Chemistry and Metabolism of 3-Deoxy-\(\beta\)-mannooctulosonic Acid

1. STEREOCHEMICAL DETERMINATION*

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SUMMARY

Ammonium 3-deoxy-\(\beta\)-glucooctulosonate and ammonium 3-deoxy-\(\beta\)-mannooctulosonate (KDO) have been synthesized. \(\beta\)-Arabinose was condensed with potassium di-t-butyl oxalacetate to give 3-deoxy-3-\(\beta\)-(t-butoxycarbonyl)-2-en-2-ol-d-mannooctulosonate \(\gamma\)-lactone (Compound II) and 3-deoxy-3-\(\beta\)-(t-butoxycarbonyl)-2-en-2-ol-\(\beta\)-glucooctulosonate \(\gamma\)-lactone (Compound I), which were hydrolyzed and decarboxylated to form 3-deoxy-\(\beta\)-glucooctulosonate \(\gamma\)-lactone (Compound I) and 3-deoxy-\(\beta\)-mannooctulosonate \(\gamma\)-lactone (Compound IV). Compounds III and IV were converted to V and VI. Structures were assigned on the basis of ultraviolet, infrared, and nuclear magnetic resonance spectra, optical rotation, and chemical properties. Ozonolysis of Compounds III and IV gave glucose and mannose, respectively. KDO aldolase cleaved the \(m\)ano isomer (KDO), but was inactive toward the \(g\)lucos isomer.

Since its discovery in 1959 (1), 2-keto-3-deoxyoctonate has steadily grown in importance as a natural product of bacterial origin. It is an integral component of the cell wall of gram-negative bacteria and is believed to provide the linkage, via a ketosidic bond, between the antigen and lipid A of the lipopolysaccharide (2). Eds-rom and Heath (3) and Osborn (4) have shown that, KDO occupies the reducing terminal position of the somatic (O) antigen. Since KDO has a central role in the linkage of the somatic (O) antigen to the bacterial surface, an understanding of its chemistry and metabolism is important to understanding its biological function.

Studies to determine the chemical properties and structure of KDO (5-7) have resulted in the proposal that naturally occurring KDO is 3 deoxy \(\beta\)-mannooctulosonic acid. This conclusion was based on the rate of oxidation with periodate (6) and on ceric sulfate oxidation following sodium borohydride reduction (7). This report describes the chemical synthesis and structural determination of ammonium 3-deoxy-\(\beta\)-mannooctulosonate and ammonium 3-deoxy-\(\beta\)-glucooctulosonate based on the method of Kuhn and Basch anding (8, 9). KDO aldolase isolated from Aerobacter cloacae NCTC 5920 utilizes the \(m\)ano isomer but is inactive toward the \(g\)lucos isomer.

MATERIALS AND METHODS

Materials—All compounds and reagents were obtained from commercial sources except where noted otherwise. Potassium di-t-butyl oxalacetate was synthesized by the procedure of Heidelberger and Hartley (10) and Bacher and Homan (11). A crude, cell-free solution of KDO aldolase activity was prepared by the procedure of Ghabrial and Heath (12). KDO aldolase was assayed by a modification of the two-step lactic dehydrogenase procedure (18). Each incubation mixture contained the following in a final volume of 1.1 ml: KDO aldolase, KDO salt (30 \(\mu\)moles), and phosphate buffer (40 \(\mu\)moles), pH 7.0. Aliquots of 200 \(\mu\)l were heated for 60 sec in a boiling water bath. The heat-denatured aliquots were cooled to 0-4° and centrifuged for 20 min at 7500 rpm. One hundred-microliter samples of each aliquot were used for the second step of the assay. Each assay for pyruvate contained the following in a final volume of 1.0 ml: \(\gamma\)-DPNH (0.128 \(\mu\)mole), Sigma type I lactic dehydrogenase (2.5 units), phosphate buffer (20 \(\mu\)moles, pH 7.0), and a heat-denatured aliquot from KDO aldolase incubation mixture (100 \(\mu\)l). The incubation mixtures were heated at 37° for 2 min, and the optical densities at 340 mp were determined in 1-cm cuvettes in a Beckman DU-2 spectrophotometer. The amount of KDO aldolase activity in each assay was

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1 The abbreviations used are: KDO, 2-keto-3-deoxyoctulosonate; NMR, nuclear magnetic resonance.

