A Four-Step Enzymatic Cascade for the One-Pot Synthesis of Non-natural Carbohydrates from Glycerol

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A total of four enzymatic steps were combined, in a one-pot reaction, to synthesize carbohydrates starting from glycerol. First, phosphorylation of glycerol by reaction with pyrophosphate in the presence of phytase at pH 4.0 in 95% glycerol afforded racemic glycerol-3-phosphate in 100% yield. The L-enantiomer of the latter underwent selective aerobic oxidation to dihydroxyacetone phosphate (DHAP) at pH 7.5 in the presence of glycerolphosphate oxidase (GPO) and catalase. Subsequently, fructose-1,6-bisphosphate aldolase catalyzed the aldol reaction of DHAP with butanal. Finally, dephosphorylation of the aldol adduct was mediated by phytase at pH 4 affording 5-deoxy-5-ethyl-D-xylulose in 57% yield from L-glycerol-3-phosphate. The phytase on/off-switch by pH was the key to controlling phosphorylation and dephosphorylation.

Introduction

Enzyme-catalyzed aldol addition is a powerful methodology for building carbohydrate derivatives.[1,2] Aldolases, which in their natural function perform a retro-aldol reaction, are capable of catalyzing an extensive variety of carbon−carbon bond constituting reactions[3]. The group of aldolases which depend on dihydroxyacetone phosphate[4] (DHAP) as the donor substrate is particularly attractive as total control over the stereochemical outcome of the aldol reaction is obtained (see Figure 1). They are hampered, nevertheless, in their practical application by the lack of cheap and practical access to DHAP.

A simple and economical route to DHAP—and therefore to carbohydrates—should involve a limited number of steps from inexpensive starting materials. The reaction should preferably be enzymatic as this would obviate the need for protection and deprotection steps inherent in chemical phosphorylation.[5] Moreover, chemical phosphorylation is likely to be incompatible with subsequent enzymatic steps. Owing to the high energy content of the phosphate ester bond, DHAP synthesis generally requires an activated phosphate. For example, phosphorylation of dihydroxyacetone with ATP[6,7] calls for regeneration of the latter from an organic phosphate.

Alternatively, DHAP can be prepared by oxidation of L-glycerol-3-phosphate, mediated by glycerolphosphate oxidase (GPO)[8] in the presence of catalase to suppress the build-up of hydrogen peroxide. Although GPO is specific for L-glycerol-3-phosphate, racemic glycerol-3-phosphate can be used, making the process more cost-effective. In vivo L-glycerol-3-phosphate is produced by glycerol kinase catalyzed reaction of glycerol with ATP which would again call for a source of activated phosphate for its regeneration. Moreover, glycerol kinase has a low tolerance for oxygen.

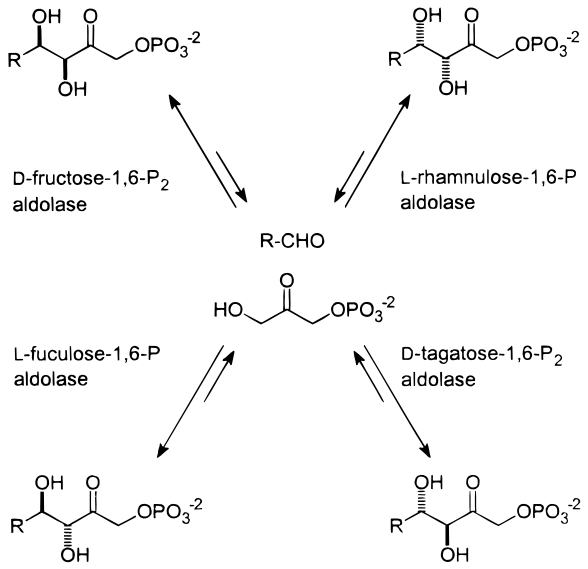

Figure 1. The four DHAP dependent aldolases.

