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THE REACTIONS OF 3-DEOXY-D-*manno*-OCT-2-ULOSONIC ACID (KDO) IN DILUTE ACID

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ABSTRACT

On heating in dilute acid, 3-deoxy-D-*manno*-oct-2-ulosonic acid (KDO) is converted into 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid and 5-(D-*erythro*-1,2,3-trihydroxypropyl)-2-furoic acid. The former is unreactive to periodic acid–thiobarbituric acid and to semicarbazide, and its formation explains the depressed estimates of KDO in lipopolysaccharides. Formation of the furoic acid can lead to high estimates using the semicarbazide assay. Neither product can be formed from 5-*O*-glycosyl-KDO.

INTRODUCTION

3-Deoxy-D-*manno*-octulosonic acid (KDO) is a characteristic component of lipopolysaccharides and polysaccharides of Gram-negative bacteria[1–3]. The extreme hydrolytic lability of its ketosidic linkage has been exploited in structural studies, but considerable losses occur even under the extremely mild acid conditions used[4,5]. We now report the characterisation of products, formed from authentic KDO under conditions typically used for lipopolysaccharide studies[5], which lead to errors in analysis.

RESULTS AND DISCUSSION

Treatment of ammonium KDO with aqueous 1% formic acid (100°, 75 min), followed by gel chromatography[6,7], gave unchanged KDO and two other acidic compounds. One of these products was unreactive to borohydride and periodate, indicating a glycosidic anhydride which lacks free vicinal hydroxyl groups. These structural requirements are uniquely satisfied by 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid (**1**).

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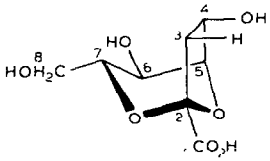

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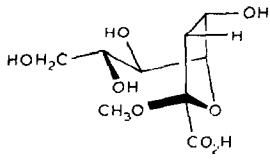

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The presence of a furanosidic ring was confirmed by the characteristic multiplet[8] for H-3 and H-3′ in the ^{1}H-n.m.r. spectrum (Table I) and resonances[8] at 44.19 (C-3), 86.42 (C-5), and 106.06 p.p.m. (C-2) in the ^{13}C spectrum (Table II, Fig. 1). The large downfield shift for C-7, relative to the methyl α-furanoside **2** (Table II), confirms C-7 as the site of the anhydro linkage. The magnitude of the chemical shift difference for C-6, compared to that for C-8 (Table II), is attributed to its involvement in the dioxane ring of **1**. Because of the configuration at C-5, the anomeric configuration must necessarily be α.

Compound **1** is analogous to the 1,6-anhydroheptofuranoses[9]. In common with these model compounds, it has a 2,8-dioxabicyclo[3.2.1]octane structure with the six-membered anhydro ring in the chair form. Of all the aldoheptose diastereomers, the D-*glycero*-D-*talo* isomer is energetically most favoured to form such an anhydride[9] **3**, as the hydroxyl groups at positions 2 and 3 and the hydroxymethyl group at position 6 are *exo* and the hydroxyl group at position 5 is equatorial. Analogously, the D-*manno* configuration of KDO favours the formation of the 2,7-anhydrofuranose **1**. Moreover, the absence of an oxygen substituent at position 3, and the decreased 3,4-*cis* interaction, adds to the stability of **1** over that of **3**. The

TABLE I

^{1}H-N.M.R. DATA FOR ARTIFACTS OF 3-DEOXY-D-*manno*-OCT-2-ULOSONIC ACID (KDO)

Proton	*Chemical shift*		*Spin coupling (Hz)*		
	1	**5**	J	**1**	**5**
3	2.11	6.97	3,3′	−15.0	
3′	2.67		3,4	1.3	3.4
4	4.61	6.54	3′,4	7.3	
5	4.35		3,5	0.8	
6	3.71	4.71	4,5	0.9	
7	3.52	4.07	5,6	4.5	
8	3.66	3.66	5,7	0.8	
8′	3.80	3.80	6,7	9.7	7.6
			6,8	0.9	
			6,8′	0.6	
			7,8	5.2	2.7
			7,8′	2.0	6.4
			8,8′	−12.3	−12.4

TABLE II

COMPARISON OF ^{13}C-N.M.R. CHEMICAL SHIFTS FOR 2,7-ANHYDRO-3-DEOXY-α-D-*manno*-2-OCTULOFURANOSONIC ACID (**1**) AND METHYL 3-DEOXY-α-D-*manno*-2-OCTULOFURANOSIDONIC ACID (**2**)

Carbon atom	**1**	**2**	*Difference*
1	176.0	176.3	−0.3
2	106.1	107.6	−1.5
3	44.2	44.3	−0.1
4	69.9	72.0	−2.1
5	86.4	85.5	−0.9
6	63.3	70.1	−6.8
7	76.3	70.4	5.9
8	62.1	63.2	−1.1

formation at equilibrium of 34% of **1**, compared[9] with 12.2% of **3**, is consistent with this analysis. Formation of the isomeric 2,8-anhydropyranose **4** from KDO is not favoured because of the destabilising interaction[9] between the *endo* substituents at positions 4 and 8.

