylammonium salt monohydrate. L-*glycero*-Tetrulose 1-phosphate (L-erythrulose 1-phosphate) was obtained from the acetal by mild acid hydrolysis. It was reduced to L-threitol 1-phosphate by glycerol phosphate dehydrogenase and reduced nicotinamide-adenine-dinucleotide at a rate about 1% of that found with dihydroxyacetone phosphate as the substrate. Rabbit muscle aldolase cleaved the L-erythrulose 1-phosphate at a rate about 0.021 times that of D-fructose 1,6-diphosphate, and the equilibrium constant for the reaction was 0.24 at *p*H 7.2.

Recent investigations have indicated that L-*glycero*-tetrulose 1-phosphate (L-erythrulose 1-phosphate) may be an intermediate in the metabolism of four-carbon sugars (Batt *et al.*, 1960; Wawszkiewicz, 1961). Of particular interest is the reversible reaction of dihydroxyacetone phosphate and formaldehyde, catalyzed by aldolase, to yield L-erythrulose 1-phosphate (Meyerhof *et al.*, 1936; Taylor *et al.*, 1948; Mueller *et al.*, 1955). Charalampous (1954) has presented evidence that this reaction is catalyzed by a specific enzyme from rat liver, "phosphoketotetrose aldolase," whereas Peansky and Lardy (1958) found that highly purified bovine liver aldolase was twice as active in catalyzing the addition of formaldehyde to dihydroxyacetone phosphate as it was in splitting D-fructose 1,6-diphosphate. Rutter *et al.* (1961) found that rabbit muscle and yeast aldolases differed in their specificities for a number of substrates, including the condensation of formaldehyde with dihydroxyacetone phosphate.

Other indications of a possible metabolic role for L-erythrulose 1-phosphate have been obtained from studies of the metabolism of erythritol by *Propionibacterium pentosaceum* (Wawskiewicz, 1961) and of L-threitol and L-erythrulose in the rat (Batt *et al.*, 1960). Wawskiewicz found in extracts of *P. pentosaceum* some oxidizing activity toward D-erythritol 4-phosphate with 2,6-dichlorobenzylene-indophenol as a redox indicator. Tracer studies in the rat, by Batt *et al.*, suggest that the main pathway of L-threitol and L-erythrulose metabolism does not proceed *via* the tetrulose phosphate. However, the labeling patterns in the glucose formed are consistent with a portion of the L-threitol being oxidized to L-erythrulose, which could, in turn, be phosphorylated to L-erythrulose 1-phosphate. The latter would be split to dihydroxyacetone phosphate and formaldehyde. Synthetic D-erythrulose 1-phosphate was found by Chü and Ballou (1961) to be reduced to D-erythritol 1-phosphate by reduced nicotinamide adenine dinucleotide and glycerol phosphate dehydrogenase.

The literature records one previous attempt to synthesize L-*glycero*-tetrulose phosphate. Gorin *et al.* (1955) obtained 1,2-isopropylidene-2-(hydroxyethyl)-L-*glycero*-tetrulose from 1,2-isopropylidene-D-fructopyranose, but they were unable to isolate a phosphorylated product after treatment of the former with diphenylphosphorochloridate. Recently, however, Chü and Ballou (1961) achieved the synthesis of D-*glycero*-tetrulose 1-phosphate and 4-phosphate, and Taylor and Ballou (1963) have prepared D-*glycero*-tetrulose 1,4-diphosphate. Condensation of 2,3-di-*O*-benzoyl-D-glyceronyl chloride and diazomethane provided 1-deoxy-1-diazo-3,4-di-*O*-benzoyl-D-*glycero*-tetrulose. Hydrolysis of the latter to the free ketose dibenzoate, followed by successive ketalation and phosphorylation, gave D-*glycero*-tetrulose 1-phosphate after removal of the blocking groups. The success of this procedure suggested that a parallel synthesis of the L-isomer would be fruitful.

Results and Discussion

Since the source of D-glyceronic acid is D-mannitol

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(Fischer, 1915; Müller, 1932; Brigl and Grüner, 1933), an improved method for the synthesis of L-mannitol was devised. This used procedures which were employed in the preparation of D-*manno*-hexodialdose from (+)-inositol (Ballou and Fischer, 1953) and in the elucidation of the structures of the optically active inositols by Angyal and MacDonald (1952) and Anygal *et al.* (1953). (−)-Inositol (I), derived from quebrachitol [2-*O*-methyl-(−)-inositol], was acetonated to produce 1,2:5,6-di-*O*-isopropylidene-(−)-inositol (IIb) in 40% yield from quebrachitol. This was oxidized with sodium periodate and the resulting diisopropylidene-L-*manno*-hexodialdose (IIIa) was reduced with sodium borohydride to 2,3:4,5-di-*O*-isopropylidene-L-mannitol (IIIb), which was benzoylated to give 1,6-di-*O*-benzoyl-2,3:4,5-di-*O*-isopropylidene-L-mannitol (IV). This compound was partially hydrolyzed and rearranged to 1,6-di-*O*-benzoyl-3,4-*O*-isopropylidene-L-mannitol (V), which was benzoylated and hydrolyzed to yield 1,2,5,6-tetra-*O*-benzoyl-L-mannitol (VI).