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selected so that the decrease in optical density at 340 m\(\mu\) was 40 to 60\% of the initial optical density of 0.8. Protein concentration was measured by the procedure of Waddell (19).

**Chromatographic Methods**—Thin layer chromatography was performed on Whatman thin layer Chromedia SC-41 at a thickness of 0.20 mm. The plates were developed by ascending chromatography and removed from the chamber when the solvent had run 10.0 cm. Organic compounds were visualized by spraying the chromatograms with potassium dichromate-sulfuric acid spray and heating gently with a Meeker burner. Paper chromatography was carried out in a descending manner on Whatman No. 1 filter paper. KIO\(_4\) was detected on the chromatograms with Warren's spray reagent (20), compounds with a glycol structure were detected with periodate-benzidine reagent (21), and reducing compounds were detected by dipping the paper into an acetone-silver nitrate solution, developing with an ethanolic potassium hydroxide solution, fixing in 6% ammonium hydroxide, and washing with water. The irrigation systems used for thin layer and paper chromatography were: A, 1 butanol ethanol-water (5:1:4); B, 1-butanol-ethanol-water (4:1:1); C, water-saturated phenol; and D, acetone-1-butanol-water (7:2:1). Methanol solutions of the aldonic acids were decationized immediately prior to thin layer or paper chromatography on a column (0.5 \(\times\) 5 cm) of AG50W-X8, 200 to 400 mesh, hydrogen form resin suspended in methanol. Cation exchange chromatography was effected on a column (3 \(\times\) 30 cm) of AG50W-X8, 20 to 80 mesh, hydrogen form resin, also in methanol. Separations involving partition chromatography were carried out on cellulose columns (1.2 \(\times\) 37 cm) which had been packed in 85% acetone, the solvent to be used as the eluting agent.

**Determination of Physical Constants**—Corrected melting points were determined on a Laboratory Device's Mel-Temp unit with the temperature increasing at a rate of 2° per min. Optical rotations were determined on a model 70 No. 1002 Rudolph polarimeter with a 1.3 to 3.5% solution in a 1-cm cell, with the sodium D-line as the light source. Infrared spectra were recorded with a Perkin-Elmer Infracord spectrophotometer and interpreted by first order analysis. All samples were run as 15 to 20% solutions in dimethyl sulfoxide with tetramethylsilane as an internal reference.

**Oxonolysis**—Oxonolysis was carried out at 16-20° with a Weisbach T-23 oxonizer which had the following settings: voltage, 80 volts; air pressure, 4.5 p.s.i.; oxygen pressure, 8.0 p.s.i.; ozone flow rate, 0.005 cu ft per min. The ozone was decomposed by reduction with hydrogen with 5% palladium on asbestos as a catalyst. Hydrogenation was continued until the uptake of hydrogen ceased.

**EXPERIMENTS AND RESULTS**

**Synthesis of 3-Deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-\(\alpha\)-manno-octulosonate \(\gamma\)-Lactone and 3-Deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-\(\alpha\)-glucosoctulosonate \(\gamma\)-Lactone (Fig. 1)—**Potassium di-t-butyl oxalacetate (1.25 g) was suspended in 50 ml of anhydrous methanol, and 1.5 g of D-arabinose was added immediately. The reaction flask was flushed with anhydrous argon and sealed with a ground glass stopper, and the suspension was shaken on a wrist action shaker or stirred on a magnetic stirrer for 72 hours. AG50W-X8, 20 to 80 mesh, hydrogen form resin, previously washed with anhydrous methanol, was added with stirring until the pH of the purple suspension had dropped from approximately 11 to approximately 4. During this treatment, the suspended solid dissolved and the solution became light yellow. The resin was removed by filtration and washed with anhydrous methanol. The filtrate and wash were decationized by column chromatography, and the effluent was concentrated to a yellow syrup on a rotary evaporator.

