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One-pot Cascade Reactions using Fructose-6-phosphate Aldolase: Efficient Synthesis of D-Arabinose-5-phosphate, D-Fructose-6-phosphate and Analogues

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Abstract. One-pot multienzymatic reactions have been performed for the synthesis of 1-deoxy-D-fructose-6-phosphate, 1,2-dideoxy-D-*arabino*-hept-3-ulose-7-phosphate, D-fructose-6-phosphate and D-arabinose-5-phosphate. The whole strategy is based on a fructose-6-phosphate aldolase (FSA) mediated aldol key step as a part of three or four enzymes-catalysed cascade reactions. The four known donors for FSA dihydroxyacetone (DHA), hydroxy acetone (HA), 1-hydroxy-2-butanone (HB) and glycolaldehyde (GA) were used with D-glyceraldehyde-3-phosphate as the acceptor substrate. The target phosphorylated sugars were obtained in good to excellent yields and high purity.

Keywords: D-arabinose-5-phosphate; D-fructose-6-phosphate; phosphorylated sugars; Fructose-6-phosphate aldolase; cascade reactions

Phosphate esters are well established to play a dominant role in the physiology of cells and hence are essential to any organism. They provide the extremely stable backbone for the biopolymers that encode the genetic information (RNA then DNA) and on the other hand, they play an important role for protein regulation that is largely under the control of kinases and phosphatases. They are also involved in the generation, distribution, and application of free energy throughout the cell by using anhydrides of phosphoric acid and its esters, notably adenosine triphosphate.[1]-[3] Thus, phosphate groups are found in a wide variety of structurally diverse natural and biologically active molecules such as phospholipids, nucleic acids, nucleotides, proteins, coenzymes, steroids and in particular carbohydrates. Selectively phosphorylated chiral sugars are of importance as central metabolites in the non-oxidative pentose phosphate pathway, in the Calvin cycle and of particular interest for the study of various metabolic diseases.[4] Given the vital roles for such compounds many methods have been developed for their synthesis. Enzymatic methods ensure mild reaction conditions and furthermore, tedious protection and deprotection of functional groups often resulting in low yields can be circumvented.[5] For the preparative synthesis of monophosphorylated sugars two approaches are mainly found in the literature depending on the enzymes used: (i) by selective phosphorylation of an alcohol function and (ii) by C-C bond formation. The first method is usually completed through the use of kinases albeit they often display narrow substrate specificity. Kinases require ATP as phosphoryl donor which generally needs to be regenerated *in situ* for two main reasons: (i) the resulting ADP is usually a strong inhibitor of the kinase and (ii) for use in large scale applications.[6] As an example, D-arabinose-5-phosphate, an important metabolite involved in the biosynthetic pathway of the outer membrane component lipopolysaccharide (LPS), was prepared *via* a kinase catalysed reaction.[7] Direct phosphorylation of a hydroxyl group using phosphatases or phosphotransferases and inorganic pyrophosphate have also been described by Wever *et al.*[5] In such process the key intermediate in the mechanism is an activated phosphorylated enzyme intermediate that may react with a nucleophile like for example primary alcohol function to yield a phosphorylated product. Water being a competitive nucleophile, the intermediate may also be hydrolysed, resulting in formation of free phosphate. In addition, the main drawback of such methodology relies on the reversibility of these phosphatase catalysed reactions causing dephosphorylation of the newly formed product. Thus reaction progress must be carefully monitored for quantitative production of the desired phosphorylated compound. However efficient

applications were illustrated in the preparative synthesis of glucose-6-phosphate[5a] and dihydroxyacetone phosphate (DHAP).[5b-c] Another alternative is the use of C-C bond formation catalysed by transketolase[4] alone, combined in cascade reaction with an aldolase[8] or catalysed by an aldolase in cascade reaction.[8c] Thus, efficient multi-enzymatic syntheses of D-xylulose-5-phosphate were achieved.[4a],[8b] From the pioneer Fessner's work, non natural 1-phosphate ketoses were synthesised in an artificial metabolic reaction cascade involving *in situ* formation of DHAP.[8a] Numerous methods based on enzymatic reaction cascades were described for the preparation of DHAP, as well as chemical methods.[8c,9] Recently, two thermophilic aldolases (D-fructose-1,6-bisphosphate aldolase (FBA) and deoxyribose aldolase (DERA)) were combined *in vivo* (*E. coli*) for the synthesis of deoxyribose-5-phosphate in an artificial biosynthetic pathway.[10] Also synthases such as D-deoxyxylulose-5-phosphate synthase (DXP synthase)[4h],[11] or D-3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase)[4h,i],[6] were used for the preparation of DXP and DAHP respectively.