These obstacles can, in principle, be circumvented by using a phosphatase as the phosphorylation catalyst and pyrophosphate as an inexpensive source of phosphate.[9,10] Combination of such a phosphorylation of glycerol with in situ oxidation to DHAP and subsequent enzymatic

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(1) Gijsen, H. J. M.; Qiao L.; Fitz W.; Wong C.-H. *Chem. Rev.* **1996**, *96*, 443−469.

(2) Fessner, W.-D.; Walter, C. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 614−616.

(3) Wang, P. G., Fitz, W.; Wong, C.-H. *Chemtech* **1995**, April 22−23.

(4) Meyerhof, O.; Lohmann, K. *Biochem. Z.* **1934**, *271*, 89.

(5) (a) Colbran, R. L.; Jones, J. K. N.; Matheson, N. K.; Rozema, I. *Carbohydr. Res.* **1967**, *4*, 355−358. (b) Effenberger, F.; Straub, A. *Tetrahedron Lett.* **1987**, *28*, 1641−1644. (c) Pederson, R. L.; Esker, J.; Wong, C.-H. *Tetrahedron* **1991**, *47*, 2643−2648. (d) Jung, S.-H.; Jeong, J.-H.; Miller, P.; Wong, C.-H., *J. Org. Chem.* **1994**, *59*, 7182−7184.

(6) Wong, C.-H.; Whitesides, C. M. *J. Org. Chem.* **1983**, *48*, 3493−3497.

(7) Crans, D. C.; Whitesides, G. M. *J. Am. Chem. Soc.* **1985**, *107*, 7019−7027.

(8) Fessner, W.-D.; Sinerius, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 209−212.

(9) Pradines, A.; Klaébé, A.; Périé, J.; Paul, F.; Monsan, P. *Tetrahedron* **1988**, *44*, 6386-6386.

(10) Pradines, A., Klaébé, A.; Périé, J.; Paul, F.; Monsan, P. *Enzyme Microb. Technol.* **1991**, *13*, 19−23.

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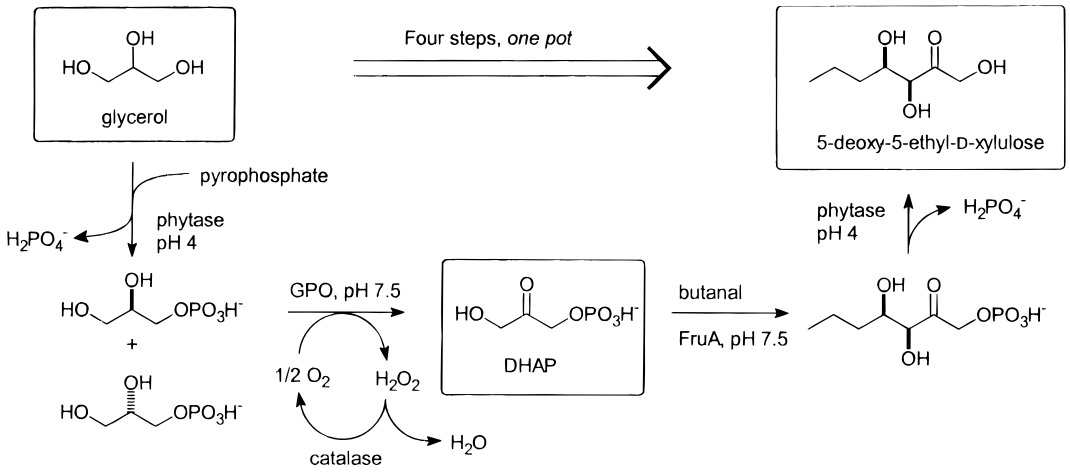

Figure 2. In situ DHAP preparation and subsequent aldol reaction with butanal and dephosphorylation constructs a four-step one-pot reaction leading to carbohydrates directly from glycerol. Phosphorylation and dephosphorylation is carried out at pH 4.0, the remainder at pH 7.5.

aldol condensation and dephosphorylation would provide a one-pot procedure from glycerol to carbohydrates.

We herein report[11] on the successful application of this concept of a one-pot procedure, comprising a cascade of four enzymatic steps, for the synthesis of carbohydrates from two inexpensive and readily available starting materials: glycerol and pyrophosphate.