The syrup was triturated with successive samples of ethyl ether until no further color was removed. The white powder (2.3 g) was triturated with small portions of ethyl acetate (total, 250 ml), followed by trituration with small portions of absolute ethanol (150 ml were used). The ethyl acetate and ethanol solutions were each concentrated under reduced pressure to effect crystallization. Colorless needles (0.92 g or 28.8% yield) were isolated from the ethyl acetate (designated as Compound I). Colorless plates (1.2 g or 37.5% yield) were isolated from the ethanol (designated as Compound II). After three recrystallizations from ethyl acetate, 0.61 g (19% yield) of Compound I and 0.41 g (15% yield) of Compound II were isolated. Compound I had a melting point of 138.5-140° and \([\alpha]_D^{25} +61.4° (c, 1.6, in methanol). Compound II had a melting point of 152-154° and \([\alpha]_D^{25} -5.2° (c, 3.0, in methanol). On thin layer chromatography, with Solvent B, Compound I had an \(R_F\) of 0.64 and Compound II had an \(R_F\) of 0.66; with Solvent A, the respective \(R_F\) values were 0.44 and 0.34.

\[
\text{C}_{10}\text{H}_{14}\text{O}_5
\]

**Calculated:** C 48.77%, H 6.25%  
**Found:**  
Compound I: C 48.60%, H 6.37%  
Compound II: C 48.64%, H 6.36%

**Structural Proof of Compounds I and II—**Each of the two
compounds gave a positive ferric chloride (22) test, indicative of the presence of an enolic hydroxyl. The ultraviolet absorption, as predicted on the basis of A. T. Nielsen's rule (23) and as observed, are given in Table I. The close agreement between these spectra was strong evidence for the 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol carboxyl structure of each compound. A band at 5.61 μ in the infrared spectra (Fig. 2) of each compound indicated the presence of a γ-lactone (24, 25). Compound II exhibited bands at 5.87 and 5.96 μ. These bands are characteristic of β keto esters exhibiting hydrogen bonding of the enolic proton to the ester carboxyl oxygen (24). Compound I exhibited a band at 5.87 μ, and the expected longer wave length band appeared as a shoulder. The infrared spectra did not suggest the presence of a free carboxyl group; however, the enolic proton was expected to be strongly acidic. The determined neutralization equivalents were 321 and 322, compared to a theoretical value of 320.2.

Confirmation of the size of the lactone rings was obtained by periodate oxidation. Theoretically, the γ-lactone should have consumed 3 eq of periodate with the production of 2 eq of formic acid, and the δ-lactone should have consumed 2 eq of periodate with the production of 1 eq of formic acid. The experimentally determined results, shown in Fig. 3, clearly confirmed the presence of a γ-lactone ring in each compound. Based on the existence of the γ-lactone ring and Hudson's lactone rule (26), tentative stereochemical assignments about carbon atom 4 were made from the optical rotatios. Compound II was tentatively identified as 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-manno-octulosonate γ-lactone, and Compound I as 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-glucosyl-octulosonate γ-lactone.

