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Borate as a Phosphate Ester Mimic in Aldolase-Catalyzed Reactions: Practical Synthesis of L-Fructose and L-Iminocyclitols

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Abstract: Dihydroxyacetone phosphate (DHAP)-dependent aldolases have been widely used for the organic synthesis of unnatural sugars or derivatives. The practicality of using DHAP-dependent aldolases is limited by their strict substrate specificity and the high cost and instability of DHAP. Here we report that the DHAP-dependent aldolase L-rhamnulose 1-phosphate aldolase (RhaD) accepts dihydroxyacetone (DHA) as a donor substrate in the presence of borate buffer, presumably by reversible *in situ* formation of DHA borate ester. The reaction appears to be irreversible, with the products thermodynamically trapped as borate complexes. We have applied this discovery to develop a practical one-step synthesis of the non-caloric sweetener L-fructose. L-Fructose was synthesized from racemic glyceraldehyde and DHA in the presence of RhaD and borate in 92% yield on a gram scale. We also synthesized a series of L-iminocyclitols, which are potential glycosidase inhibitors, in only two steps.

Keywords: aldol reaction; aldolases; borate; iminocyclitols; L-sugars; synthetic methods

Dihydroxyacetone phosphate (DHAP)-dependent aldolases catalyze aldol reactions using DHAP as a nucleophilic donor substrate, and have been synthetically useful due to their ability to form carbon-carbon bonds with high stereoselectivities and enantioselectivities.[1] A major drawback of DHAP-dependent aldolases is their strict donor substrate specificity toward DHAP, and non-phosphorylated dihydroxyacetone (DHA) cannot be utilized by the DHAP-dependent aldolases. The crystal structure of rhamnulose 1-phosphate aldolase (RhaD), which is a class II DHAP-dependent aldolase, has been reported in complex with a DHAP analogue, showing that the phosphate moiety forms hydrogen bonds with four residues in the active site, and is the major determinant for binding affinity between the aldolase and DHAP.[2] This likely explains why unphosphorylated analogues such as DHA are not accepted by these enzymes. Although several chemical[3] or chemo-enzymatic[4] syntheses of DHAP have been described, the high cost and instability of DHAP, as well as the requirement of a phosphatase to remove the phosphate ester from the product, makes these aldolases less than ideally practical.

Previous efforts to overcome the DHAP dependence of aldolases have involved *in situ* formation of arsenate or vanadate esters of DHA, which act as phosphate ester mimics.[5–7] Although arsenate has been used effectively for synthetic applications,[5,6] the high toxicity of arsenate is an issue from a practical standpoint. Vanadate can also be used even at lower concentrations than arsenate, but vanadate may have problematic redox activity and its expense limits its practical utility.[5]

Here we report that using inorganic borate buffer allows DHA to be accepted as a substrate by RhaD aldolase, presumably by reversibly forming a borate ester with dihydroxyacetone (DHA) *in situ*. We have used this discovery to develop a practical, inexpensive one-step synthesis of L-fructose (Figure 1). The unnatural non-caloric sweetener L-fructose has been the target of several chemical[8] and chemoenzymatic syntheses,[9] and we recently reported a one-pot synthesis of L-fructose from DHAP and DL-glyceraldehyde using RhaD aldolase and acid phosphatase.[10] We describe in this communication the development of RhaD-catalyzed one-step synthesis of L-fructose from DL-glyceraldehyde and DHA in the presence of borate on a gram scale. We also developed two-step syntheses of a series of L-iminocyclitols, which are promising as glycosidase inhibitors.

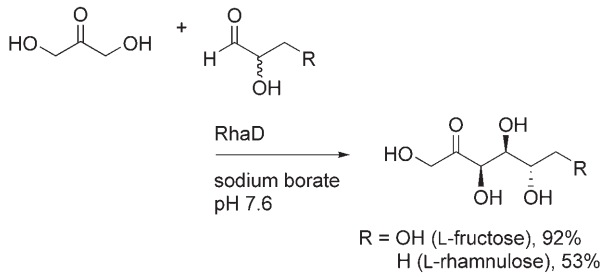

Figure 1. One-step synthesis of L-fructose and L-rhamnulose, from dihydroxyacetone and DL-glyceraldehyde or lactaldehyde using RhaD aldolase in the presence of borate.