Results and Discussion

Our synthesis scheme comprises kinetically controlled phosphorylation of glycerol by pyrophosphate, aerobic oxidation of L-glycerol-3-phosphate, aldol addition of DHAP, and finally dephosphorylation of the aldol adduct (see Figure 2). The key to its success is the judicious use of pH control to switch the activities of the various enzymes on and off during the cascade.

The oxidation and aldol reactions are optimally performed at pH 7.5; hence, to avoid interference by the phosphatase its hydrolytic activity should be zero at neutral pH. This restricted our choice of phosphatase to the acid phosphatases, because the use of an alkaline phosphatase would introduce the problem of base-catalyzed background hydrolysis of glycerol phosphate. The phosphatase of choice was phytase[12] from *Aspergillus ficuum*, which is a cheap and readily available industrial enzyme used in animal feed. It has two pH optima for the hydrolysis of its natural reactant, phytic acid: pH 2.2 and 5. The pH profile of the hydrolysis of L-glycerol-3-phosphate (see Figure 3) shows an optimum at pH 3−4. At pH 7.5 phytase is stable, but unreactive toward either L-glycerol-3-phosphate or DHAP, which makes it possible to combine the phosphorylation, oxidation, and aldol addition steps into a one-pot procedure by pH control. Phytase exhibited the same hydrolytic activity toward DHAP as to L-glycerol-3-phosphate.

Phosphorylation of Glycerol. The production of L-glycerol-3-phosphate by phytase-mediated reaction with pyrophosphate was pH dependent with a very broad optimum (see Figure 4). At pH 2 phosphorylation was substantial, and it decreased above pH 4 to become zero at pH 7. The pH optimum did not change significantly with increasing glycerol concentration. As long as the pH was kept between pH 3 and 4, good results were obtained. No change in pH was observed during the reaction.

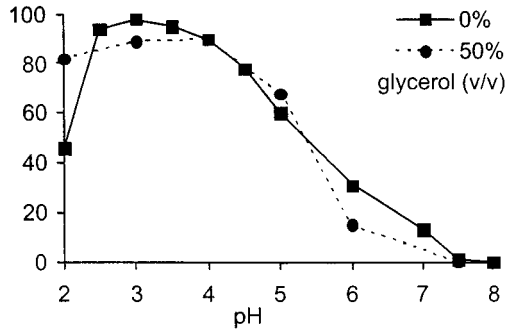

Figure 3. Hydrolysis of L-glycerol-3-phosphate (L-G-3-P) (initial concentration 50 mM) after 2 h of incubation at room temperature catalyzed by phytase (1 mg/mL) in 0 and 50% glycerol (v/v) at different pH.

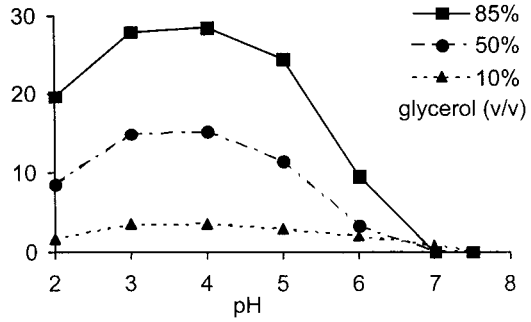

Figure 4. Production of L-glycerol-3-phosphate by phytase (1 mg/mL) in 10, 50 and 85% glycerol (v/v) at different pH after 2.5 h incubation with 150 mM pyrophosphate. Presumably an equal amount of D-glycerol-3-phosphate is formed.

Since only L-glycerol-3-phosphate can be oxidized to DHAP, the D-isomer (which is presumably produced in equal amounts) will remain unused in solution. In the final dephosphorylation step it is converted back to glycerol and phosphate. This also implies that the yield of the reaction, based on pyrophosphate, cannot surpass 50%.

Effects of Glycerol Concentration. The time to reach maximum conversion, based on the amount of

(11) Schoevaart, R.; Van Rantwijk, F., Sheldon, R. A. *Chem. Commun. (Cambridge)* **1999**, 2465−2466.

(12) Dvorakova, J. *Folia Microbiol.* **1998**, *43*, 323−338.