Modification of two steps used in the parallel synthesis of D-*glycero*-tetrulose phosphates from D-glyceronic acid dibenzoate (Chü and Ballou, 1961) gave greatly improved yields. A lower temperature during coupling of L-glyceronyl chloride dibenzoate (IX) with diazomethane, and a simplified procedure for the purification of the resulting diazotetrulose dibenzoate, gave a 50% increase in the yield of pure 1-deoxy-1-diazo-3,4-di-*O*-benzoyl-L-*glycero*-tetrulose (X). Difficulties encountered in the acetalation of 3,4-di-*O*-benzoyl-L-*glycero*-tetrulose (XI) were overcome by the use of sulfuric acid as the catalyst, which provided 3,4-di-*O*-benzoyl-L-*glycero*-tetrulose dimethyl acetal (XII) of high purity and in good yield.

Phosphorylation of the dibenzoyl-L-tetrulose dimethyl acetal gave L-*glycero*-tetrulose 1-phosphate dimethyl acetal in yields of 50 to 100% over that obtained by the procedure used for the enantiomorph. The average improvement of over two-fold in the yield in the third stage of the synthesis (from L-glyceraldehyde dibenzoate to L-*glycero*-tetrulose dimethyl acetal) may be attributed to the higher purity of the diazotetrulose and the improved acetalation procedure.

The properties of L-*glycero*-tetrulose 1-phosphate dimethyl acetal identify it as the enantiomorph of the known D-isomer (Chü and Ballou, 1961). The dimethyl acetal consumed one mole of sodium periodate per mole and had a rotation opposite in sign and equal in magnitude to its enantiomorph.

The acidic dissociation constants of L-*glycero*-tetrulose 1-phosphate dimethyl acetal are $pK_1 = 1.8 \pm 0.05$ and $pK_2 = 6.45 \pm 0.02$, whereas the free tetrulose phosphate has a secondary dissociation of $pK_2 = 6.24 \pm 0.02$. Dihydroxyacetone phosphate, a homolog of L-erythrulose 1-phosphate, has $pK_2 = 6.45$ (Kumler and Eiler, 1943).

The free tetrulose phosphate was reduced slowly to L-threitol 1-phosphate by glycerol phosphate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH); this indicates that the stereospecificity of the reaction is the same for dihydroxyacetone phosphate and the D- and L-erythrulose 1-phosphates. The rate of reduction of L-erythrulose 1-phosphate was about 1% that of dihydroxyacetone phosphate and about 10% that of D-erythrulose 1-phosphate. The synthetic L-erythrulose 1-phosphate was 97% pure on the basis of the NADH oxidized. The Michaelis constants (K_m) and maximal velocities found for rabbit muscle glycerol phosphate dehydrogenase with dihydroxyacetone phosphate, L-erythrulose 1-phosphate, and D-erythrulose 1-phosphate were, respectively, 6.5×10^{-5} M and 1.9×10^{-4} moles/min./g enzyme, 8.3×10^{-5} M and 2.01×10^{-5} moles/min./g enzyme, and 2.29×10^{-4} M and 9.15×10^{-3} moles/min./g enzyme. No evidence of inhibition of the reduction of dihydroxyacetone phosphate by either of the two tetrulose 1-phosphates was obtained.

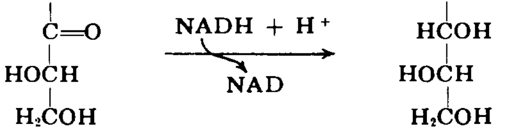

TABLE I

EFFECT OF L-*glycero*-TETRULOSE 1-PHOSPHATE ON THE RATE OF CLEAVAGE OF D-FRUCTOSE 1,6-DIPHOSPHATE BY RABBIT MUSCLE ALDOLASE

In addition to the indicated concentrations of substrate, each reaction mixture contained 150 μmoles of diethylamine buffer, *p*H 7.2, 1.0 μmole of NADH₂, 10 units of glycerol phosphate dehydrogenase, and 1.3 units of rabbit muscle aldolase in a total volume of 3.0 ml. The aldolase was the last component added, and the rates were measured for 15 minutes by the decrease in absorbancy at 340 mμ.

