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SCOPE AND LIMITATIONS OF THE ALDOL CONDENSATION CATALYZED BY IMMOBILIZED ACYLNEURAMINATE PYRUVATE LYASE

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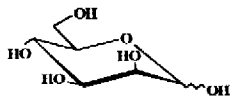
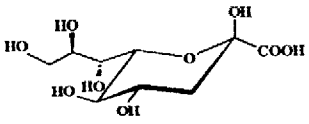
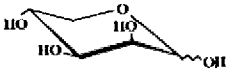
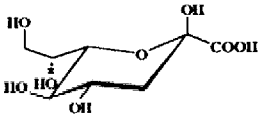
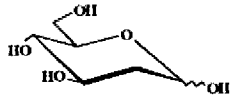
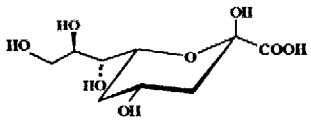
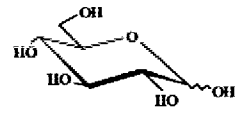
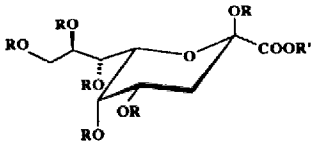
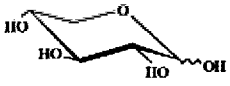
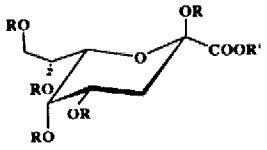
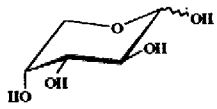
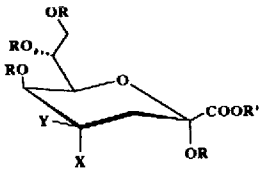
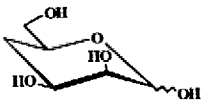
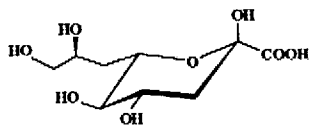
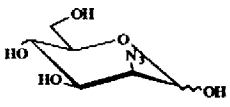
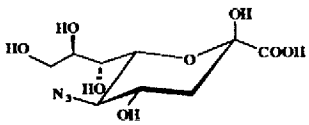
Summary : New substrates have been used in the aldol condensation catalyzed by immobilized acylneuraminate pyruvate lyase : hexoses **3**, **4**, **7**, **8** and pentoses **2**, **5**, **6**. Condensation of pyruvate with **2** -**5**, **7** and **8** led to pure compounds related to 3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN). Surprisingly, condensation with D-arabinose **6** gave a mixture of diastereomers, 3-deoxy-α-D-manno-2-octulosonic acid (KDO) and 4-epi KDO.

Acylneuraminate pyruvate lyase (EC 4.1.3.3) catalyzes the reversible aldol condensation of pyruvate with N-acetylmannosamine:

In a previous paper, we took advantage of the broad specificiy of the enzyme towards the acceptor; starting from the corresponding mannosamine derivatives, we prepared six naturally occuring sialic acids using this immobilized enzyme.[1] The same method allowed us to achieve the first synthesis of 3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN) (**9**) from D-mannose (**1**).[2]
We wish now to report further investigations on the specificity of this enzyme. We used 7 new substrates : D-lyxose (**2**), 2-deoxy-D-glucose (**3**), D-glucose (**4**), D-xylose (**5**), D-arabinose (**6**), 4-deoxy-D-mannose (**7**) and 2-azido-2-deoxy-D-mannose (**8**).[3] For some of them, enzyme kinetic parameters have recently been reported in the literature .[4]
In a typical experiment a 0.1 M solution of sugar (**2**-**8**) (1 mmol) in 0.05 M potassium phosphate buffer, pH 7.2, containing 0.01 M dithiothreitol and 0.02% sodium azide was treated with 10 equiv. of sodium pyruvate. The mixture was gently stirred in the presence of acylneuraminate pyruvate lyase covalently bound to 4% agarose,[1] at 37° under nitrogen for 1 to 5 days. After filtration of the gel the products were isolated by anion exchange chromatography according to method a or b; method a : the products were eluted from