Fructose-6-phosphate aldolase (FSA) has been established over the last few years as a robust and powerful biocatalyst for stereoselective C-C bond formation.[12] Thus several efficient syntheses of sugars and analogues were readily described.[12c,13] Nevertheless using FSA as biocatalyst, no synthesis of phosphorylated sugars has been described in the literature to date.

Cascade enzymatic pathways, which allow multi-step conversions to take place in one reaction vessel, are crucial for the development of highly efficient new methods of chemo-enzymatic synthesis.[8],[14] Inspired by natural cascade procedures, we explored two straightforward multi-enzymatic strategies for the synthesis of several sugar monophosphates summarised in Figure 1. In the first one, all the substrates were achirals. Thus the chirality was elegantly introduced by biocatalysed asymmetric synthesis using two enzymes, an isomerase fixing one stereogenic center and an aldolase establishing two contiguous centers.

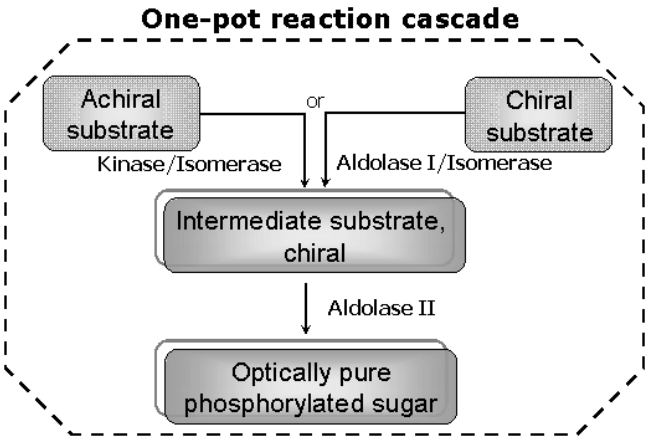
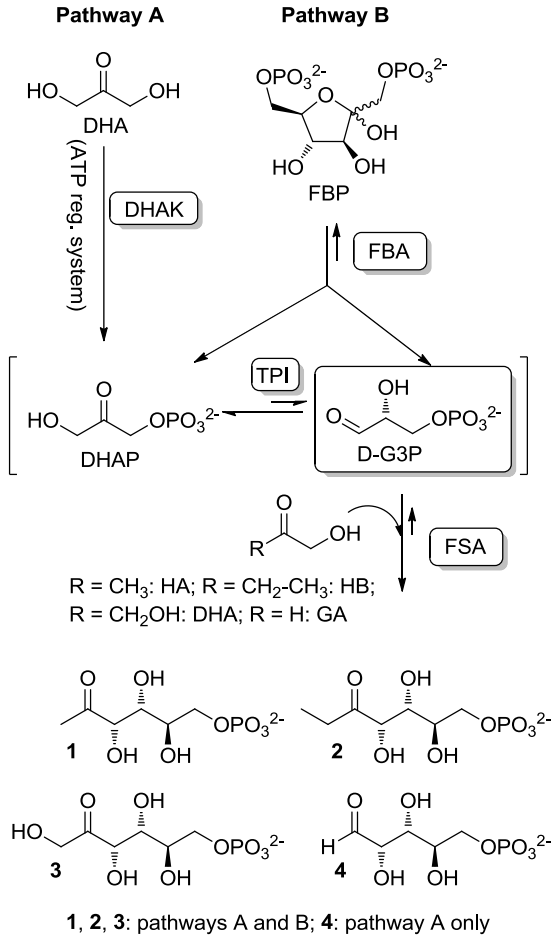


Figure 1: Enzymatic cascade reactions.

In the second one, the asymmetric centers were governed by two aldolases, combined in a multienzymatic process. Like in the biomimetic pathway described by Ohtake and coll.,[10] a dihydroxyacetone phosphate (DHAP) dependent aldolase was associated in tandem with a non-phosphorylated donor dependent one.

The first strategy (pathway A, Scheme 1) consisted in a one-pot two-steps reaction starting from dihydroxyacetone (DHA) in the presence of a cocktail of four biocatalysts. FSA acceptor substrate D-glyceraldehyde-3-phosphate (D-G3P) was obtained *in situ* by isomerisation of DHAP using triosephosphate isomerase (TPI). Interestingly, DHAP was readily synthesised from DHA by direct phosphorylation catalysed by dihydroxyacetone kinase[15] (DHAK) with ATP regenerated *in situ* by the phosphoenolpyruvate (PEP)/pyruvate kinase (PK) system.