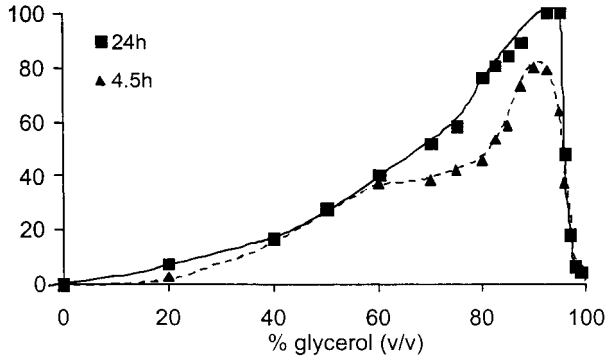

Figure 5. Phosphorylation of glycerol with pyrophosphate (150 mM) by phytase (1 mg/mL) at pH 4.0 at 37 °C in glycerol/water (v/v) mixtures. Racemic glycerol-3-phosphate is obtained in 100% yield (based on pyrophosphate) in 95% glycerol after 24 h.

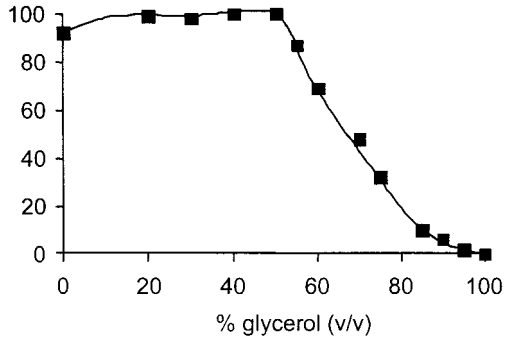

Figure 6. Oxidation of L-glycerol-3-phosphate to DHAP by GPO in varying concentrations of glycerol at pH 7.5. Conversion was measured after 1 h. Reaction rates were expressed relative to the highest rate obtained at 55% glycerol (v/v).

pyrophosphate, was hardly affected by glycerol concentrations between 10 and 85% (v/v), but at 95% the rate had dropped by a factor of 10 (data not shown). At still lower water concentrations the enzyme activity decreased to zero in pure glycerol, although some activity still could be observed when only 0.25% water was present.

The yield of D,L-glycerol-3-phosphate increased with increasing glycerol concentration (see Figure 5), presumably because the competing hydrolysis of pyrophosphate and glycerol-3-phosphate is suppressed at low water concentrations. At 95% glycerol concentration a quantitative conversion of pyrophosphate into glycerol-3-phosphate was obtained, after a 24 h reaction time.

Selectivity. Phytase is believed to be nonstereoselective in the phosphorylation of glycerol.[9] Since only L-glycerol-3-phosphate is a substrate for GPO, the production of D-glycerol-3-phosphate cannot be monitored by this enzymatic assay. The consumption of pyrophosphate and production of phosphate and glycerol phosphate could be monitored using ^{31}P NMR, however. A reaction mixture with 95% glycerol and an initial pyrophosphate concentration of 150 mM was analyzed for ^{31}P before 100% conversion was reached. This revealed that the phosphate concentration was 100 mM whereas the pyrophosphate concentration was 50 mM, a decrease of 100 mM (67% conversion). This observation demonstrates that, under these conditions, pyrophosphate was converted to one equivalent of glycerol phosphate and one equivalent of phosphate, without any hydrolysis of pyrophosphate or glycerol-3-phosphate. The concentration of D,L-glycerol-3-phosphate therefore was 100 mM. Enzymatic detection of L-glycerol-3-phosphate with GPO and GDH gave a concentration of 50 mM, exactly matching the NMR results and confirming that phytase is not stereoselective. But, since glycerol-2-phosphate was absent in the reaction mixture phytase is completely regiospecific.

