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Simple Enzymatic in situ Generation of Dihydroxyacetone Phosphate and Its Use in a Cascade Reaction for the Production of Carbohydrates: Increased Efficiency by Phosphate Cycling

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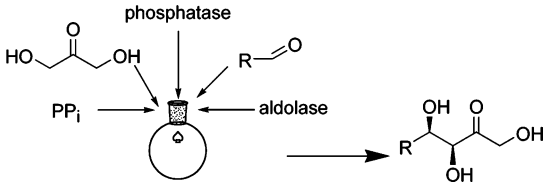

A new enzymatic method for the generation of dihydroxyacetone phosphate (DHAP) using the acid phosphatase from *Shigella flexneri* (PhoN−Sf) and the cheap phosphate donor pyrophosphate (PP_i) is described. The utility of this method was demonstrated in an aldolase-catalyzed condensation carried out in one pot in which DHAP was generated and coupled to propionaldehyde to give a yield of 53% of the isolated dephosphorylated end product.

As a result of the broad substrate tolerance and predictable product stereochemistry, dihydroxyacetone phosphate (DHAP)-dependent aldolases have been frequently utilized in the formation of carbon−carbon bonds[1] of highly functionalized molecules (^{13}C labeled,[2] heterosubstituted,[3] deoxy,[4] fluoro,[5] and high carbon sugars[6]) from simple precursors. Additionally, this enzymatic approach has been extended to a variety of non-carbohydrate natural products.[7] Several synthetic procedures of DHAP precursors have been developed based on protected DHAP dimers,[8] DHAP dimethylacetals,[9] a cyclic DHAP dimethyl acetal,[10] or benzyl-protected DHAP.[11] Many of these chemical procedures are low yielding and complicated by multistep purification procedures, unstable intermediates, and/or expensive toxic reagents. An enzymatic method would obviate the need for protection and deprotection steps inherent in chemical phosphorylation. Enzyme-catalyzed reactions are often compatible with each other, and this makes it, in principle, possible to combine several enzymes in a one-pot, multistep reaction sequence. The easiest enzymatic method is the in situ formation of 2 equiv of DHAP from fructose-1,6-bisphosphate (FDP) by FDP−aldolase and triose phosphate isomerase,[1a,12] but incomplete conversion complicates isolation of products. Other methods are based on the phosphorylation of dihydroxyacetone (DHA) using either glycerol kinase[2,13] or recombinant DHA kinases,[14] which need in situ regeneration of ATP. Oxidation of L-glycerol phosphate by glycerophosphate oxidase (GPO)[15] also results in DHAP. L-Glycerol phosphate can be generated by phosphorylation of glycerol using glycerol kinase.[13a,16] It is also possible to phosphorylate glycerol by phytase using cheap PP_i as a phosphate donor;[17] however, racemic glycerol phosphate results and D-glycerol phosphate is not oxidized by GPO.

Recently, we reported that the acid phosphatase from *Shigella flexneri* (PhoN−Sf) is able to phosphorylate a wide variety of alcoholic compounds regioselectively using cheap PP_i as phosphate donor.[18] Here we describe a new and easy method for the generation of DHAP in which DHA is phosphorylated by the phosphatase and PP_i. The in situ generated DHAP can be coupled to an aldehyde in an aldolase-catalyzed condensation using rabbit muscle aldolase (RAMA), resulting in a C−C

