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Higher-carbon Sugars by Enzymatic Chain Extension Oxidative Generation of Aldol Precursors *in situ* # 1

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Abstract: A novel multienzymatic approach for the preparation of complex carbohydrates by enzymic aldolization is presented in which both the aldol donor and acceptor components are generated *in situ* by air oxidation using microbial oxidases. The L-*threo* stereochemistry generated by the recombinant rhamnulose 1-phosphate aldolase has been established by X-ray structural analysis.

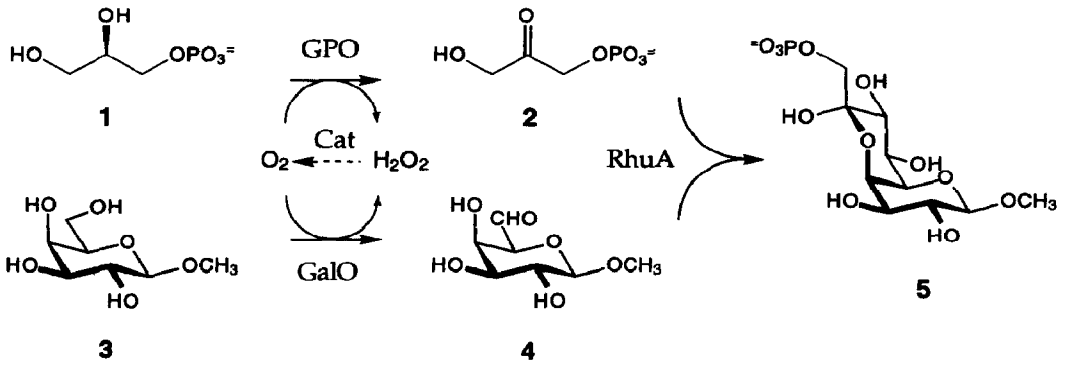
Complex sugars and their derivatives with backbones longer than the standard five or six carbon atoms have received considerable attention lately because of their interesting biological activities. Due to the scarcity of these materials various methods have been developed for their preparation by (repetitive) chain extension of common aldoses.[2] Chemical syntheses, however, are usually hampered by the fact that the latent aldehyde functionality of the carbohydrate has to be unmasked from its hemiacetal structure or a second aldehyde be generated by selective oxidation of the nonreducing terminus, the necessity of prior implementation of suitable protecting groups, and the distinct influence of the preexisting carbohydrate structure on the stereoselectivity of the addition which mandates an individual tuning of the reaction conditions to minimize separation efforts.

A number of aldolases has been recruited for the stereoselective coupling of certain nucleophiles to the aldehyde carbonyl of unprotected monosaccharides.[3] Particularly, a bacterial sialic acid aldolase has been employed in the preparation of higher 3-deoxyulosonic acids related to KDO or sialic acid,[4] whereas the fructose 1,6-bisphosphate aldolase from rabbit muscle requires sugar substrates to be phosphorylated in order to be acceptable for addition of dihydroxyacetone phosphate (DHAP) and formation of 2-ulose bisphosphates.[5]

Following a complementary, "inverse" approach,[6] in this letter we demonstrate the feasibility of an enzymatic chain extension of unprotected aldoses or glycosides at their (formally) non-reducing terminus, utilizing a highly tolerant recombinant L-rhamnulose 1-phosphate aldolase (RhuA; EC 4.1.2.19) from *Escherichia coli* for the addition of DHAP.[7] The one-pot, all-enzymic method is based on the *in situ* oxidation of the sugar precursor to a dialdose derivative, and on a recently developed protocol for oxidative formation of the labile DHAP intermediate.[8]

D-Galactose oxidase (GalO; EC 1.1.3.9) secreted by the fungus *Dactylium dendroides* is a monomeric Cu(II) enzyme that catalyzes the oxidation of D-galactose to the corresponding D-*galacto*-hexodialdose. Its X-ray crystal structure[9] has been long awaited because of the unusual involvement of covalently linked

