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Combined Chemoenzymatic Synthesis of 2O-6-Deoxy-α-L-Sorbofuranosyl-D-Glucose

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Abstract: *The chemoenzymatic synthesis of 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose has been performed by coupling four enzymatic and one simple chemical step on a preparative scale with 17% overall yield. At first, pyruvic aldehyde dimethyl acetal was reduced stereospecifically to L-lactaldehyde dimethyl acetal by the carbonyl reductase from Candida parapsilosis with approximately 100% ee. Subsequently, the product was converted to L-lactaldehyde by simple treatment with a strong cation exchanger. Secondly, stereoselective condensation between dihydroxyacetone phosphate and L-lactaldehyde was catalyzed by fructose 1,6-bisphosphate aldolase from Staphylococcus carnosus. The resulting sugar phosphate was dephosphorylated by acid phosphatase treatment and further purified by cation exchange chromatography. The structure and stereochemistry of the product, 6-deoxy-L-sorbose, was confirmed by* ^{1}H- *and* ^{13}C-*NMR analysis. In the last step sucrose synthase from rice grains was utilized to catalyze the transfer of glucose from UDP-glucose to 6-deoxy-L-sorbose. The structure and stereochemistry of the disaccharide formed, 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose, was also confirmed by* ^{1}H- *and* ^{13}C-*NMR analysis.*

The use of enzymes in the synthesis of highly functionalized compounds, such as carbohydrates, is of increasing importance because of the high optical yields, regioselectivity, mild reaction conditions and lack of protection groups. Aldolases and transketolases are utilized to catalyze C–C bond formation yielding natural and unnatural sugar derivatives. Deoxy sugars have gained attention due to their role in biological systems as glycosidase inhibitors[1], as constituents of secondary metabolites[2] and unnatural substrates of glycosyltransferases. In the latter case the combination of aldolases and glycosyltransferases seems to be possible but has been shown only in few examples[3].

Fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus*[4] has already been applied in the synthesis of a variety of carbohydrates[5]. Like other aldolases[6] this enzyme is restricted to dihydroxyacetone phosphate as methylene component, but shows a very broad substrate range with respect to the aldehyde compound. In order to prepare chiral hydroxyaldehydes as substrates for the aldolase reaction, reduction of bifunctional ketals (i.e. pyruvic aldehyde dimethyl acetal) by the novel carbonyl reductase isolated from *Candida parapsilosis*[7a] was attempted. The possible utilization of sucrose synthase for the synthesis of unnatural disaccharides has been mentioned[7b], but only limited information on the acceptor specificity was available until now. In this paper the combined use of the carbonyl reductase and formate dehydrogenase, fructose 1,6-bisphosphate aldolase, acid phosphatase and sucrose synthase in the chemoenzymatic synthesis of an unnatural disaccharide on a preparative scale is described.

In **Figure 1** the chemoenzymatic route for the synthesis of 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose is presented. The reaction sequence involved four enzymatic and only one simple chemical step. First, pyruvic aldehyde dimethyl acetal **1** was converted into L-lactaldehyde dimethyl acetal **2** by reduction with a novel carbonyl reductase from *Candida parapsilosis*. Coenzyme regeneration was achieved by coupling to the

formate dehydrogenase reaction[7c,d]. Subsequently, the product was easily deprotected by incubation with a strong cation exchanger. The third step involved fructose 1,6-bisphosphate aldolase for the C-C-bond formation between L-lactaldehyde **3** and dihydroxyacetone phosphate forming 6-deoxy-L-sorbose-phosphate **4**. The next step was the enzymatic dephosphorylation by acid phosphatase treatment yielding 6-deoxy-L-sorbose **5**. The transfer of glucose from UDP-glucose was catalyzed by sucrose synthase from rice and yielded the disaccharide 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose **6** in 17% overall yield. All reactions were performed at room temperature.