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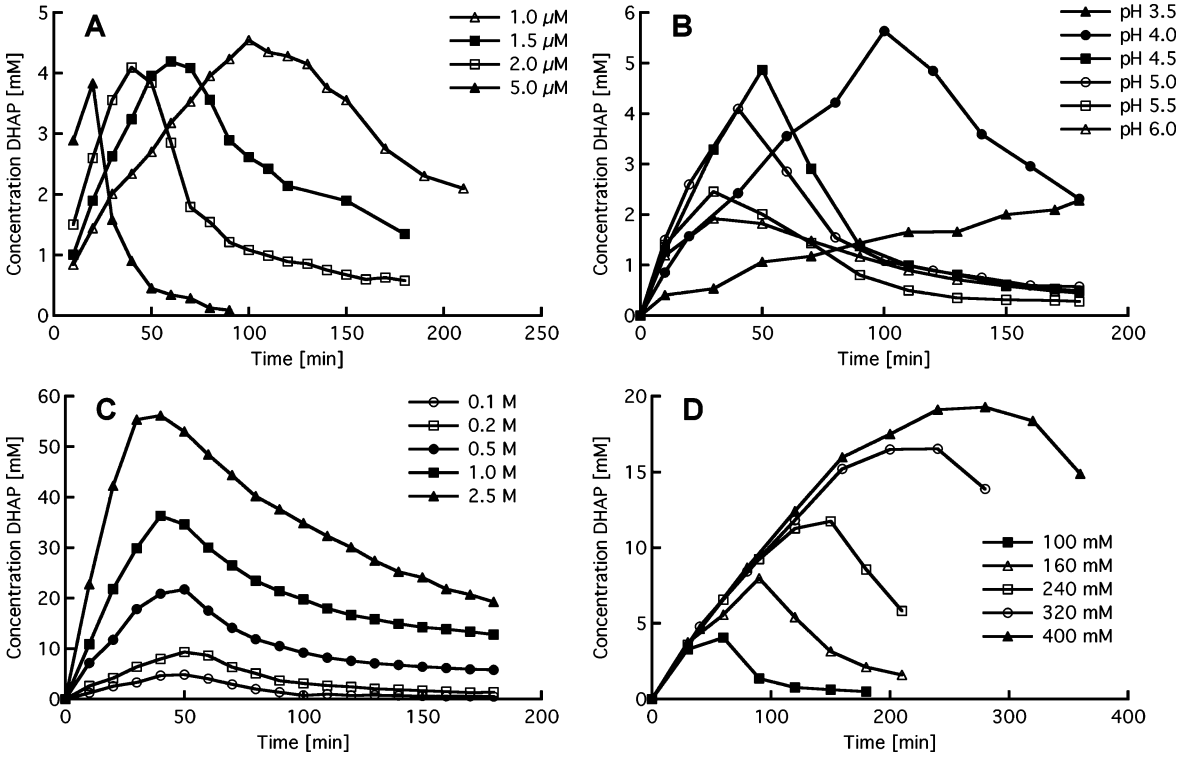
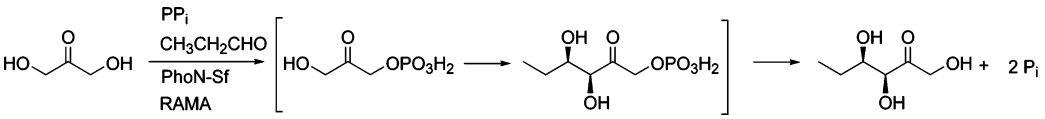


FIGURE 1. Optimization of DHAP formation. Standard reaction mixture contains 100 mM PP_i, 100 mM DHA in 100 mM acetate buffer at 30 °C. The reaction was started by addition of 2 μM (54 μg/mL) PhoN−Sf. DHAP concentration was determined by the coupled assay as described in the Supporting Information. (A) PhoN−Sf concentration dependency at pH 5.3. (B) pH dependency. (C) DHA concentration dependency at pH 4.5. (D) PP_i concentration dependency at pH 4.5.

SCHEME 1. One-Pot Reaction: Formation of Dephosphorylated Aldol Adduct from DHA, PP_i, and Propionaldehyde



coupled product with two new stereocenters with high stereospecificity (Scheme 1).