The second strategy (pathway B, Scheme 1) differed from the first one by means of D-G3P formation and corresponded to a one-pot one-step reaction. In this case, the acceptor aldehyde and DHAP were generated *in situ* from the split of D-fructose-1,6-*bis*phosphate (FBP) catalysed by FBA .



Scheme 1: Enzymatic cascade reactions using fructose-6-phosphate aldolase (FSA) with *in situ* generated D-glyceraldehyde-3-phosphate as the acceptor substrate.

Hence, one-pot multienzymatic reactions (pathway A or B) have been set up for hundred mg scale synthesis of 1-deoxy-D-fructose-6-phosphate (**1**), 1,2-dideoxy-D-*arabino*-hept-3-ulose-7-phosphate (**2**), D-fructose-6-phosphate (**3**: D-F6P) and D-arabinose-5-phosphate (**4**: D-A5P) respectively from hydroxyacetone (HA), hydroxybutanone (HB), dihydroxyacetone (DHA) and glycolaldehyde (GA) as donors. Reaction with GA was not considered following pathway B. Indeed, GA was reported to be a good acceptor substrate for FBA that would lead to the formation of D-xylulose-1-phosphate[16] as by-product.

As the common substrate for each target compound was D-G3P, we first started with the study of FSA in the presence of this enantiopure aldehyde. The best acceptor substrate of FSA reported to date was the racemic D,L-G3P as indicated by the following kinetic parameters: K_M=0.80 mM; k_{cat}=173 s^{-1}, k_{cat}/K_M=216s^{-1}.mM^{-1}).[13a] Those measured in this work for D-G3P were found relatively close to the above cited: K_M=0.56 mM; k_{cat}=195 s^{-1}, k_{cat}/K_M=348 s^{-1}.mM^{-1}. The FSA catalytic efficiency (k_{cat}/K_M) is slightly higher for D-G3P than for the racemic substrate.
This aldehyde is known to be quite unstable under different conditions of pH and temperature.[4a-d],[10] Thus, for optimal reaction conditions determination for the one-pot multi-enzymatic system, stability of D,L-G3P was tested (see supplementary materials). As the results have shown that glycylglycine buffer usually employed for FSA over-expression [12] was really unsuitable, it was displaced in the FSA preparation by water through gel filtration. FSA in water displayed a good stability at 4ºC, retaining close to 100% of its initial activity after 1 month. Finally, TRIS-HCl buffer, which is known to be compatible with all the enzymes involved, was chosen for the cascade enzymatic reactions.

We have then focused our work on the formation of D-G3P at a corresponding hundred mg scale. Chemical syntheses are tedious, giving low yields[17] or requiring laborious purification steps.[10],[18] Consequently, we decided to investigate described enzymatic routes. Two methods were selected: (i) one published by Whitesides[19] where DHAP[8c,9] was isomerised to D-G3P using TPI (pathway A) and (ii) using FBP as starting material and the sequential action of FBA and TPI (pathway B).[8],[10],[20] Initially, we have chosen to optimize the one-pot two-steps strategy (pathway A) based on DHAP accumulation before its transformation into D-G3P. In fact, the reaction parameters were controlled more easily as these two steps were independent. The equilibrium of the isomerisation reaction is in favour of DHAP (96%),[21] thus the formation of D-G3P had to be directly coupled with the FSA catalysed aldol reaction in order to obtain the best yields.

Pathway A:

Despite the fact that in our lab we were used to produce DHAP chemically or enzymatically,[21] the protocol described by García-Junceda's group using DHAK for enzymatic phosphorylation of DHA was chosen for its ease of handling and final high yield and purity of DHAP.[15] Nevertheless, the regeneration of ATP based on acetate kinase, was changed and replaced for the phosphoenolpyruvate (PEP)/pyruvate kinase (PK) system. This latter system displays two interesting advantages for large scale biosynthesis of DHAP: (i) PEP is a more stable phosphate donor than the originally used acetylphosphate, and (ii) PK is a cheaper enzyme than acetate kinase. Applying the best determined 1.0/0.8 DHA/PEP substrate ratio (see supplementary materials), DHAP could be synthesised in 95% yield, corresponding to a 1.4 g scale. Thus, a one-pot two-steps reaction was carried out where DHAP was accumulated prior to the addition of TPI and FSA in order to complete DHA consumption. Indeed, a one-pot one-step reaction was not considered to avoid FSA catalysed DHA conversion, as a side reaction.

For each FSA donor substrate, the time course of the aldolisation reaction was spectrophotometrically monitored by either following monosaccharide appearance or DHAP disappearance, using GPDH (α-glycerophosphate dehydrogenase)/TPI NADH dependent assay with or without FSA respectively (see supplementary materials).