Oxidation of L-Glycerol-3-phosphate. After complete conversion of pyrophosphate by phytase, the pH was raised to 7.5 to suppress the hydrolysis of L-glycerol-3-phosphate (and DHAP) and maximize GPO's activity. The concentration of glycerol must also be adjusted since GPO activity is low at 95%. To achieve maximum space−time yields, a balance must be struck between a high L-glycerol-3-phosphate concentration and compatibility with GPO activity. At pH 7.5 the oxidation rate of L-glycerol-3-phosphate was increased 50-fold by lowering the glycerol concentration (Figure 6) from 95 to 55%, which convincingly shows that dilution is highly advantageous.

A 1.5-mmol (pyrophosphate) reaction was carried out in 95% glycerol. The time to reach full conversion at this concentration was 1 day. After phosphorylation was completed the mixture was diluted with water to 55% glycerol and the pH was adjusted to 7.5. Addition of GPO/catalase and entrainment with oxygen for 3 h afforded DHAP in a concentration of 43 mM corresponding to 50% yield based on pyrophosphate.

Aldol Reaction and Dephosphorylation. Aldol reactions can be performed with the in situ prepared DHAP in glycerol. To this end we used the fructose-1,6-bisphosphate aldolase (FruA) from *Staphylococcus carnosus* to prepare the aldol adduct of DHAP and butanal.[13] Butanal and aldolase were added to the mixture containing DHAP, phytase, glycerolphosphate oxidase, catalase, and 55% glycerol. In fact, it is not necessary to do this step separately; the oxidation and aldol reaction can be carried out simultaneously,[8] thus saving hours of reaction time. The consumption of DHAP was used to monitor the course of the reaction. Under these conditions the aldolase displayed normal activity and modification of the mixture to enhance its performance was therefore unnecessary. In another example we used L-rhamnulose-1-phosphate aldolase from *Escherichia coli* and phenylpropionaldehyde as acceptor substrate. Now the oxidation and aldol reaction were effectively conducted simultaneously. These illustrations demonstrate that the combination of the relaxed acceptor specificity of DHAP dependent aldolases with the one-pot reaction opens the way to the synthesis of a wide spectrum of possible structures.

The addition of extra phosphatase for removal of the phosphate group is not necessary since phytase is still present and active. After oxidation and aldol reaction, phytase-mediated dephosphorylation of the adducts was carried out by simply lowering the pH to 4.0. Apparently phytase exhibits a wide substrate specificity in the hydrolysis reaction and, hence, is expected to be generally applicable for a broad range of substrates in this final step.

(13) Schoevaart, R.; Van Rantwijk, F., Sheldon, R. A. *Tetrahedron: Asymmetry* **1999**, *10*, 705−711.

Conclusion

Phytase-catalyzed phosphorylation of glycerol with pyrophosphate afforded D,L-glycerol-3-phosphate in quantitative yield. L-Glycerol-3-phosphate underwent GPO-catalyzed oxidation to DHAP after adjustment of the pH to 7.5. The D-enantiomer was not converted but its presence had no effect on the succeeding steps. Under the conditions used for oxidation and aldol reaction (pH 7.5) phytase did not hydrolyze L-glycerol-3-phosphate or DHAP. Manipulation of pH thus gives total control over phytase's activity; it can be "switched off" by increasing pH from 4 to 7.5 and "switched on" again by lowering back to pH 4.0.

This alliance of four different enzymes in a one-pot cascade of four enzymatic transformations provides an attractive procedure for performing aldol reactions with DHAP aldolases starting from the cheap, readily available glycerol and pyrophosphate. Combined with the broad substrate specificity of DHAP aldolases toward acceptor substrates it constitutes a simple procedure for the synthesis of a wide variety of carbohydrates from glycerol.

Experimental Section

General. UV spectroscopy was performed with a temperature controller. The aldolase and oxidase were obtained from Roche Diagnostics. All other enzymes and chemicals were purchased from Sigma.

Assay for DHAP and L-Glycerol-3-phosphate. DHAP was assayed with a coupled enzyme system: reduction with NADH-consuming glycerol-3-phosphate dehydrogenase enables determination of DHAP by measuring NADH concentration with UV spectroscopy. From a diluted DHAP solution, 50 μL was added in a quartz cuvette containing 1.95 mL of 50 mM TRIS pH 7.6, 0.16 mM NADH, 1.25 U glycerol-3-phosphate, and 12.5 U triose-1-phosphate isomerase. The absorption was monitored at 340 nm at 20 °C. Blank DHAP: 0.0047 mM/min. The molar adsorption coefficient taken was 6.22 L $mmol^{-1}$ cm^{-1}.

