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AN EFFICIENT SYNTHESIS OF CYTIDINE MONOPHOSPHO-SIALIC ACIDS WITH FOUR IMMOBILIZED ENZYMES.

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Summary : Four CMP-sialic acids, among them the new derivative CMP-KDN, have been prepared straightforward from CMP, phosphoenolpyruvate and sialic acid by using four immobilized enzymes.

Enzymic synthesis of sialyloligosaccharides seems to be a valuable alternative[1] to chemical synthesis. It involves sialyltransferases which catalyze the transfer of neuraminic acid (1a) or more generally of sialic acid from the nucleotide sugar to the oligosaccharide acceptor :

CMP-sialic acid + oligosaccharide ⟶ sialyloligosaccharide + CMP

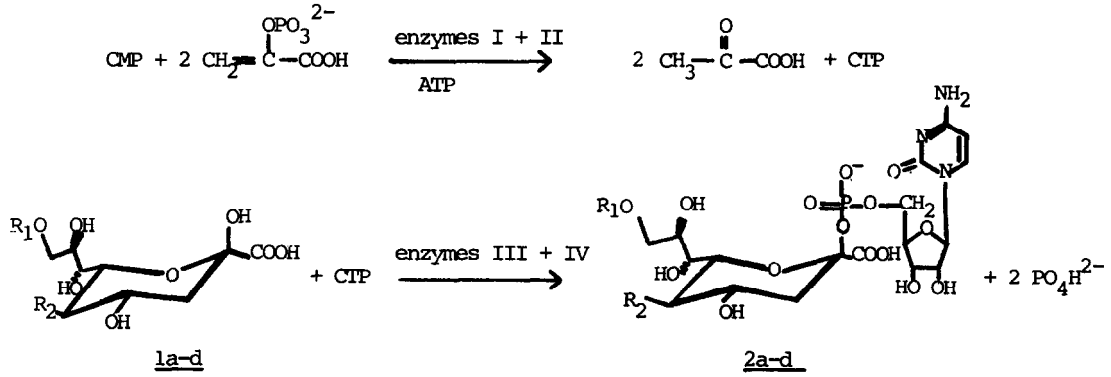
CMP-sialic acid can be obtained enzymatically by the CMP-sialic acid-synthetase-catalyzed condensation of sialic acid with CTP :

CTP + sialic acid ⟶ CMP-sialic acid + PPi

Several enzymatic syntheses of CMP-NeuAc (2a) using either soluble[2] or immobilized[3] CMP-sialic acid synthetase have been reported in the literature. In all cases a large excess of CTP was necessary. Sialic acid being now readily available,[4] the limiting factor in the preparation of the nucleotide sugar is henceforward CTP.

We report here a straightforward preparation of CMP-sialic acids from CMP, phosphoenolpyruvate and sialic acids by using four immobilized enzymes : nucleoside monophosphokinase (I, EC 2.7.4.4), pyruvate kinase (II, EC 2.7.1.40), inorganic pyrophosphatase (IV, EC 3.6.1.1) which are commercially available, in addition to CMP-sialic acid synthetase (III, EC 2.7.7.43) that was concentrated from calf brain.[5] The enzymes were immobilized on Agarose as previously described.[6] CMP was first converted to CTP by incubation of enzymes I and II with two equivalents of phosphoenolpyruvate and a catalytic amount of ATP. Then CTP was utilized in the incubation of enzymes III and IV with a stoechiometric quantity of sialic acids.

This procedure was applied to the synthesis of four derivatives : CMP-NeuAc (2a), CMP-NeuGc (2b), CMP-9-O-Ac-NeuAc (2c) and CMP-KDN (2d) starting from the corresponding sialic acids.



1a-d 2a-d

a, R_1= H, R_2= NHAc ; b, R_1= H, R_2= $NHCOCH_2OH$
c, R_1= Ac, R_2= NHAc ; d, R_1= H, R_2= OH

Table. Preparation of CMP-sialic acids catalyzed by immobilized CMP-sialic acid synthetase and inorganic pyrophosphatase.

CMP sialic acids	scale in mmol	units of enzyme	reaction time in h	pH	yield[7] %
2a	0.5	3.7	10	9	60
2b	0.1	6.5	5	9	80
2c	0.5	6	8	7.5	52
2d	0.5	9	24	9	26

Conversion of CMP to CMP-sialic acids 2a, 2b, 2c was achieved on a 0.5 mmol scale with an average 50-60% yield. These compounds have been already prepared by Paulson who used soluble enzyme.[5] NMR data were identical with those reported.

Of great interest was the conversion of the newly discovered deaminated neuraminic acid[8] (KDN) (1d) to CMP-KDN (2d).[9] From the kinetic parameters we measured [KM(KDN) : 3 mM, KM(NeuAc) : 1.8 mM, $V_{max}(KDN)/V_{max}(NeuAc) = 1/12$] for 1d, it turned out that 1d was not a substrate as efficient as the other sialic acids. More enzymatic activity would have been necessary in order to increase the moderate yield obtained for compound 2d.

A phosphatase, contaminating the preparation of enzyme III and hydrolyzing CMP to cytidine, made the use of a four enzymes system impossible. Hence, conversion of CMP to CMP-sialic acid was achieved in two steps. The same immobilized CMP-sialic acid synthetase preparation was used for the four syntheses and was still active at the end.

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7. As CMP-sialic acids are fragile compounds which degrade during purification, the yield is estimated by the thiobarbituric acid assay. Only aliquots of 2a, 2b, 2c and 2d were purified, first on a silica gel column (elution with 1-propanol - water : 7:3) and then on a biogel P_2 (200-400 mesh) (elution with 0.1 M triethylammonium hydrogenocarbonate pH 7.8).
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9. ^1H NMR for 2d (D_2O, HOD = 4.80 ppm) δ : 1.57 (m, 1 H, $J_{3eq,3ax} = J_{3ax,4} = 12.5$ Hz, $J_{3ax,P} = 6$ Hz, H-3ax, 2.41 (dd, 1 H, $J_{3ax,4} = 5$ Hz, H-3eq), 3.58 (t, 1 H, $J_{4,5} = J_{5,6} = 9$ Hz, H-5), 3.60 (dd, 1 H, $J_{9,9'} = 11$ Hz, $J_{9,8} = 6$ Hz, H-9), 3.70 (m, 1 H, H-8), 3.84 (d, 1 H, $J_{8,7} = 9$ Hz, H-7), 3.89 (d, 1 H, H-9), 3.97 (m, 1 H, H-4), 4.05 (d, 1 H, H-6), 4.17-4.36 (m, 5 H, H-2, H-3, H-4, H-5, H-5' ribose), 5.96 (d, 1 H, $J_{1,2} = 4.5$ Hz, H-1 ribose), 6.10 (d, 1 H, $J_{5,6} = 7.5$ Hz, H-5 cytosine), 7.95 (d, 1 H, H-6 cytosine).

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