Substrate	Activity Alone	Activity when Combined	Expected Activity if Additive	% Inhibition
D-Fructose 1,6-diphosphate (2.6×10^{-5} M)	1.218			0
Tetrulose 1-phosphate (2.0×10^{-4} M)	0.178	1.080	1.398	22.5
Tetrulose 1-phosphate (5.0×10^{-4} M)	0.264	0.750	1.482	49.5

was 0.24. Charalampous and Mueller have reported values of 0.1–0.33 (Charalampous and Mueller, 1953; Charalampous, 1962).

On the basis of the rate of oxidation of NADH in the presence of excess glycerol phosphate dehydrogenase (at levels insufficient to produce measurable reduction of the tetrulose phosphate alone) the relative rate of cleavage of L-erythrulose 1-phosphate by rabbit muscle aldolase was found to be 0.021 times that of D-fructose 1,6-diphosphate. The apparent K_m of L-erythrulose 1-phosphate was 5.6×10^{-4} M. Since this value approaches that of D-fructose 1,6-diphosphate, 1.5×10^{-5} M (Rutter *et al.*, 1961), competitive inhibition should be observed if the same enzyme is acting on both substrates. Alternatively, if two different enzymes are responsible for the splitting of the two substrates, the amounts of dihydroxyacetone phosphate formed should be additive. The results in Table I indicate that only one aldolase is present or that all aldolases act on both substrates to a similar degree. The equilibrium constant for the reaction

$$\text{L-Erythrulose 1-phosphate} \rightleftharpoons \text{Formaldehyde} + \text{Dihydroxyacetone phosphate}$$

EXPERIMENTAL

All reagents were of the highest commercial grade or were purified by published procedures (Riddick and Topps, 1955). Melting points were determined in sealed capillary tubes and were uncorrected. Analyses were by the Microchemical Laboratory, University of California, Berkeley.

1,2:5,6-Di-O-isopropylidene-(−)-inositol (IIb).—

Impure quebrachitol (a gift of the American Rubber Company) was dissolved in an equal weight of boiling water, and the hot solution was decolorized with Darco G60 charcoal. Absolute ethanol, in two portions equal in volume to the decolorized solution, was added over several hours while the solution was stirred rapidly and cooled. The product crystallized and the recovery was about 90% of the initial weight, m.p. 192–193°. The recrystallized quebrachitol was dried at 100° for 4 hours and then demethylated by refluxing in 50% hydriodic acid, 2.5 ml per g of material, for 2 hours (Ballou and Fischer, 1953). The dark solution was poured into boiling ethanol (16 ml per g of quebrachitol) in the hood behind a safety shield. After the solution had cooled at 4° overnight, the (−)-inositol (I) was filtered off, washed well with absolute ethanol, and air-dried to provide I in 95% yield, m.p. 238–242°.

The (−)-inositol, 97 g, was acetonated in 1850 ml of dry acetone (distilled from potassium carbonate) containing 450 g of fused zinc chloride by shaking the mixture until all the solids had dissolved (about 36 hours) (Angyal and MacDonald, 1952). The reaction mixture was poured into a rapidly stirred suspension of 500 g of potassium carbonate in 500 ml of water and 1850 ml of diethyl ether. After the solution had stirred for 30 minutes, during which time the walls of the vessel were continuously scraped to resuspend the solids, the suspension was filtered and the filtrate was dried immediately with potassium carbonate. The potassium carbonate was removed by filtration and washed with a mixture of equal portions of diethyl ether and acetone. Evaporation of the combined filtrates by flash distillation left 105 g of crude partially acetonated material (II), which was fractionated by extracting with three 125-ml portions of boiling water. The water-insoluble residue, triisopropylidene-(−)-inositol, was recrystallized from absolute ethanol (10 ml/g) to give 34 g of 1,2:3,4:5,6-tri-*O*-isopropylidene-(−)-inositol (IIa), m.p. 215.5–217.5°. The aqueous extract was evaporated to dryness and extracted with about 3 liters of hot benzene in 500-ml portions. After filtration, the filtrate was cooled to 5°, yielding 54 g of crude diisopropylidene-(−)-inositol. This was recrystallized from water to yield 39.2 g of 1,2:5,6-di-*O*-isopropylidene-(−)-inositol (IIb), m.p. 154–155°. The benzene-insoluble fraction was recrystallized from ethyl acetate to yield 1.2 g of 1,2-*O*-isopropylidene-(−)-inositol (IIc), m.p. 155–156°.

