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Activated α,β-Unsaturated Aldehydes as Substrate of Dihydroxyacetone Phosphate (DHAP)-Dependent Aldolases in the Context of a Multienzyme System

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Abstract: The utility for carbon-carbon bond formation of a multienzyme system composed of recombinant dihydroxyacetone kinase (DHAK) from *Citrobacter freundii*, the fructose bisphosphate aldolase from rabbit muscle (RAMA) and acetate kinase (AK) for adenosine triphosphate (ATP) regeneration has been studied. Several aldehydes with great structural diversity, including three α,β-unsaturated aldehydes, have been analysed as acceptor substrates. It was found that α,β-unsaturated aldehydes bearing an electron-withdrawing group in the β position to the double bond with a *trans* configuration are good acceptors for RAMA in this multienzyme system. The aldol reaction proceeds with excellent D-*threo* enantioselectivity and the aldol adduct is obtained in good overall yield. The L-*threo* and D-*erythro* enantiomers are also accessible from rhamnulose 1-phosphate aldolase (Rha-1PA) and fuculose 1-phosphate aldolase (Fuc-1PA) catalysed reactions, respectively.

Keywords: aldol reaction; aldolases; biotransformations; C–C bond formation; multienzyme processes

Introduction

Aldolases have been extensively used in chemoenzymatic syntheses because of their ability to catalyse the formation of C–C bonds with a high degree of stereochemical control. The main group of aldolases is the one that uses dihydroxyacetone phosphate (DHAP) as donor.[1] Their major drawback is their strict specificity for the donor substrate. Besides the limitations imposed by this fact, DHAP is expensive to be used in large-scale synthesis and labile at neutral and basic pH. Efforts to overcome the DHAP-dependence of aldolases have involved the *in situ* formation of arsenate or borate esters of dihydroxyacetone (DHA) which act as phosphate ester mimics,[2] the modification of the substrate specificity of aldolases by *in vitro* enzyme directed evolution strategies[3] and the use of newly discovered enzymes.[4] In addition to these efforts, an efficient method of DHAP preparation is still essential and several chemical and enzymatic routes for DHAP synthesis have been described.[5]

A straightforward strategy for DHAP preparation is the kinase-catalysed DHA phosphorylation, using ATP as phosphoryl donor. This approach was first described in 1983 by Wong and Whitesides using the enzyme glycerol kinase.[6] ATP-dependent DHA kinases have also been used for the simple and efficient preparation of DHAP. Itoh et al.,[7] have shown that DHAK isoenzyme I, from *Schizosaccharomyces pombe* strain IFO 0354, is a useful biocatalyst for the production of DHAP.

Our research group has described a multienzyme system for one-pot C–C bond formation,[8] based on the use of a recombinant ATP-dependent DHAK from *Citrobacter freundii* CECT 4626[9] for *in situ* DHAP formation and fuculose 1-phosphate aldolase (Fuc-1PA) for the catalysis of the aldol reaction. The multienzyme system was completed with the *in situ* regeneration of ATP from acetyl phosphate catalysed by acetate kinase (AK). This regeneration system allows the use of ATP in catalytic amounts and avoids the accumulation of ADP, which is a strong inhibitor of the DHAK activity.

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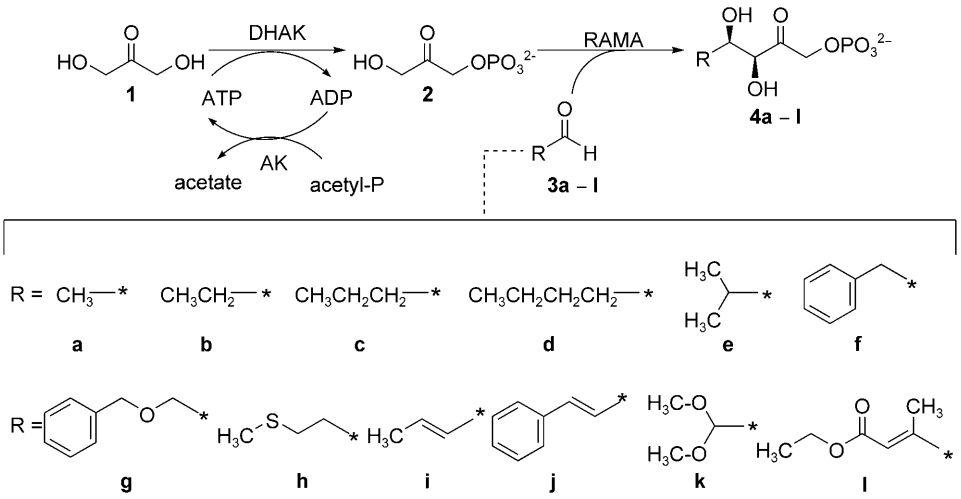