L-Glycerol-3-phosphate was detected by oxidizing it to DHAP with GPO and subsequent assay for DHAP. Samples of 125 μL containing L-glycerol-3-phosphate were diluted 4-fold with 200 mM Tris buffer, pH 8.0. This buffer neutralizes acidic samples thus preventing hydrolysis by phytase. In an 8-mL vial with gastight Teflon cap 5 μL GPO/catalase mixture (25 units/250 units) was added and oxygen was applied for 30 s while the vial was shaken vigorously. After 1 h of incubation at room temperature, the mixture was assayed for DHAP.

Phosphorylation. To a 10 mL solution containing 95% (v/v) glycerol and 150 mM pyrophosphate pH 4.0 (adjusted with 2 N HCl) was added freeze-dried phytase (10 mg). The mixture was incubated for 24 h at 37 °C in a 30-mL flask with Teflon cap and shaken gently.

Oxidation. After cooling to room temperature the pH was raised to 7.5 by addition of 2.0 N sodium hydroxide. Water was added (7.4 mL) to obtain a final glycerol concentration of 55%. Then 100 μL GPO/catalase mixture (50 units/500 units) was added, and oxygen was applied for 3 min. This was repeated after 30 and 60 min. The flask was shaken at room temperature. Oxidation was stopped after 3 h. The yield of DHAP (from pyrophosphate) was 50% (0.75 mmol). Since phytase produces equal amounts of the enantiomers the yield of D,L-glycerol-3-phosphate is 100%.

Aldol Reaction and Dephosphorylation. To a 10-mL mixture containing 43 mM DHAP, 88 μL of butanal (100 mM), and 12.5 units of fructose-1,6-bisphosphate aldolase from *S. carnosus* were added. Conversion was 78% after 4 h. Dephosphorylation by phytase still present in the mixture started by lowering the pH to 4 with 2 N HCl and stirring overnight. The glycerol/water mixture was extracted with ethyl acetate; the ethyl acetate layer was extracted with water and dried with sodium sulfate. After evaporation of the solvent 39 mg of 5-deoxy-5-ethyl-D-xylulose was isolated (analyzed using ^{1}H NMR spectra of authentic samples[13]). The yield was 57% from l-glycerol-3-phosphate (or 29% from pyrophosphate).

Simultaneous Oxidation and Aldol Reaction. To a 100-mL solution containing 55% glycerol and 43 mM L-glycerol-3-phosphate (based on the expected yield of the phosphorylation) was added 0.574 mL GPO/catalase mixture (287 units/2870 units), 125 units of l-rhamnulose-1-phosphate aldolase from *E. coli* and 1.57 mL (100 mM) of phenylpropionaldehyde. Oxygen was then applied for 10 min. This was repeated after 30 and 60 min. The flask was stirred at room temperature. Oxidation was stopped after 3 h. Dephosphorylation by phytase still present in the mixture started by lowering the pH to 4 with 2 N HCl and stirring overnight. The glycerol/water mixture was extracted with ethyl acetate/diethyl ether (2:1); the organic layer was extracted with water and dried with sodium sulfate. After evaporation of the solvent 3.2 g of product was isolated. The yield was 79% from l-glycerol-3-phosphate. Acetylation with 20 mL of pyridine and 10 mL of acetic anhydride of 1 g of product and subsequent column chromatography (EtOAc/petroleum ether 40−60 (1:5)) yielded a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.00 (s, 2H), 5.90 (d), 4.20 (m), 1.81 (m, 2H), 2.74 (m, 2H), 7.23 (m, 5H), 2.05 (s, 3H), 2.05 (s, 3H), 2.05 (s, 3H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 77.05, 201.55, 77.47, 76.60, 30.71, 29.68, 140.50, 128.44, 126.30, 169.1, 20.94.

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