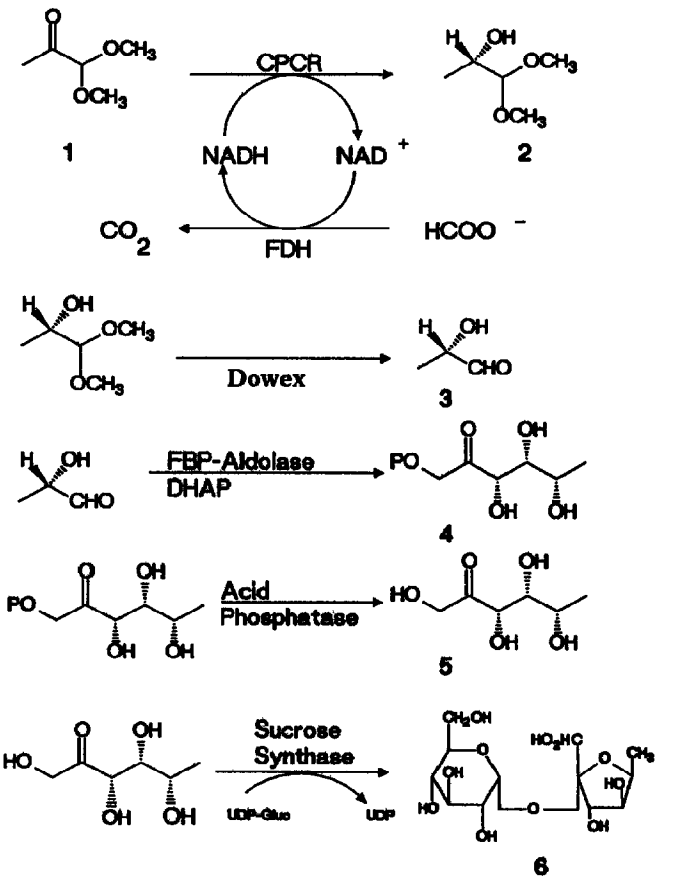

Fig. 1 Chemoenzymatic synthesis of 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose

The novel NADH-dependent carbonyl reductase from *Candida parapsilosis* shows an extremely broad substrate range including primary and secondary alcohols, aldehydes, aliphatic and aromatic ketones, cyclic ketones, diketones, halogenated ketones, keto esters and halogenated keto esters of variable chain length as substrates[7]. Besides these substrates ketals are also accepted and reduced at high reaction velocities to the corresponding (S)-hydroxy compound. The stereoselective reduction of pyruvic aldehyde dimethyl acetal yielded the corresponding L-lactaldehyde dimethyl acetal with 92% recovery and approximately 100% ee. The coenzyme, NADH, was regenerated by the well established formate dehydrogenase reaction on the expense of sodium formate. Cross inhibition of the formate dehydrogenase by pyruvic aldehyde dimethyl acetal was observed above 200 mM concentration. Thus, the concentration of pyruvic aldehyde dimethyl acetal was adjusted to 200 mM where the activity of the formate dehydrogenase was about 85%. Cross inhibition is very important with respect to the efficiency of multi enzyme systems. Vasic-Racki and coworkers[8] showed cross inhibition and activation phenomena in a coupled enzyme system of mandelic acid dehydrogenase and formate dehydrogenase.

An activity ratio carbonyl reductase/total enzyme of 0.2 has been found optimal[7]. The carbonyl reductase showed high stability under the reaction conditions employed. The activity was recovered after the reaction by ultrafiltration to 92%. To our knowledge this is the first enzymatic preparation of L-lactaldehyde dimethyl acetal with soluble enzymes. Wong et al.[9] described the enzymatic synthesis of lactaldehyde dimethyl acetal with immobilized alcohol dehydrogenase from yeast (YADH), horse liver (HLADH) and *Thermoanaerobium brockii* (TBADH), respectively. YADH and HLADH gave the (L)-enantiomer of lactaldehyde dimethyl acetal with high enantiomeric excess (ee) whereas TBADH produced only 51% ee of the (D)-enantiomer. However, YADH and HLADH tend to give poor activity yields during immobilization[10,11]. Murata et al.[12] isolated and characterized a strictly NADPH-dependent methylglyoxal reductase from *Saccharomyces cerevisiae*, but the stereochemistry of this unstable enzyme is not

known yet.

Fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus* was used for the condensation between dihydroxyacetone phosphate and L-lactaldehyde. The reaction was followed by the determination of sugar phosphate by TLC and by enzymatic determination of dihydroxyacetone phosphate. Subsequently, the sugar phosphate was dephosphorylated by acid phosphatase treatment. The free sugar, 6-deoxy-L-sorbose, was further purified and analyzed by ^{1}H and ^{13}C NMR. The ^{13}C NMR data for 6-deoxy-L-sorbose synthesized by the chemoenzymatic route are in good aggreement with data from literature[13a]. In equilibrium state the α-furanose form of 6-deoxy-L-sorbose is the predominating species.

The product of the aldolase reaction, 6-deoxy-L-sorbose, was used as acceptor for the transfer of α-D-glucose from UDP-glucose with sucrose synthase from rice. The relative activity compared to the native acceptor D-fructose was 8.8%. The disaccharide obtained by sucrose synthase catalyzed transfer of glucose from UDP-glucose to 6-deoxy-L-sorbose in preparative scale was characterized by ^{1}H and ^{13}C NMR. Signal assignment of the ^{13}C NMR spectrum was achieved by C/H correlation. Analysis of C_2 and C_1' chemical shifts revealed an α-1,2 linkage. This was established by comparison to sucrose[13b] and 2O-β-D-xylulofuranosyl-D-glucose[13c] and by calculation of C_2 values of α- and β-linkage isomers from the 6-deoxy-L-sorbose values considering a glycosylation shift of approximately 2 ppm.

In conclusion, a synthetic route for the synthesis of unnatural disaccharides has been developed based on four enzymatic and one simple chemical step. The combination of carbonyl reductase, aldolase and sucrose synthase should provide access to a series of valuable sucrose analogues.

The following procedures are representative.

Enzyme assays. The carbonyl reductase from *Candida parapsilosis* and the fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus* were assayed as described previously[4,7a]. Sucrose synthase from rice was assayed by the synthesis reaction with UDP-glucose as donor substrate. The formed UDP was detected by HPLC as described previously[14]. 6.8 mg of 6-deoxy-L-sorbose (42 μmol) in 680 μl HEPES-NaOH buffer (200 mM, pH 7.2) and 2 mM UDP-glucose were incubated with 82 mU sucrose synthase (final volume 1 ml). After 3 h at 30°C the reaction was stopped by incubation at 95°C for 5 min. An aliquot was analyzed for UDP formation on HPLC and compared with controls without enzyme and without acceptor substrate, respectively. The enzyme activity was expressed as relative activity of the enzyme with D-fructose.

Thin layer chromatography. Pyruvic aldehyde dimethyl acetal and (L)-lactaldehyde dimethyl acetal could be separated due to their different solubility in the mobile phase. Samples (2 μl) were applied on a silica plate 60 F_{254} and developed with diethyl ether/benzene$_{60-80}$ (2:1). After drying in a warm air current the plates were dipped in the following solution for ten seconds: 0.15% of 2.4-dinitrophenylhydrazine in 50% (v/v) ethanol, 50% (v/v) hydrochloric acid. Spots were visualized by heating the plates in a hot air current. Keto compounds have higher R_f-values than the corresponding hydroxy compounds. The R_f values were 0.63 for pyruvic aldehyde dimethyl acetal, 0.25 for L-lactaldehyde dimethyl acetal and 0 for L-lactaldehyde. Sugar phosphates and free sugars were analyzed as described elsewhere[5,15]. Disaccharides were separated from monosaccharides using acetonitrile/0.1 M ammonium chloride (75:25). Spots were visualized by spraying the plates with a solution of naphthoresorcinol (20 mg), diphenylamine (40 mg) in ethanol (10 ml) and sulphuric acid (0.4 ml). After drying in a hot air current the plates were heated (110°C) until spots of different colour appeared. The R_f values were 0.41 for sucrose, 0.51 for 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose and 0.79 for 6-deoxy-L-sorbose.

