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THE SYNTHESIS OF 3-DEOXY-4-*O*-METHYL-D-*arabino*-2-HEPTULOSONIC ACID AND ITS BEHAVIOUR IN THE WARREN REACTION

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SUMMARY

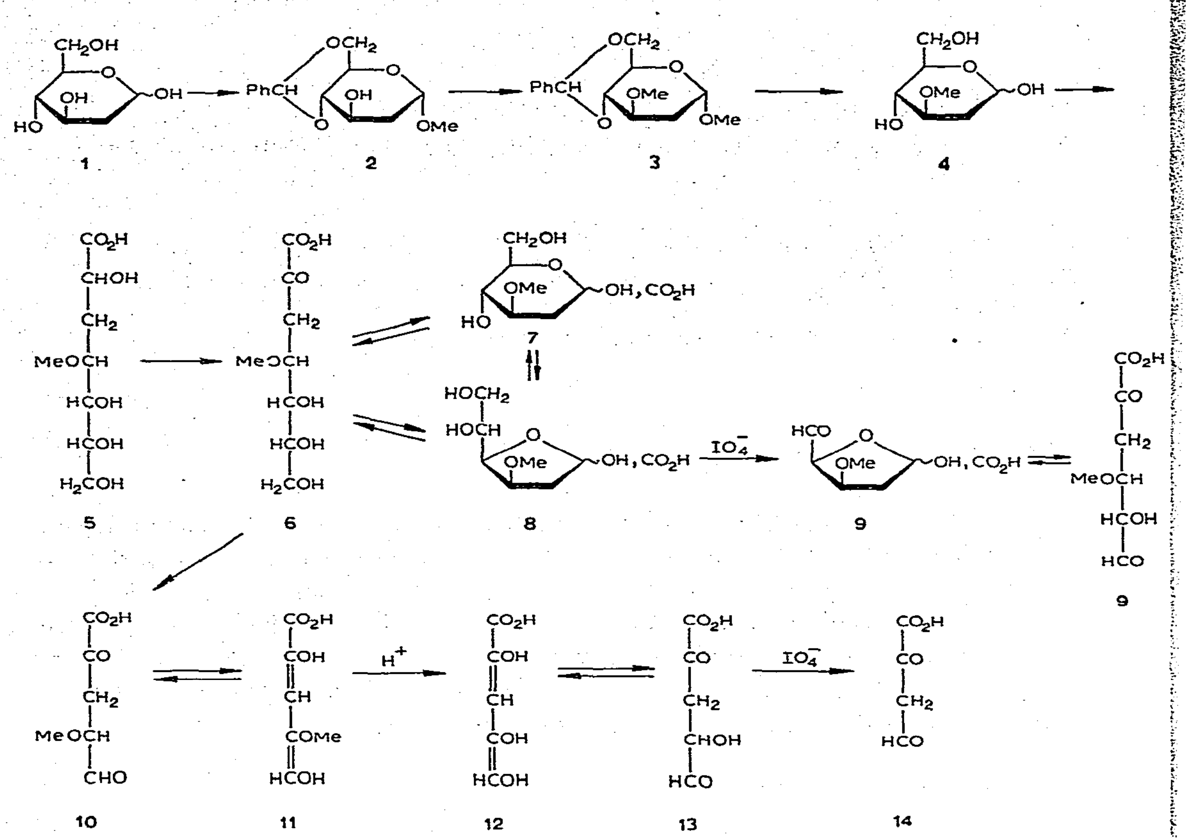
3-Deoxy-4-*O*-methyl-D-*arabino*-2-heptulosonic acid was synthesized in six steps starting from 2-deoxy-D-*arabino*-hexose. Treatment with periodate–thiobarbiturate (Warren reaction) gave a positive reaction with a molar absorption coefficient of 3700 to 5000 depending on the conditions of the periodate oxidation. It is suggested that the Warren reaction, which is eminently suitable for the detection of 3-deoxyaldulosonic acids, should not be used for the quantitative estimation of 3-deoxy-2-aldulosonic acids of undetermined substitution pattern.