Scheme 1. Multienzyme system for the facile aldol addition reaction between DHAP – *in situ* obtained by enzymatic phosphorylation of DHA – and different aldehydes, catalysed by RAMA.

In our aim to broaden the scope of the multienzyme system, we describe here its use with fructose 1,6-bisphosphate aldolase from rabbit muscle (RAMA) for the catalysis of the aldol reaction of aldehydes with diverse chemical structures as acceptor substrates (Scheme 1). Among the aldehydes assayed three α,β-unsaturated aldehydes (compounds **3i**, **3j** and **3l**) were included although it has been described that these aldehydes are not substrates of RAMA.[10]

Interestingly, ethyl 3-methyl-4-oxocrotonate (**3l**) was efficiently accepted as substrate by RAMA in the context of this multienzyme system. Therefore, we focused on the study of the reaction with this aldehyde and explored if it is also a substrate of l-fuculose 1-phosphate aldolase (Fuc-1PA) and l-rhamnulose 1-phosphate aldolase (Rha-1PA). Structure identification of the three aldol products obtained and the study of the acceptor influence on the steric outcome of the aldolase-mediated reactions are reported.

Results and Discussion

Optimization of the Multienzyme System with RAMA

In our previous paper,[8] we described the performance of our multienzyme system employing a one-pot/one-step strategy. In this approach, all the necessary components for the enzymatic phosphorylation of DHA and the aldol addition are present at the beginning of the reaction. The main feature of this process is that the DHAP must be formed at the same rate as it is consumed by the aldolase.[8] Two conditions are necessary to ensure that this multienzyme system functions optimally: (i) the pH must be maintained at 7.5 and (ii) the aldolase/kinase activity ratio must be adjusted to avoid the accumulation of DHAP. In spite of the advantages of using acetyl phosphate as phosphoryl donor, the activity of the AK is sensitive to changes in pH. Thus, continuous maintenance of the pH at 7.5 was needed to ensure the optimal functioning of the system. Since DHAP is unstable at pH 7.5, delicate adjustments of the activities of aldolase and/or DHAK were necessary to prevent the accumulation of DHAP and to minimise its non-enzymatic degradation. The optimum RAMA/DHAK activity ratio was calculated using acetaldehyde as acceptor. In our first experiment with this enzyme using a RAMA/DHAK ratio of 1/1.5, there was considerable accumulation of DHAP, but almost no formation of the aldol product after 1 hour. Thus, we decided to increase the RAMA/DHAK activity ratio to 4/1. Under these conditions, 23 μmol DHAP (23%) accumulated and only 9% of the aldol product was formed after 1 hour. At longer reaction times, the accumulation of DHAP increased but the amount of the aldol product remained constant. These results indicated that it was necessary to have an initially high RAMA/DHAK activity ratio and also that the stability of RAMA may be low under the reaction conditions. After several attempts, we established that the initial optimum RAMA/DHAK activity ratio was 11.5/1. Since, the half-life of RAMA was approximately 5 h under the reaction conditions, it was necessary to supply the reaction with an additional aliquot of 12 U of aldolase after 3 h of reaction (see the Supporting Information).