High performance liquid chromatography. Pyruvic aldehyde and L-lactaldehyde were determined by HPLC analysis on a RP_4 column, acetonitrile/water 90:10, with a flow rate of 0.4 ml/min the retention times were 7.74 min for pyruvic aldehyde dimethyl acetal, 7.12 min for L-lactaldehyde. HPLC analysis of free sugars was performed as described elsewhere[15].

L-Lactaldehyde dimethyl acetal. In a total volume of 100 ml triethanolamine-NaOH buffer, 0.1 M, pH 7.0 2.42 ml pyruvic aldehyde dimethyl acetal (20 mmol), 6.65 mg NAD^+ (10 μmol), 2.72 g sodium formate (40 mmol), 10 units of carbonyl reductase and 40 units of formate dehydrogenase were combined and stirred (150 rpm) at 25°C. At times, samples were removed and analyzed by TLC. After the reaction had reached 92% yield as determined by HPLC analysis (34 h), the enzymes were removed by ultrafiltration.

Enzymatic determination of the optical purity. The optical purity of lactaldehyde dimethyl acetal was determined after hydrolyzation of a sample with Dowex 50 (H^+ form) to lactaldehyde (see below) and aliquotes of the solution were oxidized to lactic acid with aldehyde dehydrogenase as catalyst and NAD as cofactor. Subsequently, the concentrations of D- and L-lactic acid were determined enzymatically with D- and L-lactic acid dehydrogenase[16a]. Instead of glutamate-pyruvate transaminase the pyruvate decarboxylase from *Zymomonas mobilis*[16b] was used in order to draw the equilibrium. D- and L-lactic acid were used as standard material. Determination of the optical purity of lactaldehyde indicated that the lactaldehyde obtained had the L-configuration with 100% ee.

Chemical deprotection. Deprotection of L-lactaldehyde dimethyl acetal was achieved by adding 3 g Dowex 50 WX 8 H^+ (50-100 mesh). After 12 h the deprotection was complete as judged by TLC. The cation exchanger was removed by filtration and pH was adjusted to pH 6.8 with NaOH.

Enzymatic synthesis of 6-deoxy-L-sorbose. To the reaction mixture (100 ml) containing L-lactaldehyde 100 units fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus* and 1.63 mmol dihydroxyacetone phosphate, which was prepared by the method of Effenberger and Straub[17], were added and stirred at 25°C. After 10 h the enzyme was removed by ultrafiltration. Product formation was determined by sugar phosphate TLC. Dihydroxyacetone phosphate was determined enzymatically according to Straub[18]. The chemical yield was 93%. The reaction mixture (130 ml) was adjusted to pH 8.2 and cooled in an ice bath. 5 ml barium acetate (2 M) were added and the mixture stirred for 30 min. After centrifugation at 10.000 g for 10 min the precipitate was washed with 1 N HCl (20 ml) and centrifuged once again (10.000 g, 10 min.). The supernatants were pooled and 600 ml ice cold ethanol were added. After 30 min. the mixture was centrifuged at 10.000 g, 10 min. The precipitate was dried under vacuum over silica gel. 10 ml of distilled water and Dowex was added until the precipitate was completely dissolved (pH 1.5). After 3 h the cation exchanger was removed by filtration and the pH adjusted to pH 5. Dephosphorylation was achieved by acid phosphatase treatment. 40 mg acid phosphatase (80 ml) were added and the reaction mixture was stirred at 25°C for 24 h. The disappearance of sugar phosphates was followed by sugar phosphate TLC and the appearance of free sugars was determined by sugar TLC and HPLC. The R_f value of 6-deoxy-L-sorbose was 0.79 and the retention time was 15.7 min. After ultrafiltration the pH was adjusted to pH 8.2 and free phosphate was removed by barium acetate precipitation. After centrifugation at 10.000 g (15 min.) the supernatant was evaporated under reduced pressure. The resulting oil was further purified over Dowex 50 WX 8 (Ca^{2+} from; 1.6 x 60 cm column; eluent : water; flow rate 6 cm/h) and 6-deoxy-L-sorbose containing fractions were detected by TLC and HPLC as described above. Positive fractions were pooled and lyophilized. The chemical yield of 6-deoxy-L-sorbose was 150 mg (56%).