INTRODUCTION

It has been shown[1], that 5-*O*-substituted 3-deoxy-2-octulosonic acids give, contrary to prevailing opinion, a positive reaction in the periodate–thiobarbiturate test[2] (Warren reaction) and that this reaction can, therefore, not be used as a valid criterion for establishing the substitution pattern of the 3-deoxy-D-*manno*-2-octulosonic acid residues present in the endotoxin of gram-negative bacteria. The mechanism proposed[1] for the formation of the red dye from 5-*O*-substituted 3-deoxy-2-aldulosonic acids implies that 4-*O*-substituted acids would not react in the test. To check this point, which is of importance for biochemical analysis, a 4-*O*-substituted 3-deoxy-2-aldulosonic acid, 3-deoxy-4-*O*-methyl-D-*arabino*-2-heptulosonic acid (**6**), was synthesized, and its behaviour in the Warren reaction examined.

RESULTS AND DISCUSSION

The starting material for the synthesis of 3-deoxy-4-*O*-methyl-D-*arabino*-2-heptulosonic acid (**6**) was commercial 2-deoxy D-*arabino*-hexose (**1**) which, after glycosidation and condensation with benzaldehyde to the 4,6-*O*-benzylidene derivative[3] (**2**), was methylated to the 3-*O*-methyl ether (**3**). Upon acid hydrolysis, the methylated free sugar (**4**) was obtained and was transformed into the 7-carbon acid (**5**) by cyanohydrin synthesis. Oxidation of the hydroxyl group at C-2 was then



achieved by Regna and Caldwell's method[4] and, after ion-exchange chromatography, the aldulosonic acid was isolated as its crystalline ammonium salt (probably the pyranose form 7). The presence of the α-keto acid system was demonstrated qualitatively by reaction with *o*-phenylenediamine[5] and semicarbazide[6]. It is worthwhile noting that in the latter test, when carried out under conditions used for the quantitative estimation of α-keto acids[6], this aldulosonate gave an apparent molar absorption coefficient of only about 1800, the value of 10 200 generally observed for α-keto acids being attained only after 5 h of heating (Fig. 1). A similarly slow reaction has previously been observed for 3-deoxy-D-*manno*-2-octulosonic acid[7].

When treated with periodate at room temperature in unbuffered solution, the compound reduced two molar equivalents of the reagent, one molar equivalent of formaldehyde being simultaneously formed. The kinetics of the reaction (Fig. 2) strongly suggest that, in solution, the aldulosonic acid is a mixture of the pyranoid (7, 80–90%) and the furanoid (8, 10–20%) forms. This conclusion was deduced from the fact that while the total amount of formaldehyde, *i.e.* one molar equivalent, is released only when the total amount of periodate, *i.e.* two molar equivalents, is reduced, the ratio of formaldehyde formed to periodate reduced drops from 0.8 to 0.5 in the early stages of the reaction and remains constant thereafter. Indeed, it can