By initially adding three equivalents of the donor substrate, equilibrium was rapidly reached in favour of the desired sugar. Indeed, a mixture of mono-saccharide/DHAP (ratio from 90/10 to 95/5) was obtained and confirmed by NMR spectroscopy, depending on the donor used. To completely shift this equilibrium, we decided to increase the quantity of donor by portion wise addition. Thereby, after adding a total amount of 6 to 12 equivalents depending on the donor substrate, no DHAP could be detected by both the spectrophotometric assay and the NMR spectra analysis, revealing a DHAP total conversion (see supplementary materials).

Then, we performed the reactions at a hundred mg scale varying the donor substrate but using the same quantity (w/w) of FSA (13U) or FSA A129S (optimised for DHA donor substrate: 50U).[12b] For completion, the reactions were run for 30 minutes or 1.5 h (Figure 2, pathway A). HB being the worst donor substrate as shown in figure 2, the reaction with this donor was slower than in the presence of other donors. When assaying for sugar concentration by retroaldolisation, 100% analytical yield was reached for the phosphorylated sugar formed, except when GA was used as donor where only traces or no D-A5P could be detected. This result could be due to the large excess of GA used, as mentioned above. When using 9 equivalents of GA, FSA has also catalysed GA self-addition to give D-threose as by-product. As this compound was known to have a slightly higher affinity for FSA (K_M=0.3 mM) compared to D-A5P

(K_M=0.56 mM),[13a] probably a competition in favour of D-threose (present in higher concentration than Ara-5-P) could occur in the active site, hampering the retroaldolisation of D-A5P, which disturbs the assay.

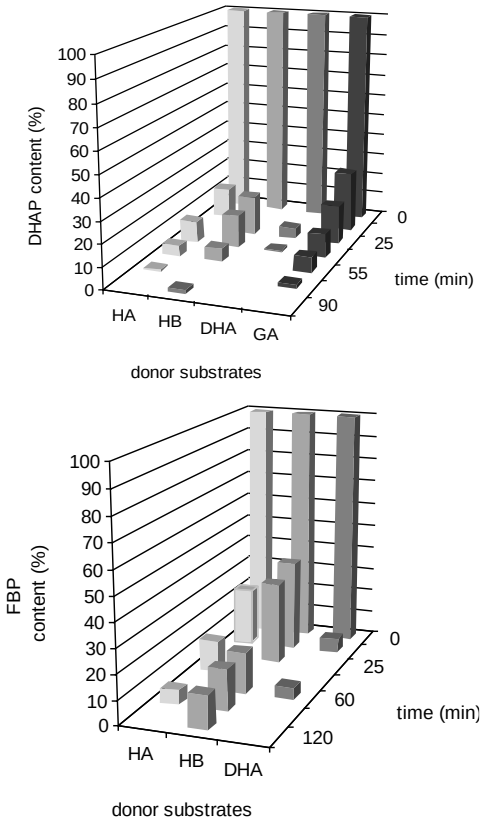


Figure 2. FSA mediated reaction progresses with the four tested donors and either DHAP (above, pathway A) or FBP (below, pathway B) as the source of D-G3P acceptor substrate. For method A, DHAP disappearance was monitored using a spectrophotometric GPDH/TPI assay while for method B, FBP disappearance was monitored using a spectrophotometric FBA/GPDH/TPI assay.

Pathway B:

Following the same set of conditions for pathway A, *i.e.* using 3 to 12 equivalents of donor substrate, reactions were carried out from FBP as the source of D-G3P and using FBA, TPI and FSA concomitantly. Reaction progress was monitored by assaying FBP upon time using a discontinuous spectrophotometric assay with FBA, GPDH and TPI. For the 3 reactions tested with HA, HB and DHA (Figure 2, pathway B), FBP consumption reached 95, 90 and 95% respectively after 90, 120 and 60 minutes respectively. Again, HB revealed to be the worst donor substrate of the three tested. Reaction times were lower in pathway A than in pathway B because in the former DHAP is directly converted to D-G3P by mean of TPI, whereas in the latter the FBP retroaldolisation catalysed by FBA is required before the isomerisation step. Despite HB was not fully converted, compound 2 was not contaminated by FBP as attested by NMR spectra. We would propose that a sequential barium phosphate precipitation would have occurred due to structurally different phosphate groups.