Hydrolysis of IIa, 197 g, in a mixture of 845 ml of chloroform, 280 ml of water, and 985 ml of glacial acetic acid for 24 hours provided an additional amount of IIb and IIc (Angyal and MacDonald, 1953). The reaction mixture was evaporated to dryness and fractionated as described above. No triisopropylidene-(−)-inositol remained, while 75 g of IIb, 12.4 g of IIc, and 96.7 g of crude I were isolated. The yield of IIb was 60–75% from I, but it could be increased by use of the mother liquors for further preparations and by recycling the recovered I. The IIb was not recrystallized before further use and had m.p. 148–152°; reported for the (−)-isomer, m.p. 149–151° (Ballou and Fischer, 1953; for the (+)-isomer, m.p. 153° (Angyal and MacDonald, 1952).

2,3:4,5-Di-O-isopropylidene-L-mannitol (IIIb).—Diisopropylidene-(−)-inositol (IIb), 189.5 g (0.734 mole), was dissolved in 2665 ml of 0.303 M sodium periodate (0.803 mole, 10% excess). The *p*H of the solution was adjusted immediately to 7.0 with 9.3 g of solid sodium carbonate, and 335 ml of water was added to prevent premature precipitation of the salts. The reaction mixture was stirred mechanically at room temperature for 30 minutes. After it was cooled to 5°, 3 liters of absolute ethanol was added to precipitate the sodium salts. The solution was filtered, and the salt was washed with 50% aqueous ethanol. The filtrate and washings were combined, cooled to 1° in an ice salt bath, and refiltered. The filtrate was cooled to 1° again and 135 g of sodium borohydride in 1 liter of water was added with smooth mechanical stirring. The solution was stirred at 1–5° for 2 hours and was then left standing at 20° in the hood overnight. The reaction mixture was concentrated to about 1.5 liters by flash distillation, and the resulting solution was extracted with 4 liters of chloroform in 500-ml portions. The combined chloroform layer was dried with sodium sulfate and evaporated to a crystalline residue that contained 153.3 g of crude IIIb. The yield was 80.5%. This was recrystallized to yield 137.2 g of IIIb, m.p. 91–92°, $[\alpha]_D$ −12.8° (*c* 2, chloroform), +1.0° (*c* 2, ethanol). The D-isomer is reported to have m.p. 90–92° and $[\alpha]_D$ +13.4° (chloroform) (Ballou and Fischer, 1953), and the L-isomer, m.p. 91.5°, $[\alpha]_D$ +1° (*c* 1.1, ethanol) (Angyal and MacDonald, 1953).

1,6-Di-O-benzoyl-2,3:4,5-di-O-isopropylidene-L-mannitol (IV).—To a solution of 130 g of IIIb (0.5 mole) in 500 ml of anhydrous pyridine, 130 ml of benzoyl chloride (1.1 mole) was added dropwise over a period of 1 hour at 0–5° with good stirring. After the mixture was stirred at room temperature for an additional 30 minutes and allowed to stand overnight at 5°, about 3 ml of water was added to decompose the excess benzoyl chloride. After 30 minutes the reaction mixture was poured into 4 liters of ice water, from which the product came out initially as an oil. Soon it solidified, and it was then filtered off, crushed in a mortar and pestle, washed well with water, and then crystallized from absolute methanol to give 213.7 g of dibenzoyl-diisopropylidene-L-mannitol (IV). The yield was 91%. Another preparation was recrystallized from methanol to give fine needles, m.p. 77.5–78°, $[\alpha]_D$ −24.6° (*c* 2, chloroform).

Anal. Calcd. for $C_{26}H_{30}O_8$ (470.5): C, 66.4; H, 6.4. Found: C, 66.0; H, 6.51.

3,4-O-Isopropylidene-1,2,5,6-tetra-O-benzoyl-L-mannitol (V).—IV, 283 g, was dissolved in 2660 ml of dry acetone and a mixture of 8.5 ml of concentrated sulfuric acid and 11.0 ml of water was added. The suspension was shaken for 4 hours at room temperature, then 107 g of anhydrous cupric sulfate was added and the reaction returned to the shaker for an additional 36 hours, during which time it became highly colored. The cupric sulfate was filtered off and the sulfuric acid was neutralized with ammonia gas. After refiltration to remove ammonium sulfate, the filtrate was concentrated to a sirup (289 g), which was benzoylated in 800 ml of dry pyridine at 0–5° by the slow addition of 210 ml of benzoyl chloride (50% excess). The mixture was left at room temperature overnight, after which the excess reagent was destroyed with water. The reaction was poured into 5 liters of ice water, from which the product crystallized. Recrystallization of the crude V from absolute ethanol gave 297.3 g of isopropylidene-tetrabenzoyl-L-mannitol, m.p. 119–120°, in 74% yield from IV.