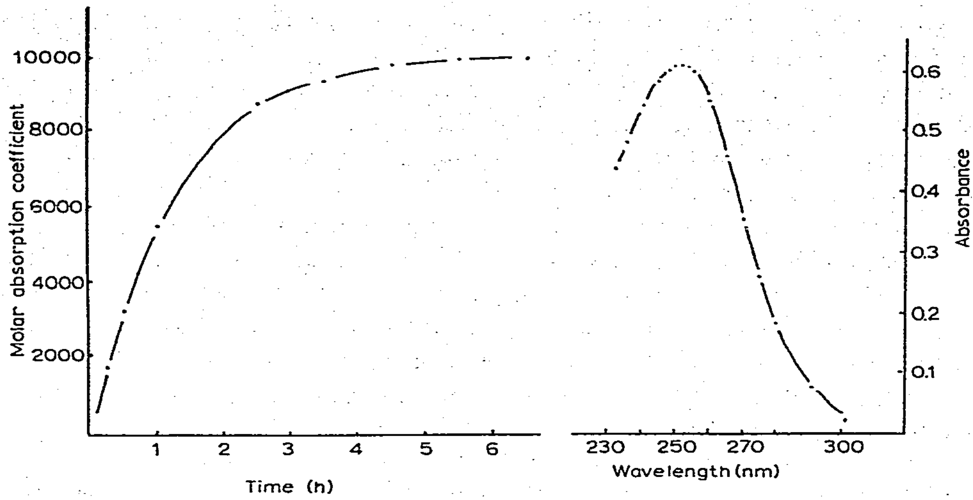


Fig. 1. Absorption spectrum (right) and rate of formation of the semicarbazone of 3-deoxy-4-*O*-methyl-D-*arabino*-2-heptulosonic acid (3 μmoles in 20 ml) at pH 4.5 and 37°; 2-ml samples were diluted with 3 ml of water for optical measurements.

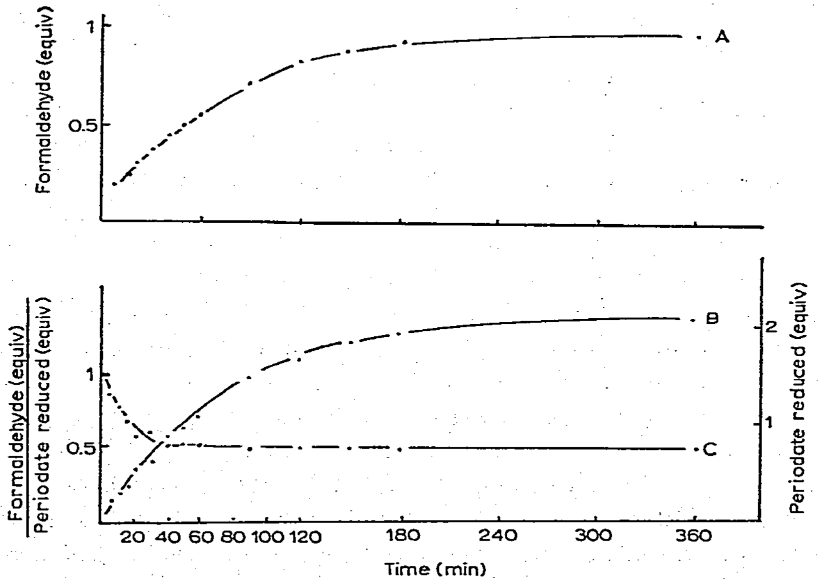


Fig. 2. Kinetics of formaldehyde formation (A), periodate reduction (B), and evolution of the ratio formaldehyde formed: periodate reduced (C) when ammonium 3-deoxy-4-*O*-methyl-D-*arabino*-2-heptulosonate was treated with periodate in unbuffered solution at room temperature. The concentration of the aldulosonate was 2mM and that of the sodium periodate 10 mM.

be expected that while the furanose form (**8**) will react immediately with one molar equivalent of periodate and release, concomitantly, one molar equivalent of formaldehyde and thus give a ratio of formaldehyde formed to periodate reduced of 1, the pyranose form (**7**) must undergo ring opening before it can be oxidized; it will then reduce two molar equivalents of periodate at similar rates and lead to the observed ratio of formaldehyde formed to periodate reduced of 0.5.

Surprisingly, the compound gave a positive reaction in the thiobarbituric acid test performed under Weissbach and Hurwitz's conditions[2]. However, its molar absorption coefficient, about 5000, was only approximately 5% of that of unsubstituted 3-deoxy-D-*manno*-2-octulosonic acid, and only about 40% of that[1] of 3-deoxy-5-*O*-methyl-2-octulosonic acids (13×10^3). The dye formed is the same in all cases, as judged by its absorption spectrum; consequently 2,4-dioxobutyric acid and/or a derivative thereof must be formed from the primary fragments **9** or **10**.