His-tagged RhaD aldolase was overexpressed in *E. coli* and purified as reported previously.[11] Alternatively, *E. coli* BL21 (DE3) cells harboring pETDR-haD were used directly as a whole cell biocatalyst without enzyme purification. A typical reaction mixture contained borate buffer (100 mM, pH 7.6), DHA (50 mM), DL-glyceraldehyde (100 mM), and RhaD aldolase. To confirm that this is an aldolase-catalyzed reaction, an omission test was performed under these conditions. The results showed that all components (borate, DHA, glyceraldehyde, and RhaD) were necessary for formation of L-fructose, indicating that this is a borate-dependent RhaD-catalyzed aldol reaction. We believe that inorganic borate reversibly forms esters with DHA *in situ* in aqueous solution, and thus formed DHAB can be accepted by RhaD aldolase as a donor substrate. After the aldol reaction with L-glyceraldehyde to give L-fructose borate ester, the metastable borate esters were readily hydrolyzed during reaction work-up.

The effect of borate concentration on the aldol reaction with constant DHA (50 mM) and DL-glyceraldehyde (100 mM) was examined (Figure 2a). The reaction reached maximum yield at about 200 mM borate. Although this reaction could conceivably be catalytic in borate, excess equivalents of borate were required for increasing L-fructose yield. In the case of the aldolase reaction with arsenate ester, D-fructose 1,6-diphosphate (FDP) aldolase also needed a 6-fold excess of arsenate (120 mM) against 20 mM DHA.[6] The excess borate may play a role in trapping the L-fructose product and preventing the reverse reaction, as described later in this communication. From a practical standpoint, sodium borate is a common and inexpensive buffer, and the one-step synthesis with RhaD from DHA can be performed simply by replacing traditionally used buffers with sodium borate. Next, DHA concentrations were varied at constant concentration of 100 mM DL-glyceraldehyde and 200 mM borate (Figure 2b). L-Fructose production was increased as the initial DHA concentration increased, and reached maximum at 4 equivalents of DHA relative to L-glyceraldehyde.

A gram-scale synthesis of L-fructose was performed under the optimized reaction conditions, resulting in an isolated yield of 92% L-fructose based on L-glyceraldehyde (see Experimental Section). We used inexpensive DL-glyceraldehyde instead of enantiomerically pure L-glyceraldehyde as the acceptor substrate. If the enzyme were to accept D-glyceraldehyde as a substrate, the expected product would be D-sorbose. However, D-sorbose was not observed in the crude product NMR analysis. The unreacted D-glyceraldehyde was easily separated from L-fructose product by chromatography.

A one-pot synthesis of L-rhamnulose, the unphosphorylated form of the natural substrate of RhaD, was also performed (Figure 1). *In situ* formation of DL-lactaldehyde by ozonolysis of commercially available 2-hydroxy-3-butene was followed by aldol reaction with DHA in the presence of borate and RhaD, to afford L-rhamnulose in 53% yield after purification.

Enzyme kinetics in the presence of borate were determined in both the forward and reverse direction, and compared to the kinetics for the phosphorylated