A sample of V was recrystallized from ethanol for analysis and showed m.p. 121–123°, $[\alpha]_D$ −1.56° (*c* 2, chloroform). The D-isomer is reported to have m.p. 122–123°, $[\alpha]_D$ +2° (chloroform) (Fischer, 1890).

Anal. Calcd. for $C_{37}H_{34}O_{10}$ (638.6): C, 69.4; H, 5.37. Found: C, 69.17; H, 5.34.

1,2,5,6-Tetra-O-benzoyl-L-mannitol (VI).—One hundred grams of unrecrystallized isopropylidene-tetrabenzoyl-L-mannitol, V, was dissolved in 1 liter of glacial acetic acid on the steam bath. After the solution had cooled to room temperature, 120 ml of concentrated hydrochloric acid was added, and the mixture was shaken to suspend the precipitate that formed. At the end of 24 hours, the solution was poured into a rapidly stirred mixture of 5 liters of water and 250 ml of benzene. The phases were separated after 15 minutes and the aqueous phase was reextracted twice with 250-ml portions of benzene. The combined benzene layer was washed with water, dried with sodium sulfate, and concentrated to a sirupy white mass. The crude tetrabenzoyl-mannitol, VI, was crystallized from a 1:1 mixture of benzene and light petroleum ether to give 90 g of VI (97%), m.p. 121–122°, $[\alpha]_D$ +28.1° (*c* 2, chloroform); reported for the D-isomer, m.p. 127–128°, $[\alpha]_D$ −27.9° (chloroform) (E. Fischer, 1890).

Anal. Calcd. for $C_{34}H_{30}O_{10}$ (598.6): C, 68.22; H, 5.05. Found: C, 67.96; H, 5.21.

2,3-Di-O-benzoyl-L-glyceraldehyde (*VII*).—To a mechanically stirred solution of 90.5 g of VI (0.15 mole) in 750 ml of glacial acetic acid was added 110 g (6% excess) of lead tetraoxide (Varga, 1948) in small portions over a 1-hour period. After 4 hours, the excess oxidizing agent was destroyed with 0.5 ml of ethylene glycol. The solution was poured into a suspension of 5 liters of water and 400 ml of benzene. The suspension was separated after being stirred rapidly for 15 minutes, then the aqueous layer was reextracted with two 400-ml portions of benzene. The combined benzene layer was washed with three 50-ml portions of water, dried with sodium sulfate, and concentrated to a sirup which crystallized when triturated with light petroleum ether. The yield of crude dibenzoyl-L-glyceraldehyde was 81.5 g, m.p. 75–77°, being 91% of the theoretical yield from VI. Attempts to recrystallize the VII were unsuccessful. The D-isomer is reported to have m.p. 80° (Brigl and Grüner, 1933).

2,3-Di-O-benzoyl-L-glyceronic acid (*VIII*).—Crude dibenzoyl-L-glyceraldehyde (VIII), 81.5 g, was dissolved in 500 ml of ethyl acetate, 250 ml of 4 M peroxypropionic acid (Barker and MacDonald, 1960) was added, and the solution was refluxed for 1 hour. After the solution had been cooled, it was concentrated to a sirup which was dissolved in 400 ml of benzene and evaporated to dryness again. The resulting sirup was dissolved in 1 liter of benzene and the solution was extracted five times with 30-ml portions of water. The benzene layer was dried with sodium sulfate, and then it was evaporated to a sirup which was reevaporated three times after addition of dry benzene. Crystallization occurred when the crude product was dried under vacuum, yielding 80.7 g (99%) of VIII, m.p. 77–82°. Attempts to recrystallize the compound by mixed solvent precipitation failed. The D-isomer had been crystallized from a 1:1 mixture of toluene and light petroleum ether to yield a product with m.p. 88–89° (Chü and Ballou, 1961).

1-Deoxy-1-diazo-3,4-di-O-benzoyl-L-glycerotetrulose (*X*).—A solution of 70.3 g of crude VIII in 900 ml of dry benzene and 90 ml of redistilled thionyl chloride was refluxed for 3 hours, then cooled and evaporated to a sirup, followed by reevaporation three times after addition of dry benzene. After being degassed overnight on the vacuum pump, a golden sirup (74.6 g), representing the theoretical yield of IX, was obtained. Attempts to crystallize the acid chloride were unsuccessful.