Dowex-1 ($HCOO^-$) with a 0 - 2 M HCOOH gradient and freeze-dried; method b : the products were eluted from Dowex-1 (HCO_3^-) with a 0 - 0.2 M HCO_3NH_4 gradient, freeze-dried, deionized with Dowex-50 (H^+) and again freeze-dried. The gel which retained good enzymatic activity was reused in the next run.
Results are summarized in the table. This includes KDN (**9**) (run 1), already described,[2] which is the best substrate of the series. Pure compounds were obtained from runs 2, 3, 7 and 8 .[6] Compounds **10** (C_8 homologous of KDN) , **16** (7-deoxy KDN) and **17** (5-azido KDN) were isolated in high yields. Incidentally N-acetyl-7-deoxyneuraminic acid was reported not to be a substrate of acylneuraminate pyruvate lyase.[7] We cannot explain the moderate yield we obtained for compound **11** ,whereas similar efficiency (V_{max}/KM) have been reported by Wong for the substrates **1** and **3** .[4]
In three cases (runs 4, 5, 6) (independently of the isolation procedure used) ^1H NMR spectra were too complex to be interpreted. These products were therefore characterized as their peracetylated methyl esters. Acetylation was achieved according to the procedure of Unger,[8] which leads to pyranoses derivatives. Esterification was then performed by treatment with iodomethane in N,N-dimethylformamide for 3 h at room temperature.[9] The structures **12b** and **13b** could be assigned on the basis of the ^1H NMR data [10] for the peracetylated methyl esters issued respectively from runs 4 and 5. However ^1H NMR spectra revealed the presence of some contaminants (<10 %) , hence we are not able to assert in these cases the diastereomeric purity of the products.
The reaction with D-glucose (**4**) was of particular interest since N-acetylglucosamine is not a substrate of the enzyme. Complexity of ^1H NMR spectra for free compounds **12a** (5-epi KDN) and **13a** (C_8 homologous of 5-epi KDN) might be due to 1-5 lactone formation as it has been suggested for 3-deoxy-α-D-manno-2-octulosonic acid.[12]
Surprisingly, run 6, involving D-arabinose (**6**) led to a mixture of two diastereomers **14b** and **15b** in a 56:44 ratio (HPLC analysis), which could be partially separated by flash chromatography (SiO_2, 1:1 ethylacetate/hexane).**15b** was identified as the derivative of 3-deoxy-α-D-manno-2-octulosonic acid (KDO) and **14b** as the one of 4-epi KDO.[13] **14a** is the compound we expected in the enzymic condensation, whereas **15a** is the anomalous one. Nevertheless **15b** was unambiguously identified by comparison with an authentic sample of peracetylated methyl ester of KDO (prepared according to Unger,[14] acetylated and esterified as described,[8,9] m.p.:157-159°). Epimerization at C-4 could not arise from the work up. Moreover the optical rotation we measured from the analytically pure mixture of **14a** and **15a** corresponded to an intermediate value (+27°) between the optical rotation of KDO (+40.3°)[14] and 4-epi KDO (+12.5°).[15] Therefore we think we are able to conclude in this case that the acylneuraminate pyruvate lyase exhibits a lack of specificity. This is the first time we observed such a behaviour for the enzyme.
We thank Professor S. David for helpful discussions.

Table: Aldol condensation catalyzed by immobilized acylneuraminate pyruvate lyase.[5]

Run	Substrates	U/mmol of substrate	Reaction time (days)	Products	Yield (%)
1	1 	15	1	9 	84
2	2 	14	2	10 	66
3	3 	6	5	11 	36
4	4 	16	5	12 	28
5	5 	20	5	13 	18
6	6 	12	5	14 X=OR, Y=H 15 X=H, Y=OR 	35 (15a + 14a)
7	7 	12	3	16 	67
8	8 	12	1	17 	78

a R=R'=H

b R=Ac, R'=CH_3

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3 The synthesis of compound **7** will be reported elsewhere. The synthesis of compound **8** is going to be published: C. Augé, S. David, A. Malleron Carbohydr. Res. 1989

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5 On table, compounds **9 - 17** are represented in their predominant anomeric form deduced from N.M.R. spectra.

6 ^{1}H N.M.R. (250 MHz, D_2O, HOD = 4.80 ppm) compound **10** : δ 1.78 (t, $J_{3ax,3eq} = J_{3ax,4}$ = 12.5 Hz, H-3 ax), 2.16 (dd, $J_{3eq,4}$ = 5 Hz, H-3 eq), 3.52 (t, $J_{4,5} = J_{5,6}$ = 9.5 Hz, H-5), 3.60 (d, H-8, H-8'), 3.70 (d, H-6), 3.89 (m, H-4), 4.00 (t, $J_{7,8} = J_{7,8'}$ = 6 Hz, H-7); compound **11** : δ 1.57 (t, $J_{3ax,3eq} = J_{3ax,4}$ = 12 Hz, H-3 ax), 1.62 (q, $J_{5ax,5eq} = J_{5ax,6} = J_{5ax,4}$ = 12 Hz, H-5 ax), 1.88 (m, H-5 eq), 2.17 (dd, $J_{3eq,4}$ = 4 Hz, H-3 eq), 3.48 (dd, $J_{7,8}$ = 8 Hz, $J_{7,6}$ = 1.5 Hz, H-7), 3.62 (dd, $J_{9,9'}$ = 11 Hz, $J_{9,8}$ = 6 Hz, H-9), 3.74 (m, H-8), 3.82 (dd, $J_{9',8}$ = 2.5 Hz, H-9'), 4.20 (m, H-4, H-6); compound **16** (ddd, $J_{7,7'}$ = 15 Hz, J = 3 Hz, J = 10 Hz, H-7), 1.82 (dd, $J_{3ax,3eq}$ = 13 Hz, $J_{3ax,4}$ = 12 Hz, H-3ax), 1.92 (o, J = 2 Hz, J = 10 Hz, H-7'), 2.23 (dd, $J_{3eq,4}$ = 5 Hz, H-3 eq), 3.18 (t, $J_{4,5} = J_{5,6}$ = 9 Hz), 3.45 (dd, $J_{9,9'}$ = 12 Hz, $J_{9,8}$ = 7 Hz, H-9), 3.57 (dd, $J_{9',8}$ = 4 Hz, H-9'), 3.87 (m, H-4, H-6, H-8); compound **17** : δ 1.86 (dd, $J_{3ax,3eq}$ = 13 Hz, $J_{3ax,4}$ = 11.5 Hz, H-3 ax), 2.26 (dd, $J_{3eq,4}$ = 5 Hz, H-3 eq), 3.51 (t, $J_{5,6} = J_{4,5}$ = 10 Hz, H-5), 3.62 (dd, $J_{9,9'}$ = 11.2 Hz, $J_{9,8}$ = 6 Hz, H-9), 3.73 (m, H-8), 3.80 (dd, $J_{6,7}$ = 1 Hz, $J_{7,8}$ = 8 Hz, H-7), 3.84 (dd, $J_{9',8}$ = 2.5 Hz, H-9'), 3.92 (dd, H-6), 4.07 (m, H-4).