The mechanism[1] by which the same red dye can be formed from 5-*O*-substituted 3-deoxy-2-heptulosonic and homologous, higher acids as from unsubstituted 3-deoxy-2-pentulosonic and homologous, higher acids can not be operative for 4-*O*-substituted 3-deoxy-2-aldulosonic acids. A 2,4-dioxobutyric acid derivative could, however, be formed from the furanose form **8** of 3-deoxy-4-*O*-methyl-D-*arabino*-heptulosonic acid: cleavage of the C-6–C-7 bond leads in this case to a fragment (**9**) which, after condensation with thiobarbituric acid, can be expected to yield the red dye by the mechanism proposed for 5-*O*-substituted 3-deoxy-2-heptulosonic and higher acids.

2,4-Dioxobutyric acid could also be formed if the 2,5-dioxovaleric acid derivative (**10**) underwent enolisation leading to compound **11** which, being an enol ether, would easily undergo hydrolysis to give the enediol (**12**); periodate cleavage of the aldehyde form (**13**) of this would then give the required 2,4-dioxobutyric acid (**14**). This hypothesis is in keeping with the observation that when 3-deoxy-4-*O*-methyl-2-heptulosonic acid was treated with periodate, after adjustment of the pH to 7, an absorption band appeared in the u.v. spectrum of the solution whose position coincided with that of authentic 2,4-dioxobutyric acid, obtained by periodate treatment of 3-deoxy-D-*manno*-2-octulosonic acid under similar conditions. The low yield of 2,4-dioxobutyrate could be due to an unfavourable position of the equilibria **10**$\rightleftarrows$**11** and **12**$\rightleftarrows$**13**. In this connection, it should be noted that periodate cleavage of the enediol **12** leads to 2-oxosuccinic acid and not to the chromogen **14**. In solutions of 3-deoxy-4-*O*-methyl-2-heptulosonic acid treated with periodate at room temperature in unbuffered solution (under these conditions the molar absorption coefficient in the thiobarbiturate test is 3700), free iodine appears after the reduction of two molar equivalents of the reagent: it is known[8] that enediols liberate iodine under these conditions. If the periodate treatment is performed under the conditions of the cold acid method[9], no iodine is liberated and the molar absorption coefficient in the thiobarbiturate test rises to 4700 (Fig. 3).

In view of these and previous[1] results, it appears that a positive thiobarbiturate reaction will be obtained for the following *free i.e.* not glycosidically bound, substituted, 3-deoxy-2-aldulosonic acids: (a) 4-*O*-substituted 2-hexulosonic and homologous higher acids, and (b) 5,6,7 ... (etc.) mono-*O*-substituted 2-heptulosonic and homologous higher acids. 4,5-Di-*O*-substituted 2-heptulosonic and homologous higher acids are also likely to give a positive reaction.