Cys 228–Tyr 272 residues in the electron relay, acting as an endogenous cofactor. The unique radical site likely contributes to the known propensity of commercial GalO preparations to deactivation. However, the enzyme has been successfully applied to the small-scale preparation of some uncommon sugars from alditols[10] and to the large-scale synthesis of galactose based sugar aldehydes.[11] The similar oxygenating nature of the reaction conditions suggested to combine sugar dialdehyde formation with the *in situ* generation of DHAP (**2**) from L-glycerol 3-phosphate (**1**) catalyzed by a flavine-dependent glycerol phosphate oxidase from *Streptococcus* (GPO; EC 1.1.3.21). Since the known DHAP aldolases proved stable in the presence of oxygen,[8] an ensuing aldol addition could consume both products *in situ* (Scheme 1) and thus obviate problems from product inhibition that had been noted for both oxidases.[8,11]



Scheme 1. Multienzymatic oxidation–aldolization strategy for the synthesis of methyl β-L-*threo*-D-*galacto*-8-nonosulo-1,5-pyranoside 9-phosphate **5**

For initial experiments[12] we chose to use methyl β-D-galactopyranoside **3** to avoid analytical difficulties with mixed anomers of unglycosylated sugars and because of the higher conversion rate (*cf.*, Scheme 2). Enzyme deactivation by hydrogen peroxide which is formed as a byproduct in the oxidation was prevented by adding a ten-fold amount of catalase activity (Cat; EC 1.11.1.6). The recombinant L-rhamnulose 1-phosphate aldolase[15] was chosen for its superior substrate tolerance with respect to sterically demanding aldehydes. The reaction mixture was stirred at 0°C under slight oxgen pressure (1.9 bar) and product formation followed by TLC. Reaction was terminated at 30-40% conversion of **3**, and the single product was dephosphorylated using acid phosphatase and purified by chromatography to furnish crystalline adduct **6** (≡ dephospho-**5**) in 14% overall yield. In another set of experiments the GalO catalyzed reaction was performed separately because of the diverging needs of oxidase and aldolase for metal cofactors; while GalO requires Cu^{2+} ions for activity and stability,[11] the Zn^{2+}-dependent aldolases are inhibited by copper ions. Thus, the product solution obtained by exhaustive oxidation of **3** was deionized before the aldolase–GPO treatment which resulted in practically quantitative consumption of **3** and isolation of **6** in 78% overall yield after purification.

According to ^1H NMR analysis **6** prevails in solution as a single anomer of a dipyranose. The large $J_{6,7}$ *trans* diaxial coupling of 9.9 Hz indicated that C–C bond formation had occurred in the expected L-*threo* fashion typical for RhuA.[7] Confirmation of the stereochemical outcome was obtained by an X-ray analysis (Figure 1) of crystals grown from water–acetone.[14]

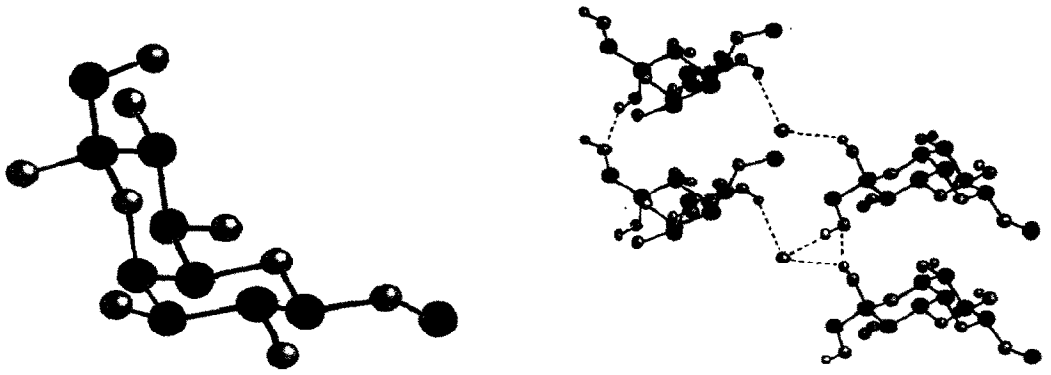
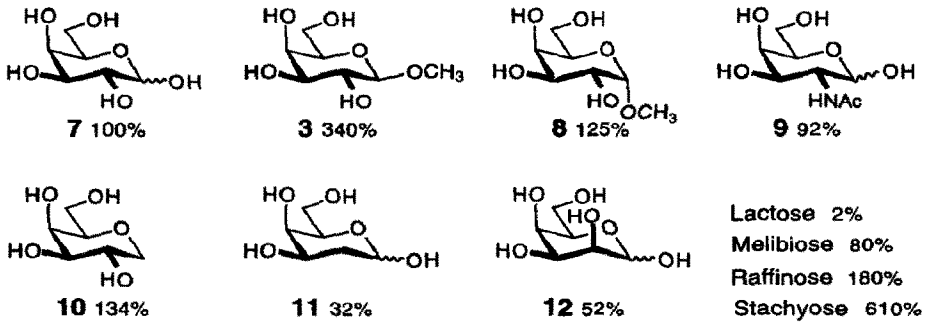