The circular dichroism spectrum of **1** displays a strong positive band at 200 nm, characteristic of the α-furanoside series. The $J_{3,4}$ value of 1.30 Hz (Table I), on the other hand, has been observed for β-furanosides[8]. Examination of models of **1** confirms that the 2,7-linkage confers on the furanose ring an E_o conformation like that inferred[8] for model β-furanosides and which has been established[10] in 1,6-anhydrohexofuranoses.

The second acidic artifact has a strong absorption maximum at 258 nm and a ^{1}H-n.m.r. spectrum corresponding to 5-(D-*erythro*-1,2,3-trihydroxylpropyl)-2-furoic acid (**5**). This compound has been identified[11,12] as a product of the acid

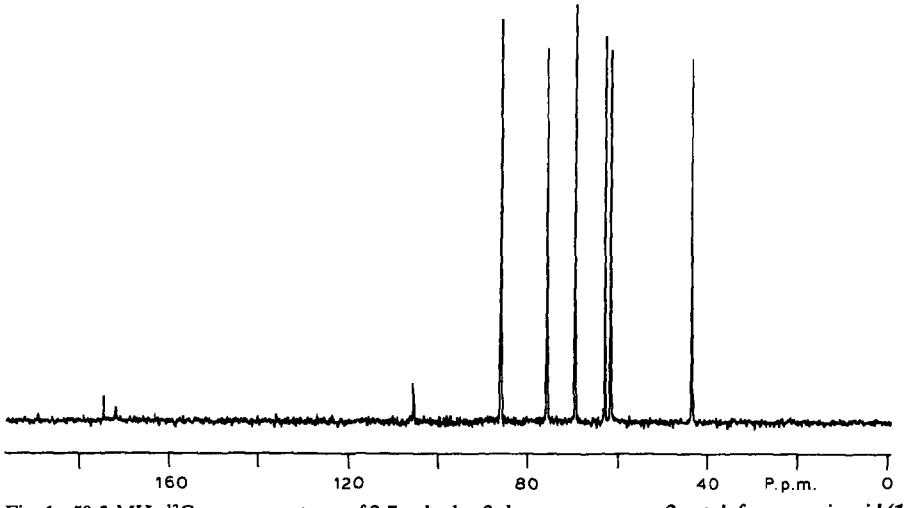
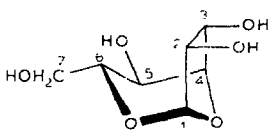


Fig. 1. 50.3-MHz ^{13}C-n.m.r. spectrum of 2,7-anhydro-3-deoxy-α-D-*manno*-2-octulofuranosonic acid (**1**).



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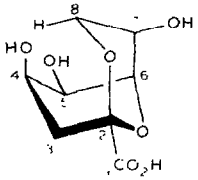

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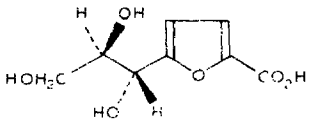

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treatment of KDO and has been isolated as the ester triacetate[8] on attempted synthesis of the methyl furanoside from the glycosyl bromide. Both artifacts derive from furanose form(s) of KDO, which are present[13] in high proportion in aqueous solution.

All peaks in the ^{13}C-n.m.r. spectrum of the crude product of acid treatment of KDO were assigned to unchanged KDO, **1**, and **5**, and their proportions determined by ^{1}H-n.m.r. spectroscopy. No evidence was found for the formation of arabinose, pyruvic acid, or 3-hydroxy-6-(D-*erythro*-1,2,3-trihydroxypropyl)-1,2-pyrone[11], or of lactones[12,14], although trace amounts of these may be present. The present conclusions are at variance with the reported conversion of KDO into periodate-sensitive 4,7- and 4,8-anhydro sugars in hot acetic acid[15]. It is to be expected that harsher acid treaments will give 1,4-dicarbonyl products as initial products of cleavage of the furan ring of **5**. This would explain the observation that, on treatment with M mineral acid, KDO gives products which, after reduction with borohydride, are reactive in the thiobarbituric acid assay[16].

After treatment with sulfuric acid (0.125M, 100°, 8 min), 34% of KDO was converted into **1**. This result is consistent with results of the thiobarbituric acid assay[5,16]. Longer treatment with formate buffer (pH 4.5, 100°, 1 h) gave 12% conversion into **1**, while formic acid (1%, 100°, 1 h) gave 31% of **1** and 5% of **5**. Pure **1**, when treated with formic acid in the same way, gave a similar mixture of products. The anhydride, therefore, is formed reversibly under these conditions.