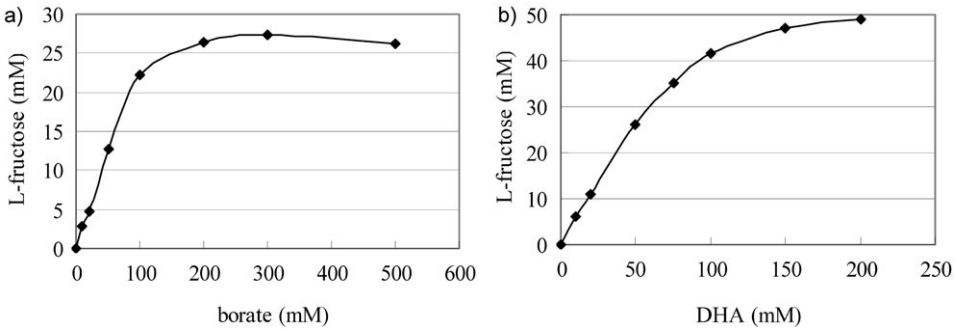

Figure 2. L-Fructose synthesis from DHA and DL-GA. (**a**) Borate-dependent formation of L-fructose; sodium borate buffer (10, 20, 50, 100, 200, 300, or 400 mM, pH 7.6), DHA (50 mM), DL-glyceraldehyde (100 mM). (**b**) DHA-dependent formation of L-fructose; sodium borate buffer (200 mM, pH 7.6), DHA (10, 20, 50, 75, 100, 150, or 200 mM), and DL-glyceraldehyde (100 mM).

substrates (Table 1). The retroaldol activity of RhaD for L-rhamnulose in the presence of 100 mM borate was approximately 60 times lower than for the natural substrate L-rhamnulose 1-phosphate. Moreover, the

Table 1. Activities of RhaD with non-phosphorylated substrates in the presence of borate.

Substrate	V_{max} (μmol/min mg^{-1})	K_m (mM)
L-rhamnulose 1-phosphate	2.2	0.96
L-fructose 1-phosphate	0.71	3.2
L-rhamnulose + borate	0.035	
L-fructose + borate	not detected	
DL-lactaldehyde + DHAP	33	
DL-lactaldehyde + DHA + borate	1.0	
DL-glyceraldehyde + DHAP	23	
DL-glyceraldehyde + DHA + borate	0.48	

retroaldol activity for L-fructose in the presence of borate was undetectable. Increasing the incubation period or the amount of enzyme did not yield the retroaldol products (DHA and L-glyceraldehyde), as checked photometrically, nor by HPLC analysis. These results suggest that L-fructose borate esters are not active substrates for the retroaldol reaction, while DHA-borate can be efficiently accepted by RhaD to go in the synthetic direction. Literature precedent from ^{11}B, ^{13}C NMR studies[12] and thermodynamic studies[13] suggest that D-fructose forms relatively stable 1:1 and 1:2 borate-β-D-fructofuranose complexes. Thus, the L-fructose product may be thermodynamically trapped in such complexes and prevented from undergoing the retroaldol reaction.

Next we applied our method to the facile synthesis of a series of L-iminocyclitols by using azido aldehyde acceptors to efficiently synthesize several L-iminocyclitols (Figure 3). Following known procedures[14] the azido ketone aldol products underwent diasteroselective reductive cyclization to produce the iminocyclitols in only two steps. Recent reports have indicated that the L-enantiomers of known D-iminocyclitol glycosidase inhibitors can also be potent glycosidase inhibitors, acting in a non-competitive mode and with unique specificity profiles.[15] While compound **1** (L-deoxymannojirimycin) is well-characterized, compounds **2**[16] and **3a**[17] and **3b**[15a] have not been extensively studied for inhibitory activity, and **4** has not been reported before. In the case of **3**, RhaD preferentially accepts the D-enantiomer of the aldehyde over the L-enantiomer by a ratio of 2:1, perhaps due to the different steric or hydrogen-bonding constraints. For **4**, only the product from the D-aldehyde was observed. Its structure was assigned by correlation with the known enantiomer of **4**.[18]