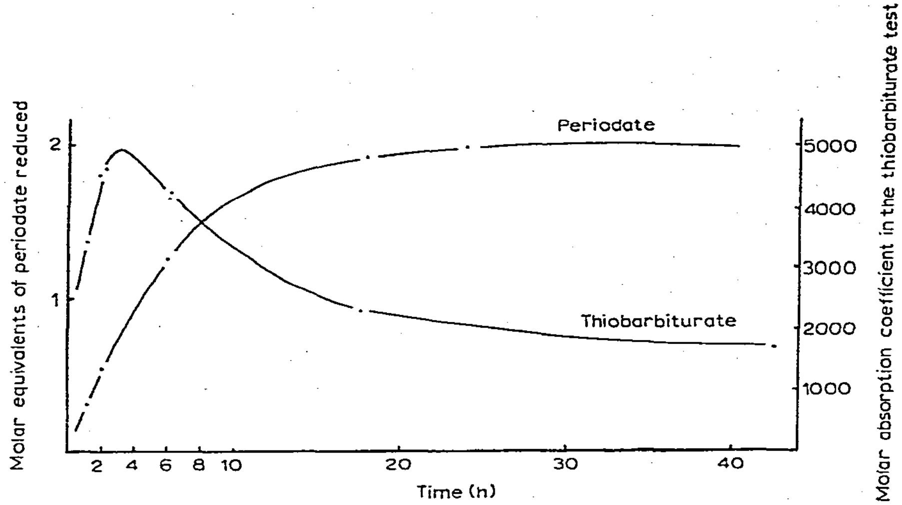


Fig. 3. Reaction kinetics of ammonium 3-deoxy-4-*O*-methyl D-*arabino*-2-heptulosonate with periodate and in the thiobarbiturate test. The reaction mixture, kept at 4°,was 2 mM with respect to the heptulosonate, 10 mM with respect to periodate and 0.125 N with respect to sulphuric acid. Periodate uptake was measured according to the method of Avigad[10].

The thiobarbiturate test is thus eminently suitable for the detection of 3-deoxy-2-aldulosonic acids, as most of the monosubstituted as well as the unsubstituted compounds give a positive reaction. On the other hand, the reaction is not reliable for the quantitative estimation of 3-deoxy-2-aldulosonic acids, unless their substitution patterns are fully established.

EXPERIMENTAL

*Methyl 4,6-O-benzylidene-2-deoxy-3-O-methyl-α-D-*arabino*-hexopyranoside* (3). — To a stirred solution of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*arabino*-hexopyranoside[3] (2, 3 g) in methyl iodide (20 ml), silver oxide (15 g) was added portionwise; the methylation was complete in about 6 h (t.l.c. on silica gel, Schleicher and Schüll E 1500 LS 254, benzene–methanol 49:1). After addition of chloroform (20 ml), the solids were removed and the solvent was evaporated *in vacuo*. The crystalline residue was recrystallised from hexane; yield 3 g; m.p. 103–104°, $[\alpha]_D^{20}$ +99° (*c* 1.5, chloroform); i.r. spectrum: no absorption band in the –OH region.

Anal. Calc. for $C_{15}H_{20}O_5$: C, 64.28; H, 7.14. Found: C, 64.56; H, 7.23.

*2-Deoxy 3-O-methyl-D-*arabino*-hexose* (4). — Aqueous acetic acid (100 ml, 10%, v/v) was added to a solution of compound 3 (2 g) in methanol (40 ml) contained in an open, 250-ml round-bottomed flask, and the mixture was maintained in a boiling water bath for 6 h, the volume being periodically readjusted to 100 ml by addition of water. The cooled mixture was extracted with ether (3 × 20 ml), and the aqueous layer, after treatment with charcoal, concentrated *in vacuo* to a thick syrup which was further dried in a desiccator to a colourless, reducing syrup (1.5 g); $[\alpha]_D^{20}$ $+14°$ (c 1.3, water).

Anal. Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.87. Found: C, 47.40; H, 7.88.

3-Deoxy 4-O-methyl-heptonic acid (**5**). — A solution of the methyl ether (**4**, 3 g) and calcium acetate monohydrate (4 g) in water (15 ml) was cooled in iced water, and a solution of potassium cyanide (1.4 g) in cold water (10 ml) was added. The mixture was kept in the cold (4°) for 72 h, and then heated for 5 h at 60°, a slow stream of air being bubbled through the mixture. The cooled solution was diluted with water (50 ml), passed through a column (20 × 4 cm) of Amberlite IR-120 (H^+) resin and the effluent and washings were concentrated to a syrup which was kept over potassium hydroxyde *in vacuo*. It was then redissolved in a small amount of water and the solution neutralized with saturated barium hydroxide, filtered through charcoal, and concentrated to about 2 ml. The barium salt of the title compound was precipitated by addition of acetone, collected by centrifugation, and dried over phosphorus pentaoxide, yield 3.1 g, $[\alpha]_D^{20}$ $+10.4°$ (c 1.2, water).