To find the optimal conditions for DHAP formation, several parameters were varied as depicted in Figure 1. Time-dependent optima are observed since, after the consumption of the PP_i, DHAP will be dephosphorylated by the phosphatase. Panel A shows the effect of the phosphatase concentration. Varying the PhoN−Sf concentration between 1 and 5 μM (27−135 μg/mL) yielded maximum DHAP concentrations around 4.3 mM between 110 and 20 min, respectively. Considering reaction time, a concentration of 2 μM (54 μg/mL) PhoN−Sf was used in further studies. The phosphorylating activity of PhoN−Sf is highly dependent on pH, as is shown in panel B. The highest DHAP concentration (5.5 mM) was reached at pH 4 after 100 min of incubation. Increasing the pH increased the initial rate of DHAP formation, but the decomposition of DHAP due to hydrolysis was also accelerated. At pH 6, only 2 mM of DHAP was formed. At pH 3.5, the transphosphorylation reaction was considered too slow to be practically viable, also because this pH deviates from the aldolase pH optimum. This pH dependency has also been observed in the phosphorylation of inosine to 5′-inosine monophosphate by PhoN−Sf.[18a] Combining yield and reaction time, pH 4.5 was considered as starting point for further optimization. As shown in panel C, the DHAP yield increases with higher initial DHA concentrations. In a reaction mixture containing 100 mM PP_i, the yield increases from 5 to 67 mM DHAP with initial DHA concentrations ranging from 0.1 to 2.5 M, respectively. From the initial rate of the reaction versus the DHA concentration (not shown), it was possible to obtain the kinetic constants. The K_m value was 3.6 M, and the k_{cat} was 40 s^{-1}. The fourth optimization factor is the effect of the PP_i concentration (panel D). Increasing the PP_i concentration causes a further increase in the DHAP formation. In a reaction mixture containing 100 mM DHA, 100 mM PP_i is converted to 5 mM DHAP and a maximal concentration of 19 mM DHAP is reached at a concentration of 400 mM PP_i.

Combining the optimization factors increases the DHAP concentration considerably. A reaction mixture containing 500 mM DHA and 240 mM PP_i at pH 4.5 and 2 μM (54 μg/mL) PhoN−Sf resulted in 52 mM of DHAP. Addition of a second portion of 240 mM PP_i after 100 min pushed the yield to 104 mM of DHAP (Figure 2).

To demonstrate the usefulness of this DHAP generating system in the synthesis of carbohydrates and other compounds, an aldolase (RAMA) was added in the presence of 100 mM propionaldehyde. The formation of DHAP under optimal conditions, as determined above, was started by the addition of PhoN−Sf and followed by ^{31}P NMR (Figure 3). After 30 min, RAMA was added, resulting in the formation of the phosphorylated aldol adduct to a maximal concentration of 33 mM. Addition of both the phosphatase and aldolase at the same time minimizes the dephosphorylation of the formed DHAP by

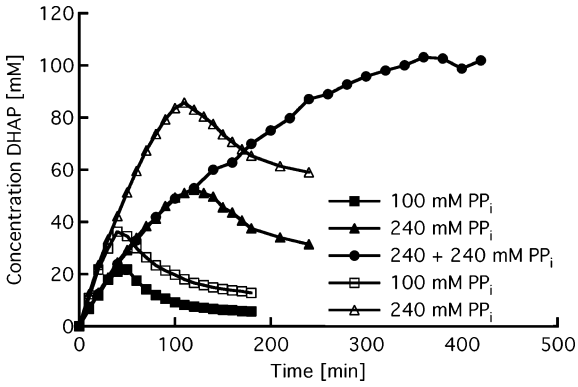


FIGURE 2. Combination of the optimization factors. Reaction mixtures contain 0.5 M DHA (closed symbols) or 1 M DHA (open symbols) and varying concentrations of PP_i in 100 mM acetate buffer (pH 4.5) at 30 °C. The reaction was started by addition of 2 μM (54 μg/mL) PhoN−Sf. The second PP_i portion (240 mM) was added after 100 min (0.5 M DHA, 240 mM PP_i).