^{1}H-NMR (300 MHz, D_2O) α-Furanose: δ = 3.55 (d, 1-H_a), 3.61 (d, 1-H_b), 4.08 (d, 3-H), 4.21 (dd, 4-H), 4.41 (q, 5-H), 1.18 (d, 6-H_3) ppm; $J_{1a,1b}$ = 12.0, $J_{3,4}$ = 4.5, $J_{4,5}$ = 5.4,

$J_{5,6}$ = 6.6 Hz; $TSPd_4$ = 0.00 ppm. ^{13}C-NMR (75 MHz, D_2O) α-Furanose: δ = 64.54 (C-1), 102.98 (C-2), 77.72 (C-3), 77.77 (C-4), 76.03 (C-5), 15.19 (C-6) ppm; β-Furanose: δ = 106.38 (C-2) ppm; $TSPd_4$ = -1.70 ppm.

Enzymatic synthesis of disaccharide with sucrose synthase. To the reaction mixture (17 ml) 8 units sucrose synthase from rice grains, 662 mg UDP-glucose, 150 mg 6-deoxy-L-sorbose were added in 50 mM HEPES buffer, pH 7.2 and incubated at 25°C. The reaction mixture was gently shaken. After the reaction was complete as judged by HPLC and TLC the mixture was applied to a Sepharose-Q column (50 ml bed volume) equiped with a refractive index monitor. The column was equilibrated previously with distilled water. Fractions containing sugar were pooled and lyophilized. Subsequently, the disaccharide was further purified by flash chromatography on a silica column using acetonitrile/0.1 M ammonium hydrogen carbonate (75:25) as solvent. Fractions containing disaccharide were pooled, lyophilized and lyophilized twice from deuterium oxide. The chemical yield was 50 mg of 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose (17%).

^{1}H-NMR (300 MHz, D_2O) δ = 3.70 (d, 1-H_a), 3.64 (d, 1-H_b), 4.17 (d, 3-H), 4.33 (t, 4-H), 4.49 (dq, 5-H), 1.19 (d, 6-H_3), 5.35 (d, 1'-H), 3.55 (dd, 2'-H), 3.72-3.86 (m, 3'-H, 5'-H, 6'-H_a, 6'-H_b), 3.42 (t, 4'-H) ppm. $J_{1a,1b}$ = 12.5, $J_{3,4}$ = 6.7, $J_{4,5}$ = 6.8, $J_{5,6}$ = 6.7, $J_{1',2'}$ = 3.9, $J_{2',3'}$ = 10.0, $J_{3',4'}$ = 9.4, $J_{4',5'}$ = 9.4 Hz.

^{13}C-NMR (75 MHz, D_2O) δ = 61.55 (C-1), 104.79 (C-2), 78.74 (C-3), 76.68 (C-4), 76.68 (C-5), 15.84 (C-6), 93.45 (C-1'), 72.26 (C-2'), 73.87 (C-3')*, 70.57 (C-4'), 73.77 (C-5')*, 61.67 (C-6') ppm.

* Assignment interchangeable

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