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10 ^{1}H NMR (250 MHz, C_6D_6), compound **12b** : δ 1.63 (s, OAc), 1.68 (s, OAc), 1.71 (s, OAc), 1.77 (s, OAc), 1.85 (s, OAc), 1.98 (s, OAc), 2.47 (m, H-3 ax, H-3 eq), 3.33 (s, CH_3), 4.13 (dd, $J_{9,9'}$ = 12 Hz, $J_{9,8}$ = 5.5 Hz, H-9), 4.36 (dd, $J_{6,5}$ = 1 Hz, $J_{6,7}$ = 8 Hz, H-6), 4.42 (dd, $J_{9',8}$ = 6 Hz, H-8), 5.22 (sex, $J_{8,7}$ = 3 Hz, H-8), 5.46 (o, $J_{4,3ax}$ = 12 Hz, $J_{4,3eq}$ = 5.5 Hz, $J_{4,5}$ = 3 Hz, H-4), 5.90 (dd, H-5), 5.95 (dd, H-7); compound **13b** : δ 1.56 (s, OAc), 1.60 (s, OAc), 1.67 (s, OAc), 1.70 (s, OAc), 1.72 (s, OAc), 2.50 (m, H3 ax, H-3 eq), 3.31 (s, CH_3), 3.90 (dd, $J_{8,8'}$ = 12 Hz, $J_{8,7}$ = 7 Hz, H-8), 4.22 (dd, $J_{6,5}$ = 1 Hz, $J_{6,7}$ = 8 Hz, H-6), 4.46 (dd, $J_{7,8}$ = 3 Hz, H-8'), 5.42 (o, $J_{4,3ax}$ = 11.5 Hz, $J_{4,3eq}$ = 6 Hz, $J_{4,5}$ = 3 Hz, H-4), 5.65 (m, H-5, H-7).

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13 ^{1}H NMR (250 MHz, C_6D_6), *compound* **14b** : 1.54 (s, OAc), 1.61 (s, OAc), 1.66 (s, OAc), 1.68 (s, OAc), 1.75 (s, OAc), 2.27 (dd, $J_{3ax,3eq}$ = 15 Hz, $J_{3ax,4}$ = 3.5 Hz, H-3ax), 2.62 (dd, $J_{3eq,4}$ = 2.5 Hz, H-3 eq), 3.33 (s, CH_3), 4.34 (dd, $J_{8,8'}$ = 12 Hz, $J_{8,7}$ = 5 Hz, H-8), 4.63 (dd, $J_{6,7}$ = 10 Hz, $J_{6,5}$ = 1.5 Hz, H-6), 4.68 (dd, $J_{8',7}$ = 2.5 Hz, H-8'), 5.10 (sex, H-4), 5.30 (dd, $J_{5,4}$ = 2.5 Hz, H-5), 5.52 (o, H-7); compound **15b** : δ 1.52 (s, OAc), 1.58 (s, OAc), 1.64 (s, OAc), 1.70 (s, OAc), 1.76 (s, OAc), 2.37 (m, H-3ax, H-3 eq), 3.34 (s, CH_3), 4.06 (dd, $J_{6,7}$ = 10 Hz, $J_{6,5}$ = 1 Hz, H-6), 4.23 (dd, $J_{8,8'}$ = 12 Hz, $J_{8,7}$ = 5 Hz, H-8), 4.56 (dd, $J_{8',7}$ = 2 Hz, H-8'), 5.41 (o, $J_{4,3ax}$ = 11 Hz, $J_{4,3eq}$ = 7 Hz, $J_{4,5}$ = 3 Hz, H-4), 5.51 (o, H-7), 5.64 (dd, H-5).

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