The acid chloride (IX), 74.6 g, in 200 ml of dry diethyl ether at −50° was added, with rapid stirring, to approximately 28 g of diazomethane[1] (about 100% excess) in 2.8 liters of dry diethyl ether at −50°. The temperature of the reaction was maintained at −50° to −35° for 1 hour by means of an acetone-dry bath, after which the temperature was allowed to rise to 20°. One liter of light petroleum ether was added and the mixture was left at −25° overnight. The majority of the product, which had crystallized, was then filtered off in the hood. By concentration of the mother liquors on a rotary evaporator in the hood (with glacial acetic acid in the condenser), an additional small portion of crude X was obtained. The yield was 66 g, m.p. 94–98° (dec.), which was 87.4% based on VIII.

The crude X was purified by dissolving it in 20 parts (w/v) of dry benzene and adding a weight of Magnesol[2] equal to that of the product. The suspension was shaken at room temperature for 30 minutes, then the Magnesol was filtered off and washed with one-half of the original volume of benzene. The filtrate and washings were evaporated to dryness and the purified X was crystallized from 5 parts of benzene (weight/volume) by the addition of 5 parts of light petroleum ether. Two crops of pale yellow needles, m.p. 102–104°, were obtained to give X (57.9 g) in a yield of 76.3% from VIII. A thrice-recrystallized sample had m.p. 103–105°, $[\alpha]_D$ +2.9° (*c* 2, chloroform); reported for the D-isomer, m.p. 105–106°, $[\alpha]_D$ −3.1 (chloroform) (Chü and Ballou, 1961).

Anal. Calcd. for $C_{18}H_{14}O_5N_2$ (338.3): C, 63.9; H, 4.17; N, 8.28. Found: C, 63.94; H, 4.26; N, 8.28.

3,4-Di-O-benzoyl-L-glycero-tetrulose (*XI*).—Purified X, 27.2 g, was dissolved in 250 ml of pure dioxane,[3] and the solution was warmed to 50° in a water bath and then mixed with 80 ml of 3 N sulfuric acid. The theoretical volume of nitrogen was evolved in the 30 minutes, during which the solution was warmed from 50° to 70°. The reaction mixture was poured into a suspension of 1 liter of water and 200 ml of benzene, the layers were separated, and the aqueous layer was reextracted with two 300-ml portions of benzene. The combined benzene layer was washed twice with 50-ml portions of water (until the water was neutral to *p*H paper), dried with sodium sulfate, and then concentrated to a sirup which weighed 27.6 g. The yield was quantitative. The infrared spectrum of the sirup, sandwiched between sodium chloride plates, showed a peak at 2.85 μ, indicating a free hydroxyl group. The compound was quite reactive toward triphenyltetrazolium chloride in methanolic potassium hydroxide (Wallenfels, 1950). It could not be crystallized from any of several solvents and showed a pronounced tendency to decompose, as evidenced by the appearance of benzoic acid and a dark color.

3,4-Di-O-benzoyl-L-glycero-tetrulose Dimethyl Acetal (*XII*).—A solution of dibenzoyl-L-erythrulose (XI), 5.4 g in 15 ml of dry methanol, was refluxed with 15 ml of redistilled trimethyl orthoformate and 0.075 ml of concentrated sulfuric acid for 8.5 hours, after which time it was still acidic. The solution was poured

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[1] Diazomethane, prepared by the second method of Arndt (1943) in a twenty-fold preparation, was dried over potassium hydroxide pellets overnight at −25°, and then the ethereal solution was decanted from the solid and used immediately. Exposure to light was avoided by covering all flasks containing diazomethane with aluminum foil.

[2] Magnesol was washed successively with benzene and 2-propanol, then dried under a vacuum at room temperature.

[3] The dioxane was purified by a modification of published procedures (Riddick and Topps, 1955) by heating it under reflux with one-tenth its volume of 1 N hydrochloric acid while a slow stream of air was passed through the solution. After 24 hours, the solution was dried over potassium hydroxide pellets and the dioxane was distilled from ferrous sulfate.

into a suspension of 800 ml of water, 0.8 ml of concentrated ammonium hydroxide, and 100 ml of benzene. The *p*H was adjusted to 7.0 with dilute sulfuric acid or ammonium hydroxide as necessary, to prevent the formation of an emulsion. The benzene layer was separated and the aqueous layer was reextracted with three 50-ml portions of benzene. The combined benzene layer was washed with three 10-ml portions of water, dried with sodium sulfate, and evaporated to a sirup weighing 5.2 g (84.4% of theory).

The product contained 16.45% methoxyl; theory for XII is 16.49%. It gave 1.6% of the color of the starting material when assayed for residual ketose by formazan formation (Wallenfels, 1950). The infrared spectrum was very similar to that of the starting material, but notable increases occurred in the peaks at 3.28 and 3.36 μ, whereas the peak at 2.85 μ remained the same after acetalation.