NMR spectra (Fig. 4) of Compounds I and II confirmed the stereochemical assignments. Interpretation of the NMR spectra was based on inspection of space-filling models. The dihedral angle between protons on carbon atoms 4 and 5 of the glucosyl epimer was approximately 70°, and for the mannose epimer it was approximately 160° (Fig. 5). The spectrum of Compound I exhibited the following signals: (a) δ = 5.22, (b) δ = 4.37, (c) δ = 4.10, (d) δ = 3.80, (e) δ = 3.40, and (f) δ = 1.46. These signals were assigned to the following respective protons: (a) on carbon atom 4 (27), (b) on the alcoholic hydroxyls, (c) on carbon atom 5 (as), (d and e) on carbon atoms 6, 7, and 8, and (f) on the t-butoxy group (28). These assignments were supported by integration of the spectra. Compound II exhibited the following signals: (a) δ = 5.10, (b) δ = 4.37, (c) δ = 4.10, (d) δ = 3.68, (e) δ = 3.51, and (f) δ = 1.46. The signals were assigned in the same respective order as with Compound I, and the assignments were supported by integration of the spectra. The coupling constant between the protons on carbon atoms 4 and 5 of Compound I was less than 0.5 cps, and the coupling constant between the protons on carbon atoms 4 and 5 of Compound II was 4.45 cps. Therefore, Compound I was concluded to be the β-glucosyl epimer, and Compound II was concluded to be the β-manno epimer (29, 30).

**Table I**

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Fig. 2. Infrared spectra of 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-glucosyl-octulosonate γ-lactone (A) and 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-manno-octulosonate γ-lactone (B).

Fig. 3. Periodate oxidation of Compounds I and II. - - , 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-glucosyl-octulosonate γ-lactone; -- - , 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-β-manno-octulosonate γ-lactone.

Synthesis of 3-Deoxy-β-glucosyl-octulosonate γ-Lactone (Compound...
and S-Deoxy-D-manno-octulosonate γ-Lactone (Compound IV) (Fig. 6)—3-Deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-D-gluc-octulosonate γ lactone (2.00 g) was dissolved in 70 ml of deionized, distilled water, and the solution was stirred with a glass rod while being heated on a steam cone. When evolution of gas ceased after approximately 15 min, the solution was cooled to room temperature. After three extractions with 70-ml portions of ethyl ether, the faintly yellow solution was slowly filtered through 2.4 g of Darco C activated charcoal. Concentration by rotary evaporation yielded a colorless syrup. The syrup was dried to a hydroscopic powder by reconcentration three times from ethanol. A colorless powder (1.03 g or 75% yield) with $[\alpha]_D^{24} +13.2^\circ$ (c 3.2, in water) was isolated. 3-Deoxy-D-manno-octulosonate γ-lactone was prepared from 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-D-manno-octulosonate γ-lactone by the same procedure. The initial reaction mixture was a suspension rather than a solution. When the charcoal filtrate was concentrated, crystallization occurred. Colorless crystals (1.10 g or 80% yield) with m.p. 192–194$^\circ$ and $[\alpha]_D^{24} +31.8^\circ$ (c 1.4, in water) were isolated.

\[ \text{C}_9\text{H}_{12}\text{O}_7 \]

Calculated: C 43.63%, H 5.50%

Found: C 43.50%, H 5.61%

**Fig. 5.** Projection formulas of 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-D-gluc-octulosonate γ-lactone (I) and 3-deoxy-3-(t-butoxycarbonyl)-2-en-2-ol-D-manno-octulosonate γ-lactone (II). The figures are drawn with carbon atom 5 to the front. R represents the arabinose side chain, and R' represents the t-butoxycarbonyl group.

**Fig. 6.** Synthesis of 3-deoxy-2-en-2-ol octulosonate γ-lactones.
Deoxy-o-mannoctulosonate γ-Lactone—The observed ultraviolet absorption bands of each compound and those predicted on the basis of Nielsen’s rules are given in Table II. The close agreement between the predicted and observed results was taken as strong evidence for the 3-deoxy-2-en-2-ol carboxyl structures. The fact that the molar extinction coefficients were slightly lower than that of 3-deoxy-2-en-2-ol-α-arabinohexitulosonate γ-lactone (31) could be explained in either of two ways. In solution, the compounds exist as a tautomeric equilibrium of the keto and enol forms, or the structural differences between the heptulosonate enol lactone and the octulosonate enol lactones may be such that the octulosonates exhibit weaker electronic interactions. Both Compounds III and IV gave positive responses to ferric chloride and decolorized bromine solution, indicating the presence of the enol structure (22). The integrity of the γ-lactone ring and presence of the enol structure were further confirmed by infrared spectra (Fig. 8). Compound III exhibited peaks at 5.61 μ and 6.01 μ. These peaks corresponded to peaks characteristically observed in the spectra of 3-deoxy-2-en-2-ol γ-lactones (31). The corresponding bands exhibited by Compound IV were at 5.61 μ and 5.95 μ. The analogous bands exhibited by 3-deoxy-D-gluc-octulosonate γ-lactone were at 5.71 μ and 6.02 μ (Fig. 8).