The results from both methods are summarised in table 1. Four phosphorylated monosaccharides were thus obtained in good to excellent yields. Simple precipitation of the monosaccharides, directly from the reaction mixture as barium salts, has led to high organic purity as depicted by the NMR spectra available in the supplementary information. Purity of salts determined spectrophotometrically in a retroaldol process by assaying D-G3P was found above at least 90%. The new sugars, 1-deoxy-D-fructose-6-phosphate **1** and 1,2-dideoxy-D-*arabino*-hept-3-ulose-7-phosphate **2** were fully characterised for the first time. ^{1}H as well as ^{13}C NMR spectra analysis revealed the number and proportion of the different forms existing in acidic water. Concerning D-F6P and D-A5P, we have found close values of chemical shifts than those from the literature.[22-23] It's noteworthy that the percentages of the α form decreased with the steric hindrance on the anomeric carbon, the bulky groups like ethyl or hydroxymethyl being preferably found in equatorial positions.

Table 1. Yields and structural forms of the monosaccharides phosphates in acidic aqueous solution (pH=1).

sugar	Yield (%)		Composition (%)		
	A	B	β	α	linear
1	95	75	43	23	34
2	94	65	42	12	46
3	80	65	81(82[22])[a]	19(16[22])[a]	0(2.2[22]) [a]
4	87	n.a	41(40[23])[a]	59(58[23])[a]	0(2[23]) [a]

[a] values in brackets are reported data

In conclusion, a new type of enzymatic one pot cascade reaction has been successfully designed, developed and applied to the preparation of four phosphorylated sugars of high biological interest. Compounds **1** and **2** have been reported for the first time in this work. A single precipitation step directly from the reaction mixture afforded the target compounds **1**-**4** as their barium salts in high yields and purities. The two methodologies described herein are complementary. On one hand, pathway A gives access to all target phosphorylated monosaccharides in very good yields but using an ATP regenerating system. On the other hand, pathway B, revealed to be an interesting alternative route, except for the D-A5P synthesis, for its ease of handling and by the commercial availability of FBA. As we could extend these two methods to other substrates and other reactions catalysed by FSA, we believe that this work represents a consequent improvement in enzymatic synthesis of important metabolites and their analogues. Other phosphorylated monosaccharides would be accessible using higher or lower homologous acceptor substrates such as glycolaldehyde phosphate, currently under

investigation in our lab. The further exploration of the synthetic methodology towards selectively phosphorylated and differentially functionalised monosaccharides is not only of interest for central metabolites, but also for remote metabolites having important biological functions. [25]

Experimental Section

Pathway A: the one-pot two-steps reaction were carried out in TRIS-HCl buffer (20 mL, 40 mM, pH 7.5) containing DHA (0.8 mmol), phosphoenol pyruvate (0.64 mmol), $MgSO_4$ (10 mM), DHAK (11 U) and PK (14 U). The reactions were initiated upon addition of ATP (52 μmol). When DHAP accumulation was closed to 95%, 3 eq of donor were added and pH was adjusted to 8.0 with NaOH 1M. 13 U of FSA wt[12a] (or 50 U FSA A129S[12b] when DHA was used as the donor substrate) and 400 U of TPI were finally added. The reaction proceeded at room temperature under gentle agitation (100-200 rpm). The reactions were run for about one hour and more. Every 25 min, 3 eq of donor were added (HA: a total of 9 eq, 55 min reaction time; HB: a total of 12 eq, one hour and a half reaction time; DHA a total of 6 eq, 30 minutes and GA: a total of 9 eq, 70 min reaction time).

Pathway B: Fructose-1,6-*bis*phosphate (0.33 mmol) was placed with FBA from rabbit muscle (250 U), and TPI (1000 U), in TRIS HCl buffer (pH 8.0, 40 mM, 25 mL). Then 6 eq of donor (2 mmol) were added. Once the solution homogenised, the reaction was initiated with 34 units of FSA wt (100 U of FSA A129S with DHA as the donor). The reaction proceeded at room temperature under gentle agitation (100-200 rpm). Every 25 min 6 more eq of donor were added (HA, 18 eq, 90 min of reaction; HB, 24 eq, 120 min of reaction; DHA 12 eq, 60 min of reaction).

General purification protocol: The reactions were stopped by dropping the pH to 3.0 resulting in partial precipitation of the enzymes. The pH was then adjusted to 6.0 and 2 eq of $BaCl_2$, $6H_2O$ were added. The solutions were centrifuged 10 min at 10,000 rpm at 4°C and the pellets were discarded. After partial concentration in vacuo, 5 volumes of ethanol were added. The solutions were let overnight at 4°C and then centrifuged. The sugar barium salts were obtained as white powders after one washing with ethanol followed by two other washings with acetone.

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