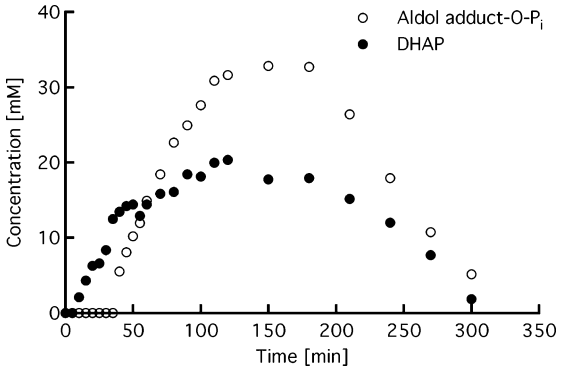


FIGURE 3. ^{31}P NMR analysis of a one-pot phosphorylation, aldol coupling, and dephosphorylation reaction. Reaction mixture contains 240 mM PP_i, 500 mM DHA, and 100 mM propionaldehyde in 100 mM acetate buffer (pH 4.5). The reaction was started by the addition of 2 μM (54 μg/mL) PhoN−Sf. After 30 min, 10 units of RAMA were added.

converting it directly into the aldol adduct and results in an increased yield. This can be explained by comparison of the K_m values for DHAP of both enzymes. RAMA has a K_m value of 49 μM,[19] whereas PhoN−Sf has a K_m value of 0.5 mM (data not shown), demonstrating a higher affinity of the aldolase for DHAP compared to the phosphatase.

To further optimize the reaction, an experiment was carried out in which RAMA was allowed to react with propionaldehyde and DHAP (commercially obtained). After reaction in which >95% of the phosphate from DHAP was transferred to the aldol adduct, the reaction mixture was split into three parts for further incubations. To the three incubations was added phosphatase, to incubations two and three was added additional aldehyde, and to incubation three was added DHA. The concentrations of the dephosphorylated aldol adduct were measured in all three incubations. To our surprise, the concentration of aldol adduct in the third incubation was significantly higher (2.3 times) compared to incubations one and two. Similar results were also obtained using butyraldehyde (1.6 times). This shows that the phosphate group from the aldol adduct can be transferred to DHA, which becomes DHAP and is subsequently used again in the aldol coupling. Scheme 2 illustrates this enzymatic phosphoryl transfer. During phosphorylation and formation of the aldol adduct, several phosphate transfer reactions are thought to occur at the same time.[18a] (1) The phosphatase reacts with PP_i to produce a binary PP_i−enzyme complex, which (2) dissociates to yield an activated phosphorylated enzyme intermediate (E_1·P). (3) Reaction of this complex with water would result in hydrolysis of the complex, but (4) the E_1·P intermediate may also transfer the phosphate group to an acceptor (in this case DHA), which (5) dissociates to form E_1 and DHAP. In the next step, the aldol condensation occurs, and DHAP is coupled to an aldehyde by RAMA to form the phosphorylated aldol adduct Z−P. As the reaction proceeds in time, PP_i becomes exhausted and dephosphorylation of Z−P occurs via (re)formation of the phosphorylated enzyme intermediate which reacts with water (hydrolysis). Our experiments show that the phosphate group from the aldol adduct (Z−P) hydrolysis is used to phosphorylate another DHA molecule when present in excess to generate more DHAP, which is used in aldol coupling. As described above, hydrolysis proceeds via the E_1·P intermediate, which is the central intermediate used in the phosphorylation. In other words, the phosphate group is transferred more than one time, and higher yields can be obtained.

A 10 mL 100 mM propionaldehyde reaction was carried out. Three portions of 240 mM PP_i were sufficient to convert >95% of the aldehyde to aldol adduct. Dephosphorylation was finished overnight. The product was isolated in 53% yield and was identical to 5,6-dideoxy-D-*threo*-2-hexulose.

When we compare this new one-pot method of in situ generation of DHAP in combination with an aldolase-coupling reaction with the method using phytase to phosphorylate glycerol and subsequent oxidation to DHAP by GPO,[17] a number of advantages can be seen. (1) Only two enzymes are necessary compared to four used in the phytase method. (2) In this new method, pH switching is not necessary. The phytase phosphorylation is carried out at pH 4.0, whereas the oxidation has to be performed at pH 7.5. Further, the dephosphorylation of the product needs a second pH switch back to pH 4.0. (3) No dilution of the reaction mixture to lower the glycerol concentration to obtain sufficient GPO activity is necessary in our method. (4) The glycerol phosphate formed is racemic in the phytase method, and since D-glycerol phosphate is not converted by GPO, only 50% of the phosphorylated product is used. (5) In our method, increased yields can be obtained due to cycling of the E−P intermediate when sufficient aldehyde and DHA are present.