Table II

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<td>228</td>
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<td></td>
<td>Observed</td>
<td>230</td>
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Definitive chemical verification of the stereochemical assignments was obtained from ozonolysis. Compound IV (41 mg) was suspended in 0.3 ml of glacial acetic acid. Ozonization was carried out for 80 to 90 min, until all of the solid dissolved and a viscous, colorless solution resulted. The solution was diluted with 0.3 ml of glacial acetic acid and hydrogenated in the presence of 11 mg of 5% palladium on asbestos as catalyst. The catalyst was removed by filtration, and acetic acid was removed by rotary evaporation. Hexose (11 mg or 33% yield) was isolated by preparative paper chromatography on Whatman No. 1 filter paper with Solvent D. Paper and thin layer chromatography with Solvents C and D revealed chromatographic identity between the product and authentic mannose (Table III). No glucose was present. For preparation of the phenylhydrazone, 11 mg of the ozonolysis product in 0.15 ml of ethanol were allowed to react with 10 μl of phenylhydrazine. An identical sample of authentic mannose was allowed to react simultaneously. The melting point of the product was 198-199°, and the melting point of mixture with the authentic mannose phenylhydrazone was 197.5-198.5°. The infrared spectra of the two phenylhydrazones were superimposable.

Compound III (33 mg) in 0.25 ml of glacial acetic acid was ozonized for 60 min, until the powder dissolved to form a colorless, viscous solution. The reaction mixture was treated by the same procedure as described for Compound IV. Paper and thin layer chromatography with Solvents C and D revealed chromatographic identity of the product with authentic glucose (Table III). No mannose was present. Reaction with glucose oxidase indicated that 1.9 mg (7% yield) of glucose had been isolated.

Synthesis of Ammonium 3-Deoxy-o-gluc-octulosonate and Ammonium 3-Deoxy-o-mannoctulosonate (Fig. 9)—Compound IV (1.00 g) was dissolved in ice water. The solution was maintained at 0-4° and at pH 9.0 with 0.1 N ammonium hydroxide.
for 24 hours. After 24 hours, 50-μl aliquots were diluted to 1 ml at pH 2.0 and pH 10.0 for recording ultraviolet spectra, which indicated that less than 10% of the enol sugar remained. The solution was adjusted to pH 6.0 with AG50W-X8 hydrogen form resin, and the resin was removed by suction filtration. After overnight refrigeration, the ultraviolet spectra showed that about 3 to 5% of the enol form of the sugar remained. The infrared spectra (Fig. 10) exhibited no peaks attributed to the lactone; however, peaks at 6.15 μ and 7.15 μ, characteristic of carboxyl salts (24), were observed. After being adjusted to pH 7.0 with 0.1 N ammonium hydroxide, the yellow solution was slowly filtered through 2.4 g of Darco C activated charcoal. The ultraviolet spectrum of the colorless filtrate was featureless above 220 μ. Ammonium's thiobarbiturate assay indicated the presence of 500 mg (48.5% yield) of ammonium 3-deoxy-o-manno-octulosonate. This solution was stable to storage in the frozen state. The solution was concentrated to a syrup and subjected to cellulose column chromatography. A single thiobarbituric acid-positive peak was eluted in Fractions 15 through 75, and after refrigeration for a few days crystallization occurred in the thiobarbiturate-positive fractions. Colorless plates (51 mg) with melting point of 125-126° and [α]D +41.3° (c, 1.9, in water) were isolated.