1,3,4-Tri-O-benzoyl-L-glycero-tetrulose Dimethyl Acetal (XVI).—A solution of 28.5 g of XII in 125 ml of anhydrous pyridine at −5° was benzoylated by the slow addition, with stirring, of 10.3 ml of benzoyl chloride (10% excess). After the solution had stood at room temperature for 3 hours, the excess reagent was destroyed with water and the mixture was poured into water, which then was extracted with benzene. The benzene layer was separated and washed with dilute hydrochloric acid, aqueous sodium bicarbonate, and water. The orange benzene solution was dried for several hours with sodium sulfate, then shaken for 5 minutes with 30 g of Magnesol prepared as previously described. The Magnesol was filtered off and washed with 100 ml of benzene. Another 30 g of Magnesol was added to the combined filtrate and washings, and the suspension was stirred for 15 minutes. This was filtered through a 30 × 50 cm column of Magnesol, which was washed with 300 ml of benzene. The total eluent was concentrated to a clear sirup which was dissolved in 500 ml of boiling methanol. After the solution was filtered and allowed to cool, two crops of fine needles separated. The first, at room temperature, m.p. 85–86°, weighed 13.6 g; the second, after 16 hours at −30°, weighed 12.9 g and had the same m.p. The yield was 73% of theory from XII. The crystalline XVI had $[\alpha]_D$ −131.5° (*c* 2, chloroform) and showed no peak at 2.85 μ in the infrared. The D-isomer is reported to have m.p. 88–90°, $[\alpha]_D$ +126° (chloroform) (Chü and Ballou, 1961).

Anal. Calcd. for $C_{27}H_{26}O_8$ (478.4): C, 67.77; H, 5.44; CH_3O, 12.97. Found: C, 67.80; H, 5.60; CH_3O, 12.70.

L-*glycero-Tetrulose 1-Phosphate Dimethyl Acetal, bis-Cyclohexylammonium Salt (XIV).*—To a solution of 5.2 g of XII in 30 ml of anhydrous pyridine at −5° was added slowly with mixing 5.0 ml of diphenylphosphorochloridate over a 30-minute period. After the reaction mixture had stood overnight at 5°, the excess reagent was destroyed by adding water, and the mixture was poured into water. The phosphorylated product XIII was extracted into benzene, which solution was washed with dilute hydrochloric acid, aqueous sodium bicarbonate, and water. The benzene solution of XIII was dried with sodium sulfate and evaporated to a sirup that weighed 6.5 g (82%). This sirup was hydrogenated overnight at 20° and atmospheric pressure in 350 ml of absolute ethanol with 2 g of platinum oxide as catalyst. The reaction consumed 3750 ml of hydrogen, the calculated amount being 3820 ml. The catalyst was removed by centrifugation and washed with absolute ethanol. The combined supernatant was added to 275 ml of 0.224 M barium hydroxide and left to saponify at room temperature for 12 hours, whereupon the ethanol was removed by evaporation. A solution of 18.3 g of *bis*-cyclohexylammonium sulfate in 200 ml of water was then added and the mixture was stirred for 15 minutes. The barium sulfate was removed by centrifugation and washed with water. The combined supernatant was concentrated to a white powder which was suspended in acetone. Filtration of the suspension yielded 5.8 g of powder. Crystallization of the acetone-insoluble material from acetone-water mixtures containing an excess of cyclohexylamine (5 to 10 parts of acetone to one of water containing a few drops of cyclohexylamine) gave 1.7 g of L-erythrulose 1-phosphate dimethyl acetal *bis*-cyclohexylammonium salt monohydrate, which was 53% based on XII.

A sample, recrystallized for analysis, had m.p. 165–167° and $[\alpha]_D$ −13.0° (*c* 2, phosphate buffer, *p*H 8.0), and −7.2° (*c* 2, acetate buffer, *p*H 4.0). Reported for the D-isomer are m.p. 160–165° and $[\alpha]_D$ +13.3° (water) (Chü and Ballou, 1961).

Anal. Calcd. for $C_{18}H_{41}N_2O_8P.H_2O$ (462.5)[4]: C, 46.7; H, 9.31; N, 6.05; P, 6.71; CH_3O, 13.4. Found: C, 46.7; H, 9.29; N, 6.22; P, 6.69; CH_3O, 12.9.

The compound, dried at room temperature, consumed 1.0 mole of sodium periodate per mole. It contained only a trace of inorganic phosphate. Titrations were carried out on aliquots which were treated with Dowex-50(H), 50–100 mesh resin for 3 minutes, filtered, and the resin washed with water to give a final volume of 3.0 ml. The titrant was 0.542 N tetrapropylammonium hydroxide. Titration data were derived directly from the recording produced by the Difunctional *p*H Titrator and showed 1.0 equivalents per mole of sample at pK_2 = 6.45 ± 0.02.