We conclude that this cascade reaction provides an attractive enzymatic procedure in which DHAP is generated in situ from DHA and cheap PP_i in an easy way and which can be coupled to aldol reactions with DHAP-dependent aldolases. Advantage is taken of the two-way action of the phosphatase. First it catalyzes the simple phosphorylation of DHA, avoiding the problems with chemical phosphorylation, and second it dephosphorylates the aldol adduct, avoiding nonenzymatic dephosphorylation which, in general, may cause decomposition of the products. Although we used only propionaldehyde and butyraldehyde as substrates for RAMA, the enzyme accepts many other natural and unnatural aldehydes.[1a] When other aldolases are used, such as L-fuculose 1-phosphate aldolase and L-rhamnulose 1-phosphate aldolase, other stereoisomers can be generated. Thus our new method may lead to the synthesis of a wide array of compounds that up to now could only be synthesized via a number of steps. A drawback to our method may be the large

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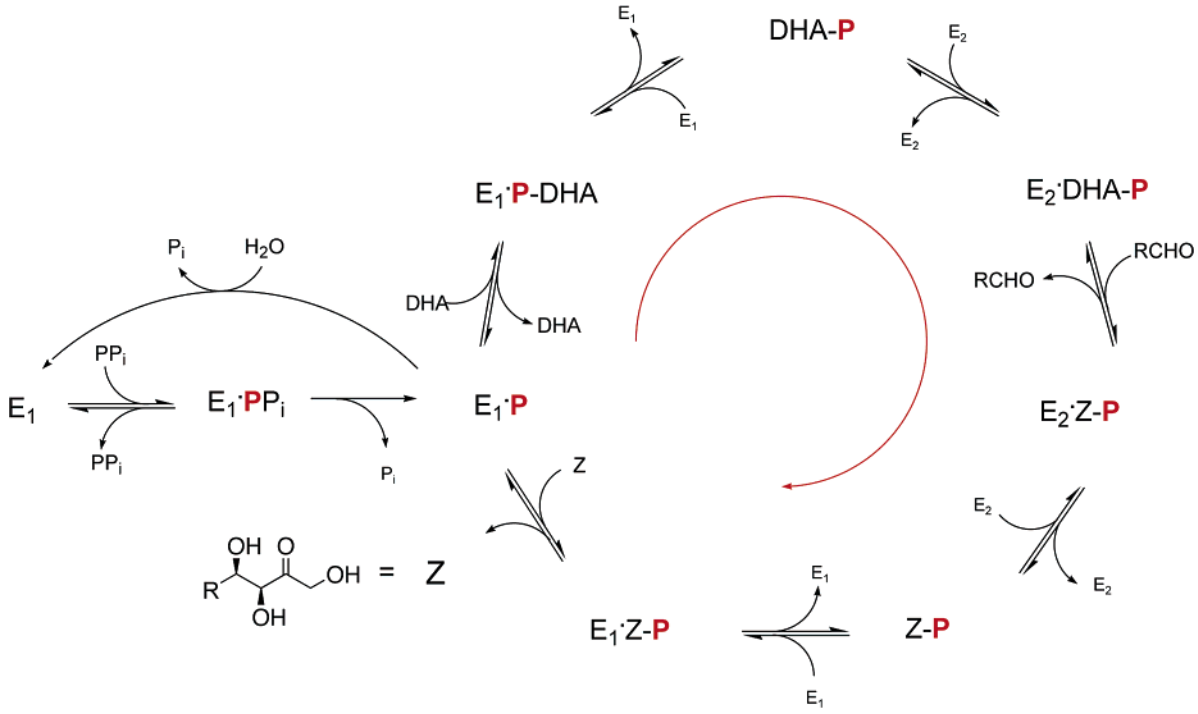
SCHEME 2. Enzymatic Phosphoryl Transfer Reactions in the Formation of Aldol Adducts[a]



