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Enzymatic Synthesis of 3-Deoxy-D-manno-2-octulosonic Acid and Analogues : a New Approach by a Non metabolic Pathway

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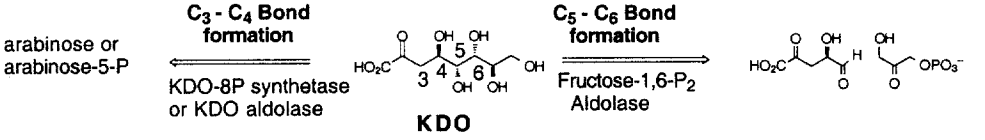
Abstract : A new approach to acceed to 4-deoxy-KDO is described. The key step is the formation of the C_5-C_6 bond catalyzed by fructose-1,6-bisphosphate aldolase which controls the stereochemistry of these two centers.

3-deoxy-D-manno-2-octulosonic acid (KDO) is a component of the cell wall of gram negative bacteria.[1] This specific occurence of KDO makes its biosynthetic pathway a possible therapeutic target. For this reason, syntheses of KDO and analogues have retained attention in the last few years. Total syntheses of KDO have been published,[2] but the most convenient procedures involve hemisyntheses starting from D-arabinose or D-mannose.[3]Some analogues have been tested as antimicrobial agents.[4]

Two enzymatic syntheses, based on the metabolic pathway of KDO have been described : the first one use KDO-8 phosphate synthetase which catalyses the condensation of phosphoenolpyruvate onto D-arabinose-5 phosphate.[5] The latter use the KDO aldolase, acting *in vivo* on the biodegradation of KDO by reversible retroaldolisation into D-arabinose and pyruvate.[6] In both cases, C_3 -C_4 bond is created with control of the configuration in C_4.

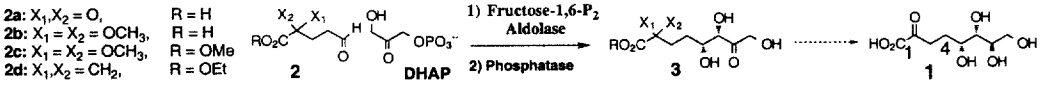
Although these methods can afford KDO or KDO-8-phosphate in moderate to good yields, they do not allow to obtain analogues of KDO, due to the specificity of the enzymes for phosphoenolpyruvate or pyrurate and close analogues of D-arabinose.[7] Particularly, 4-deoxy-KDO **1** is not accessible.

In the course of our studies on the utilisation of aldolase for the enzymatic synthesis of monosaccharide and analogues,[8] we looked for a more versatile approach. Indeed, fructose-1,6-bisphophate aldolase, which catalyses the condensation of dihydroxyacetone phosphate (DHAP) onto a variety of aldehydes,[9] can lead to KDO, according to scheme 1. In this reaction, the C_5 -C_6 bond formation as well as the configuration of these centers are controlled by the enzyme, the configuration at C_4 and C_7 can be choosed to lead to KDO or epimers, and the substitution at C_4 can be omitted to provide 4-deoxy-KDO **1**.



Scheme 1

To test the validity of this approach, we started with the synthesis of 4-deoxy analogues of KDO **3** (scheme 2). In this scheme, **3a** is already an analogue of KDO, with the right configuration in C_5 and C_6. Stereospecific reduction of the keto group in C_7 is possible but it needs the previous protection of ketone in C_2. For this reason, we tested the activity of aldolase not only towards 2,5-dioxo-pentanoic acid **2a** but also towards potential precursors of **2a**, (**2b**, **2c** and **2d**), where the 2-oxo group was protected as a dimethylacetal (**2b** and **2c**) or where a methylidene is used as its precursor (**2d**), carboxylic function being esterified in **2c** and **2d**.



Scheme 2

2a, **2b** and **2c** syntheses were based on the reaction of the Grignard reagent of 4-bromo-1-butene with diethyloxalate.[10] Hydrolysis of the resulting α-ketoester followed by ozonolysis of the double bond led to **2a**. Protection of the ketogroup provided, by the same reactions, **2c** and **2d**. The synthesis of **2d** was carried out according to Bosnich and coll.[11], by vinylation of ethyl hydroxymethacrylate followed by Claisen rearrangement.

Aldehydes **2a-d** were submitted to the action of aldolase. The reaction was monitored by enzymatic titration of residual DHAP[12], and the caracteristic constants of the reaction, the Michaelis constant Km and the maximum rate Vmax were calculated. To facilitate the comparison between different substrates, the Vmax value is given in percent of the activity of fructose-1.6-bisphosphate aldolase in the natural reaction (Vmax rel). The results are reported in the table.