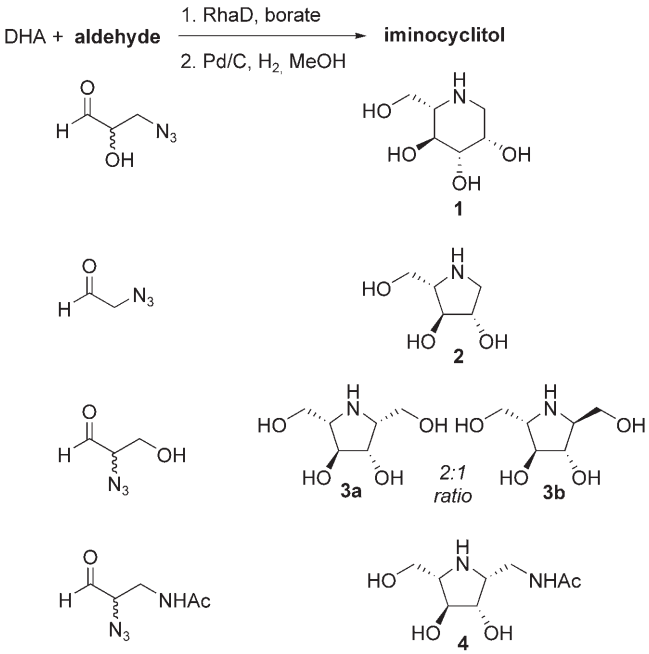

Figure 3. Two-step synthesis of iminocyclitols **1–4** from dihydroxyacetone using RhaD aldolase in the presence of borate, followed by reductive cyclization.

In conclusion, we have discovered that RhaD aldolase accepts DHA as a donor substrate in the presence of borate buffer, presumably by reversible *in situ* formation of DHA borate esters. Borate esters are formed spontaneously and reversibly in aqueous solution, resulting in a simple one-step operation in which the borate esters are formed *in situ*, and hydrolyzed during the work-up. This contrasts with the case of phosphate esters, which have to be prepared and then hydrolyzed in separate steps. In addition to reducing the synthesis to a single step, we have dramatically reduced the cost of producing L-fructose. Whereas DHAP is commercially available for *ca.* $ 2200/gram, DHA costs only $ 0.20/gram. We also applied this method to develop facile two-step syntheses of L-iminocyclitols.

Borate is a known inhibitor of some enzymes, due to esterification of hydroxyl groups of enzyme residues,[19] substrates,[20] or by mimicking tetrahedral transition states.[19,21] However, here we have demonstrated that borate can be used as a phosphate ester mimic for a practical, inexpensive enzymatic synthesis on gram scale. Investigation of new applications of borate esters with other DHAP-dependent aldolases and other classes of phosphate-utilizing enzymes is ongoing.

Experimental Section

Synthetic and analytical procedures, enzyme expression, and kinetics experiments are described in the Supporting Infor-

mation. A typical gram-scale synthesis of L-fructose is described below.

RhaD-Catalyzed Synthesis of L-Fructose in the Presence of Borate

To a solution of DL-glyceraldehyde (1.80 g, 20 mmol) and DHA (3.60 g, 40 mmol) in water (160 mL), 1 M sodium borate buffer (40 mL, pH7.6) and toluene (400 μL) were added, and *E. coli* BL21 (DE3) cells harboring pETDRhaD (2.4 g by wet weight) were suspended. The reaction mixture was shaken at 37 °C for 16 h, and cells were removed by centrifuge. The reaction mixture was passed through a column of Amberlite IR-120 (H^+) resin (50 mL) and then eluted with additional water. The resulting solution was passed through a column of Amberlite IRA-743 resin (120 mL) to remove borate.[22] After evaporation, the mixture was purified using silica gel chromatography with ethyl acetate/methanol/water (40/10/7) as eluent. Fractions containing L-fructose were collected and concentrated to afford the product; yield: 1.66 g (9.2 mmol, 92% based on L-glyceraldehyde). ^{1}H NMR and ^{13}C NMR spectra in D_2O were identical to those of authentic D-fructose spectra and L-fructose spectra (see Supporting Information). NMR samples were allowed to equilibrate for 1 hour in D_2O to allow fructose to reach its equilibrium mixture of furanose and pyranose forms; $[\alpha]_D^{23}$: +93.1° (*c* 3, H_2O).

Supporting information

For spectroscopic and analytical data, synthetic and analytical procedures, enzyme expression, and kinetics experiments, see the supporting information.

Acknowledgements

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