Figure 1. X-ray structure of dipyranoside **6** (left; hydrogen atoms omitted) and representation of selected intermolecular hydrogen bonding contacts (right; only hydroxyl hydrogens shown)[14]

The *cis*-fused bicyclic ring system carries only equatorial substituents (except the axial anomeric hydroxyl at C-8) and thus shows no significant deviations from ideal chair conformations. In the solid state the compound contains one mol of hydration water (Figure 1) which is involved in a network of several stronger (9-OH, 1.829 Å) or weaker intermolecular hydrogen bonds (6-OH, 2.476 Å; 8-OH, 2.497 Å).



Scheme 2. Substrates of galactose oxidase and relative reaction rates[15]

The synthetic scheme presented here has the advantages of simplicity of operation, ready availability of starting materials at low cost, and high diastereoselectivity in the addition (>95% for **5/6**; no contaminating diastereomer could be detected in the crude reaction mixtures within the analytical accuracy of ±3% of high-field ^{1}H NMR) combined with a broad substrate tolerance of both the aldolase[7] and GalO (Scheme 2).[15] Given the potential applicability of other DHAP aldolases having a different stereopreference this scheme should prove valuable for the stereodivergent synthesis of complex nine-carbon sugars containing two anomeric carbons that are available for further functionalization. The disadvantage of a less satisfactory stability of the GalO may likely be obviated by the use of highly purified GalO preparations which are accessible in large quantities.[11]

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References and Notes

Dedicated to Prof. Dr. C. Rüchardt on the occasion of his 65th birthday

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12. Experimental conditions: A solution of **3** (600 mg, 3 mmol) in 50 mM phosphate buffer (10 mL, pH 6.0) containing 0.5 mM $CuCl_2$ was incubated with GalO (250 U) under oxygen with stirring until total conversion was reached (3 d). After desalting was added **1** (1.5 mmol), GPO (50 U), Cat (1000 U), and RhuA (180 U), and stirring was continued until intermediate **2** was no longer detectable (6 h). Treatment with acid phosphatase, chromatography (chloroform-methanol-water 23:10:1) and crystallization from water-acetone afforded **6** (330 mg, 78%), mp 205°C; $[\alpha]_D$ 57.7° (c = 0.7, MeOH); ^{13}C NMR (D_2O) δ = 104.1, 98.2, 73.0, 71.8, 71.2, 69.3, 68.9, 68.5, 64.6, 57.5.
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14. Since crystals proved to fracture upon drying, a representative crystal was taken from solution and sealed in a capillary for X-ray measurements. Crystallographic data: $C_{10}H_{20}O_{10}$, MW 300.26, orthorhombic, $P2_12_12_1$ (# 19), a = 5.21430(10), b = 16.066(2), c = 16.3560(10) Å, V = 1370.2(2) Å^3, Z = 4, $\lambda(Cu_{K\alpha})$ = 1.54178 Å by Enraf-Nonius CAD4 diffractometer. The structure was solved and refined using the SHELXS-86 and SHELXL-93 program systems. Final R is 0.0697 for 2446 [$I>2\sigma(I)$] out of total 2878 reflections. In the crystal existed two independently hydrogen bonded rotamers of the terminal hydroxymethyl group. Tables of atomic parameters, bond lengths, bond angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.
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