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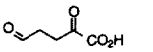
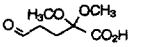
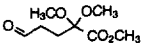
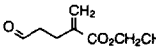
		Km (mM)	Vmax (% of the enzyme activity)	V at 50 mM (% of enz.activity)
	**2a**	47	26	13
	**2b**	63	31	14
	**2c**	380	68	8
	**2d**	127	49	14

The four aldehydes are substrates for aldolase, the best beeing **2a** and **2b**. Protection of the keto group in **2b**, although increasing the size of the molecule does not bring a large difference. The Vmax is fairly good, but, due to the high Km value, the velocity will decrease very fast at concentration under 50 mM, so that the yields relatively to **2a** and **2b** cannot be very good. The presence of the anionic charge in **2a** and **2b**, comparable to the phosphate group of the natural substrate D-glyceraldehyde-3-phosphate has a positive effect. Indeed, the esterification of the carboxylic acid in **2c** and **2d** increases the Km values to 380 and 127 mM. In these cases, the Vmax values were obtained by extrapolation and were not experimentally observed. The V measured at 50 mM in substrate are low, and the synthesis should be more difficult in that case.

In spite of this prediction and in order to test the validity of our approach, we experimented the enzymatic reaction in preparative scale starting from **2c** and **2d**, thus choosing the worse conditions in term of enzyme reactivity. The reaction were carried out on 3 mmole of DHAP and an excess of aldehyde (50 % for **2c**, 10 % for **2d**). After 48 hours, no more DHAP was present in solution, the phosphate ester was hydrolysed by action of acid phosphate and **3c** and **3d**[13] were purified by column chromatography on silicagel, and obtained with 15 and 10 % yield respectively from DHAP.

These results are really promising : aldehydes **2** are easily synthetized from commercial compounds, and lead, by a simple protocol, and a widely used methodology, to polyfunctional chiral compounds **3c** and **3d** which are close analogues of KDO. The yields are still modest but should be increased starting with **2a** and **2b** which are better substrates.This new approach using enzymes which are not involved in the metabolism of KDO could allow to reach analogues with interesting inhibitory activities.

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12. DHAP was prepared and assayed according to Gefflaut, T. ; Lemaire, M. ; Valentin, M.-L. ; Bolte, J. *J. Org. Chem.* **1997**, *62*, 5920-5922.

13. Experimental procedure for synthesis of **3c** and **3d** is as follow : 70 ml of a solution containing **2d** (700 mg ; 4.5 mmol), DHAP sodium salt (70 mg ; 0.33 mmol) and EtOH (1g ; 22 mmoles), was adjusted to pH 7.5. 200 U of fructose-1,6-bis-phosphate aldolase were added. After 24 hours 200 U of enzyme were added again, and the solution was stirred for 24 hours. The solution was extracted by 3 x 20 ml of ethylacetate. pH was adjusted to 4.7 by addition of HCl 1N, then 200 U of acid phosphatase were added. After 24 hours, the solution was adjusted to pH 7 with NaOH 0.1N, then concentrated under vacuo. The yellow residue was purified by silicagel flash chromatography (CH_2Cl_2 / AcOEt 95/5) to give 120 mg of an incolor oil (15 % yield).

3c : ^{1}H-RMN D_2O δ(ppm): 1.65 (q, 2H, CH_2, *J* = 7 Hz) ; 2.17 (m, 2H, CH_2) ; 3.40 (s, 6H, $2CH_3$) ; 3.96 (s, 3H, CH_3 ester) ; 4.08 (m, 1H, CHOH) ; 4.41 (d, 1H, ^{6}CHOH, *J* = 5 Hz) ; 4.60 (d, 1H, $^8CH_2OH$, *J* = 20 Hz) ; 4.69 (d, 1H, $^8CH_2OH$, *J* = 20 Hz). ^{13}C-RMN D_2O δ (ppm): 30.4 (*C3*) ; 33.6 (*C4*) ; 53.9 ($2CH_3$) ; 57.7 (CH_3) ; 70.3 (*C8*) ; 75.4 (*C5*) ; 81.7 (*C6*) ; 106.9 (*C2*) ; 175.2 (*C1*) ; 217.3 (*C7*).

3d : ^{1}H-RMN D_2O δ(ppm): 1.37 (t, 3H, CH_3, *J* = 7 Hz) ; 1.85 (q, 2H, CH_2, *J* = 7Hz) ; 2.50 (m, 2H, CH_2) ; 4.06 (m, 1H, CHOH) ; 4.30 (q, 2H, CH_2 ester, *J* = 7 Hz) ; 4.44 (d, 1H, ^{6}CHOH, *J* = 5 Hz) ; 4.58 (d, 1H, $^8CH_2OH$, *J* = 20 Hz) ; 4.67 (d, 1H, $^8CH_2OH$, *J* = 20 Hz) ; 5.80 (s, 1H, CH_2 oléfine) ; 6.28 (s, 1H, CH_2 olefin). ^{13}C-RMN D_2O δ(ppm): 16.0 (CH_3) ; 30.3 (*C3*) ; 33.8 (*C4*) ; 64.7 (CH_2) ; 68.8 (*C8*) ; 73.9 (*C5*) ; 80.2 (*C6*) ; 129.1 (CH_2 olefin) ; 142.4 (*C2*) ; 172.3 (*C1*) ; 215.8 (*C7*).