The infrared spectra (Fig. 11) were consistent with the proposed structure.

Compounds III (0.7 g) was dissolved in 25 ml of ice water and treated by exactly the same procedure as described for Compound IV to yield 236 mg (29.2% yield) of ammonium 3-deoxy-o-glucouronulosonate in the charcoal filtrate. The ultraviolet spectrum was featureless above 220 μ, and the infrared spectrum (Fig. 10) was consistent with the proposed structure. Upon cellulose column chromatography, a single thiobarbituric acid-positive peak was eluted in Fractions 15 through 55.

The potassium and sodium salts of Compounds V and VI were prepared by an identical procedure with potassium hydroxide and sodium hydroxide, respectively. The potassium and sodium salts, however, were not subjected to cellulose column chromatography.

**Table IV**

<table>
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<th>Substrate</th>
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<td>Compound VI</td>
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Substrate Specificity of 3-Deoxy-o-manno-octulosonate Aldolase

The assay procedure was the same as described in "Materials and Methods." The substrate concentration of potassium 3-deoxy-o-glucouronulosonate and of potassium 3-deoxy-o-manno-octulosonate was 27 mM.

The calculated and found values for the compound are as follows:

**C₆H₁₁O₅N**  
Calculated: C 34.78%, H 6.88%, N 5.07%  
Found: C 34.94%, H 6.91%, N 5.02%

The infrared spectra (Fig. 11) were consistent with the proposed structure.

Compounds V and VI were prepared by an identical procedure with potassium hydroxide and sodium hydroxide, respectively. The potassium and sodium salts, however, were not subjected to cellulose column chromatography.

Substrate Specificity of 3-Deoxy-o-manno-octulosonate Aldolase—Compounds V and VI were tested as substrates for KDO-
aldolase. The substrate concentration (27 mM) was selected so that the reaction would be detected even if it proceeded at greatly reduced rates for the less active isomer. The reaction proceeded at \( V_{\text{max}} \) for the active isomer. The results in Table IV show that the enzyme exhibited stereospecificity for 3-deoxy-
D-mannooctulosonate.

**DISCUSSION**

Synthesis of ammonium 3-deoxy-D-glucooctulosonate and ammonium 3-deoxy-D-mannooctulosonate is described. Since Ghalambor and Heath (5) established the stereospecificity of 3-deoxy-D-mannooctulosonate aldolase for the \( \alpha \)-arabinino structure about carbon atoms 5 through 8, this work establishes the absolute stereospecificity of the enzyme for 3-deoxy-D-mannooctulosonic acid. From the specificity of the enzyme, it is concluded that naturally occurring KDO is 3-deoxy-D-mannooctulosonic acid. This assignment agrees with the conclusions of Ghalambor and Heath (5) and Perry and Adams (7). The ring structure (i.e. furano, pyrano, open chain, or other) of 3-deoxy-D-mannooctulosonic acid has not been determined. This problem is the subject of further work to be carried out in this laboratory.

In addition to providing the first chemically pure source of 3-deoxy-D-mannooctulosonate, the general applicability of the methods of Kuhn and Baschag for the synthesis (8) and structural proof (9) of 3-deoxyaldulosonic acids is demonstrated. Purification and structural proof of the intermediates in the reaction sequence verify the structures of the key intermediates of the procedure.

Since the stereospecificity of KDO aldolase is absolute, it is suggested that this enzyme properly may be utilized to identify 3-deoxy-D-mannooctulosonic acid from chemical or biological sources. Utilization of the enzyme to determine quantitatively 3-deoxy-D-mannooctulosonic acid should provide enhanced specificity over the present chemical assays (13, 32), which are subject to interference by deoxy sugars and other 3-deoxyaldulosonic acids.

**REFERENCES**


Chemistry and Metabolism of 3-Deoxy-d-manno-octulosonic Acid : I.
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