[a] E_1 = PhoN; E_2 = RAMA/FruA; P_i = inorganic phosphate; R = alkyl, aryl, etc.; Z = aldol adduct.

amounts of DHA and P_i that are present during purification. However, according to the Merck Index,[20] several more polar and charged carbohydrates are soluble in methanol allowing further purification. Currently, we are performing directed evolution on the phosphatase to increase the affinity for DHA, so that, in principle, much lower concentrations of DHA and PP_i are sufficient to keep the phosphate transfer cycle going and purification of the aldol adduct may be easier.

Experimental Section

^{31}P NMR. Phosphorylated products, PP_i, and free phosphate (P_i) were quantified by phosphor nuclear magnetic resonance at 202 MHz using a 10 mm ^{15}N−^{31}P probe. Chemical shifts (δ) are expressed in parts per million relative to 85% phosphoric acid. At zero time, a spectrum was taken from the reaction mixture containing 240 mM PP_i, 500 mM DHA, 100 mM propionaldehyde, and 100 mM sodium acetate buffer (pH 4.5) in a 10 mm NMR tube at 30 °C in a total volume of 2.75 mL. The reaction was initiated by addition of 2 μM (54 μg/mL) PhoN−Sf. After 30 min, 10 units of RAMA were added. Concentrations of product and reactants were determined using 50 mM dimethylmethylphosphonate (DMMP) in deuterated water as an external standard, which was coaxially inserted in the NMR tube.

Phosphate Recycling Experiment. Sixteen units of RAMA were added to a 2 mL solution containing 300 mM DHAP and 300 mM aldehyde (either propion- or butyraldehyde) in 200 mM acetate buffer (pH 6) at 30 °C. After 5 h, another portion of 300 mM aldehyde was allowed to react overnight. Over 95% of DHAP was converted to the phosphorylated aldol adduct at this time. The mixture was split in three parts of 0.5 mL. The first portion was completed with 2 μM (54 μg/mL) of PhoN−Sf. To the second portion were added 2 μM of PhoN−Sf and 300 mM of aldehyde. The third reaction was completed with 2 μM of PhoN−Sf, 300 mM of aldehyde, and 500 mM of DHA. The volumes were adjusted to 1 mL, and 4 units of RAMA were added. After 5 h, again, 300 mM of aldehyde was added to portions 2 and 3. Reactions were allowed to react overnight and were analyzed by HPLC for amounts of dephosphorylated end product. A RAMA background reaction (500 mM DHA, 2 times 300 mM aldehyde, and 8 units of RAMA) accounted for only 3% of the total conversion.

Isolation and Characterization of Aldol Product 5,6-Dideoxy-D-*threo*-2-hexulose. To a 10 mL solution containing 500 mM DHA, 240 mM PP_i, 100 mM propionaldehyde, and 36 units of RAMA in 100 mM acetate buffer (pH 5.0) at 30 °C was added 2 μM (54 μg/mL) PhoN−Sf. After 2 and 4 h, additional PP_i was supplemented (0.53 g, 240 mM), and the pH was adjusted to 5.0 with 5 M NaOH. Dephosphorylation to the end product was complete after 24 h. The solution was freeze-dried, and the residue was extracted with methanol (overnight). Remaining phosphate salts were filtered off, and the filtrate was concentrated in vacuo. Purification was performed by silica gel column chromatography (ethyl acetate: methanol = 19:1). Product was obtained as a slightly yellow oil (79 mg; 53% based on propionaldehyde, which was shown to be >95% pure by HPLC). The isolated product was characterized[1a] by ^{1}H and ^{13}C NMR. Mass spectrometry was carried out on a JEOL JMS-SX/SX 102 A Tandem Mass Spectrometer using Fast Atom Bombardment (FAB+), (*m*/*z*): $[M + Na]^+$ calcd for $C_6H_{12}O_4Na$, 171.146; found, 171.05.

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Supporting Information Available: General experimental details, ^{31}P and ^{1}H NMR spectral data, and HPLC traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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