Anal. Calc. for $C_8H_{15}O_7Ba_{0,5}$: C, 32.93; H, 5.14. Found: C, 33.07; H, 4.95.

When water (0.5 ml) and dicyclohexylamine (0.55 ml) were added to the syrupy free acid (400 mg), the mixture stirred for 2 h, diluted with ethanol (2 ml), stirred for 1 h, diluted with acetone (about 10 ml) to very slight turbidity, and kept in the cold, a crystalline dicyclohexylammonium salt (600 mg) was obtained. After recrystallisation from methanol–acetone, it had m.p. 176–179°, $[\alpha]_D^{25}$ $+11.6°$ (c 1, methanol).

Anal. Calc. for $C_{20}H_{39}NO_7$: C, 59.25; H, 9.62; N, 3.45. Found: C, 58.45; H, 9.61; N, 3.70.

*3-Deoxy-4-O-methyl-*D-arabino-*2-heptulosonic acid* (**6**). — An aqueous solution of the barium salt of **5** (1.46 g) was treated with Amberlite IR-120 (H^+) resin and the pH of the filtered solution was brought to 8 with 2M potassium hydroxide. The solvent was removed *in vacuo* and commercial vanadium pentaoxide (15 mg), potassium chlorate (213 mg), and 3% phosphoric acid (5 ml) were added to the residue. After thorough mixing, the pH was adjusted to 4.5 with phosphoric acid or potassium hydroxide solution, and the reaction mixture stirred for 3 days: by that time, the originally orange-coloured mixture had turned deep green. It was then passed through a column (20 × 4 cm) of Amberlite IR-120 (H^+) resin and the pH of the pooled effluent and washings was brought to 7 with 2M ammonium hydroxide. The solution contained 2 mmoles (40% yield) of aldulosonic acid, as measured by the semicarbazide test[6] with an incubation time of 5 h at 37°. Two such preparations were pooled and lyophilized; the product was triturated with methanol (2 × 10 ml) and the insoluble material discarded. The solvent was removed *in vacuo*, the residue dissolved in water (10 ml), and the solution passed through a column (22.5 × 2.5 cm) of Dowex 1

(X-8) resin in the carbonate form. The column was washed with water and then eluted successively with 0.1, 0.2, and 0.3M ammonium hydrogen carbonate solution, adjusted to pH 7.7 with carbon dioxide (100 ml each). The fractions (5 ml) that gave a positive semicarbazide reaction were pooled, treated with Amberlite IR-120 (H^+) resin, and freed of carbon dioxide by bubbling air through the solution, which was then neutralized with an 0.5M ammonium hydroxide solution. The solvent was removed *in vacuo*, the residue dissolved in methanol, and acetone added to incipient turbidity. The solution was seeded (see next paragraph) and kept overnight in the cold to give the crystalline ammonium salt (360 mg) of the title compound, m.p. 135–140° (dec.); $[\alpha]_D^{20}$ $+11°$ (*c* 0.4, water) (after 5 min and 2 h).

Anal. Calc. for $C_8H_{17}NO_7$: C, 40.17; H, 7.11; N, 5.86; OMe, 12.96. Found: C, 40.42; H, 6.99; N, 5.77; OMe, 12.99.

The seed crystals were obtained by passing the solution of the ammonium salt (about 1 mmole) through a column (80 × 2.5 cm) of Sephadex G-10 gel, equilibrated with a mM ammonium hydrogen carbonate solution, and eluting the column with water. The fractions (2 ml) containing semicarbazide-positive material were pooled and acetone was added to incipient turbidity. The ammonium salt crystallized from the mixture when it was kept overnight in the cold.

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