These acid treatments are representative[1,2,5] of those used for the release of KDO from lipopolysaccharide, and provide an insight into the products of lipopolysaccharide degradation. Because **1** is unreactive to both periodic acid (used in the thiobarbituric acid assay) and to semicarbazide, its formation gives depressed estimates of KDO using either test. On the other hand, the formation of **5**, which has intense absorption at 260 nm, accounts for the high estimates given by the semicarbazide assay after extended treatment with acid[5].

Brief treatment with mineral acid, followed by the thiobarbituric acid[5,17] (for free KDO) or semicarbazide[5] (for total KDO) assay, seems preferable to more extended hydrolysis with organic acids. Treatment at pH 4.5 gives the lowest conversion into artifacts, but such mild conditions may not always be adequate[12,16,18,19] for the complete release of KDO from lipopolysaccharide.

Our observations have further important implications for lipopolysaccharide analysis. The formation of artifacts can take place only when a KDO residue has a

free 5-hydroxyl group that allows it to adopt the furanose form. Synthetic 5-*O*-glycosyl-KDO derivatives are therefore stable[20] to extended heating at pH 3.4. These compounds are representative of substituted KDO present in lipopolysaccharides[20,21]. If, then, monomeric KDO is used as a destruction control in lipopolysaccharide analysis, the assay result will be over-corrected.

The present results demonstrate that KDO is not destroyed under mild conditions of acid hydrolysis. Rather, it is converted cleanly into one or two stable artifacts, which should lend themselves to development of more satisfactory analyses of free and glycosyl-KDO. More severe acidic treatments[16,20], designed to achieve complete polysaccharide hydrolysis, bring about more complex changes and are not appropriate for KDO analysis.

EXPERIMENTAL

Materials. — 3-Deoxy-D-*manno*-oct-2-ulosonic acid (KDO) and reference methyl glycosides were synthesised[8] as previously described.

*Ammonium (methyl 3-deoxy-α-*D-manno-*oct-2-ulofuranosid)onate.* — Methyl (methyl 3-deoxy-α-D-*manno*-oct-2-ulofuranosid)onate (11 mg) was treated with sodium hydroxide (0.1M; 0.62 mL, 1.5 mol. equiv.) at room temperature for 18 h. The solution was then stirred with IR-120 (H^+) resin to pH 1.5 and filtered, the pH was adjusted to 7.0 with ammonia, and the solution was freeze-dried to give **2** (7 mg, 63%).

Acid treatment of KDO. — (*a*) *With formic acid.* A solution of ammonium KDO (0.100 g) in formic acid (1%, 10 mL) was heated at 100° for 1 h and then cooled. The pH was adjusted to 7.0 with ammonia, and the solution was freeze-dried.

(*b*) *With sulfuric acid.* Ammonium KDO (20 mg) was dissolved in water (1.0 mL), and 0.25M sulfuric acid (1.0 mL) was added. The solution was heated at 100° for 8 min, cooled, neutralised with sodium hydroxide, and freeze-dried.

(*c*) *With formate buffer.* A solution of ammonium KDO (10 mg) in ammonium formate buffer (1 mL, 25mM, pH 4.5) was heated at 100° for 1 h and then cooled. The pH was adjusted to 7.0 with ammonia, and the solution was freeze-dried.

*Treatment of 2,7-anhydro-3-deoxy-α-*D-manno-*2-octulofuranosonic acid* (**1**) *with formic acid.* — To a solution of **1** (10 mg) in water (1.0 mL) was added formic acid (10 μL). The solution was heated at 100° for 1 h, cooled, neutralised with ammonia, and freeze-dried.

A solution of the residue in trimethylammonium formate (200mM, pH 3.3, 1.0 mL) was eluted[6,7] from a column (1.5 × 95 cm) of Biogel P-2 with the same buffer. Aliquots of the 1.0-mL fractions were monitored with the phenol–sulfuric acid reagent[22]. 5-(D-*erythro*-1,2,3-Trihydroxypropyl)-2-furoic acid (**5**) was found in fractions 82–87, KDO in fractions 89–95, and **1** in fractions 97–103. Combined fractions were freeze-dried, and solutions of the residues in water were desalted with IR-120 (H^+) resin, neutralised with ammonia, and freeze-dried.

Periodate oxidation. — To a solution of **1** (10 mg) in sodium acetate buffer (40mM, pH 5.2) was added sodium metaperiodate (50 mg), and the mixture was stored in the dark at room temperature for 5 days. Ethylene glycol (50 μL) was added, and the solution was allowed to stand for a further 3 h, then neutralised with ammonia, and freeze-dried. The product was isolated as before, using a column of Biogel P-2.

N.m.r. spectroscopy. — Spectra (200 MHz for ^1H, 50.3 MHz for ^{13}C) were recorded with a Varian XL-200 spectrometer for solutions in D_2O at 21°. Chemical shifts were measured relative to that of sodium 3-(trimethylsilyl)propanesulfonate for ^1H, and methanol (at 50.04 p.p.m.) for ^{13}C. ^{13}C-N.m.r. assignments were confirmed by selective proton decoupling and measurement of heteronuclear correlation spectra[23].

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