The infrared spectrum of XIV was identical to that of the D-isomer when compared in potassium bromide pellets at 2% concentration.

L-*glycero-Tetrulose 1-Phosphate (XV).*—The lability of α-ketophosphate esters to base (Meyerhof and Lohmann, 1934) was used as a measure of the conversion of XIV to XV. A portion of XIV (13.6 mg in 3.0 ml of water) was treated with 2 g of Dowex-50(H), the solution was heated at 100° in a water bath and sampled at 1-minute intervals. The samples were placed in an equal volume of 2 N sodium hydroxide, and, after 15 minutes at room temperature, 0.4 ml of 10 N sulfuric acid was added before the solutions were assayed for inorganic phosphate. Duplicate samples were treated with sodium hydroxide and analyzed for total phosphorus. The hydrolysis of the acetal was complete in 3 minutes, as evidenced by the appearance of all the organic phosphate ester as alkali-labile phosphate.

Free L-erythrulose 1-phosphate was indistinguishable chromatographically from the D-isomer, and it traveled slightly more slowly than XIV in the 2-propanol–ammonia–water (7:1:2) solvent system of Markham and Smith (1952). A trace contaminant of some substance with the *RF* of erythrulose 4-phosphate (Chü and Ballou, 1961) was revealed by the phosphate spray of Axelrod and Bandursky (1951), the alkaline silver nitrate dip (Anet and Reynolds, 1954), and sodium periodate and benzidene sprays (Cifonelli and Smith, 1954). A sample of XV was titrated, by the same procedure as for XIV, and showed 0.929 equivalents at pK_2 = 6.24.

Reaction of Tetrulose Phosphates with Glycerol Phosphate Dehydrogenase.—Experiments were carried out

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[4] Although the D-isomer originally gave an analysis for the anhydrous salt (Chü and Ballou, 1961), both isomers dried under the same conditions have subsequently been found to give the correct analysis for a monohydrate.

with a preparation of this enzyme obtained from rabbit muscle by the method of Beisenherz *et al.* (1955), and with the commercially purified enzyme supplied by California Corporation for Biochemical Research from Boerhinger and Son. The enzyme was assayed for activity as described by Beisenherz *et al.* (1955). The change in absorbance of the reduced nicotinamide adenine dinucleotide (NADH) was measured at 340 mμ, assuming that the oxidation of 1 μmole of the nucleotide would result in a change of absorbancy of 2.074 in a total volume of 3.0 ml. The assay mixture used in the determination of K_m and $\bar{V}$ for the two erythrulose 1-phosphates and dihydroxyacetone phosphate contained 150 μmoles of diethylamine buffer, *p*H 7.2, 0.25 μmole of NADH, and sufficient dehydrogenase to give significant rates. Solutions of ketose, prepared from their acetals by acid hydrolysis (Ballou and Fischer, 1955), were assayed to determine the amount of biologically active material present.

The method outlined by Chü and Ballou (1961) for the reduction of D-erythrulose 1-phosphate and identification of the resulting D-erythritol 1-phosphate was used to study the reaction of the L-isomer. The procedure was modified to free the reaction of a trace of glycerol. A large-scale reaction, in which 97.7% of the theoretical amount of NADH was oxidized over the 24-hour period during which the reaction was run, was carried out to obtain sufficient product from the reduction of L-erythrulose 1-phosphate for a structural determination. Removal of the proteins and nucleotides left a polyol phosphate which chromatographed the same as erythritol phosphate in the 2-propanol–ammonia–water (7:1:2) system of Markham and Smith (1952). Dephosphorylation of the polyol phosphate with alkaline phosphatase gave a tetritol which was freed of a small amount of glycerol by chromatography on Whatman 3MM paper using 80% aqueous acetone as the developing solvent (Ballou and Anderson, 1953). Electrophoresis (Whatman 3MM, 0.05 M sodium borate, *p*H 9.0, 2200 v, 8 ma, 45 minutes) was used to identify the compound as threitol.

Reaction of Tetrulose Phosphates with Aldolase.—A thrice-recrystallized preparation of rabbit muscle aldolase (Taylor, 1955) was utilized. Activity of the enzyme was determined by coupling it to glycerol phosphate dehydrogenase. The complete reaction mixture for the assay contained 150 μmoles of diethylamine buffer, *p*H 7.2; 10 μmoles of fructose 1,6-diphosphate; 10 units of the dehydrogenase; and 0.25 μmoles of NADH, in a total volume of 3.0 ml. In other studies of the enzyme using the tetrulose phosphates, only the concentrations of the substrates were changed.

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