Screening of the Synthetic Applicability of the Multienzyme System. One-Pot/One-Step Approach

Under these conditions, reactions with the selection of aldehydes (**3a**–**3l**, Scheme 1) were carried out in a 0.1 mmol of DHA scale in a one-pot/one-step proto-

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Table 1. Formation of aldols **4a**–**4l** catalysed by the DHAK/RAMA multienzyme system.

	One-Pot/One-Step		One-Pot/Two-Steps	
Aldehyde	Aldol formation [%]	DHA consumption [%]	Aldol formation [%]	DHAP accumulation [%]
3a	53.2	98.0		
3b	7.8	97.4	4.5	95.0
3c	63.3	95.0	61.5	>95.0
3d	52.2	96.0		
3e	10.8	98.2	9.6	95.0
3f[a]	0.0	0.0	58.9	>95.0
3g[b]	70.6	97.1		
3h	32.1	98.0		
3i	1.5	99.4	4.1	95.0
3j[c]	1.7	22.8	3.0	>95.0
3k	70.5	95.1	81.0	>95.0
3l	7.4	61.8	48.7	>95.0

[a] Aldehyde is provided as a 50% dilution in benzyl alcohol (reaction mixture contains 3.3% of benzyl alcohol).
[b] Reaction mixture contains 10% of DMSO.
[c] Reaction mixture contains 5% of DMSO.

col. In this initial screening, yields were not optimized. For some acceptors the RAMA/DHAK activity ratio had to be experimentally optimised. The degree of substrate conversion to product was measured using the retro-aldol activity of the aldolase.[11] The results could be grouped into three categories (Table 1): (i) those where the phosphorylation of DHA worked properly, namely, the percentage of DHA consumption ranged between 95 and 100% and percentage of aldol product formation was over 25% (aldehydes **3a**, **3c**, **3d, 3g**, **3h** and **3k**); (ii) those where the extent of DHA phosphorylation was almost complete (95–100%), but the aldol formation was lower than 25% (aldehydes **3b**, **3e** and **3i**); and (iii) those where the extent of DHA phosphorylation was lower than 90% or even null reaction (aldehydes **3f**, **3j** and **3l**).

As expected, none of the three α,β-unsaturated aldehydes was substrate of the RAMA-catalysed reaction. In the case of aldehyde **3i**, the result obtained – as for other aldehydes belonging to the second category – can be explained because this compound is a poor substrate for the aldolase, however, in the case of aldehyde **3j** and even **3l**, the lack of reaction with the aldolase could be due to the low amount of DHAP formed. In fact, the results belonging to the third category show clearly that the phosphorylation system had not worked properly either because the aldehydes themselves or a component in the reaction medium interfered or inhibited with the DHAK or the AK. In this sense, the presence of benzyl alcohol in the reaction mixture with aldehyde **3f** could explain the results obtained with this substrate.

One-Pot/Two-Step Approach

In order to overcome the failures found in the reactions of the third category, we decided to explore a one-pot/two-steps strategy and apply it also to the second category reactions and to those aldehydes that were in the limit of the first category (aldehydes **3c** and **3k**). With this protocol, only the components for the enzymatic phosphorylation of DHA are added at the beginning of the reaction, allowing DHAP to accumulate. When at least 95% of DHAP was formed, we reduced the pH to 6.8 – at this pH the degradation rate of DHAP decreases significantly (see the Supporting Information) – and then, both aldolase and aldehyde were added to initiate the aldol addition as a second step.

When using this strategy, we were able to restore the functionality of the multienzyme system and, in most instances of the third category reactions, the percentage of the aldol formation increased significantly (Table 1). Thus, aldol **4f** that was unaccessible with the one-pot/one-step approach, can be obtained in good yield using this protocol. However, the most interesting result was the obtained with the activated α,β-unsaturated aldehyde **3l** that in this approach performed as a good substrate for RAMA. On the other hand, the use of the one-pot/two-steps approach produced no significant increase in the percentage of aldol formation in the reactions belonging to the second category, confirming that these aldehydes are poor substrates for RAMA. However, when the aldehyde is a good substrate for RAMA applying the one-pot/two-step approach the yield of aldol can be improved, like in the case of **4k** which yield increased from 70% to 81%.

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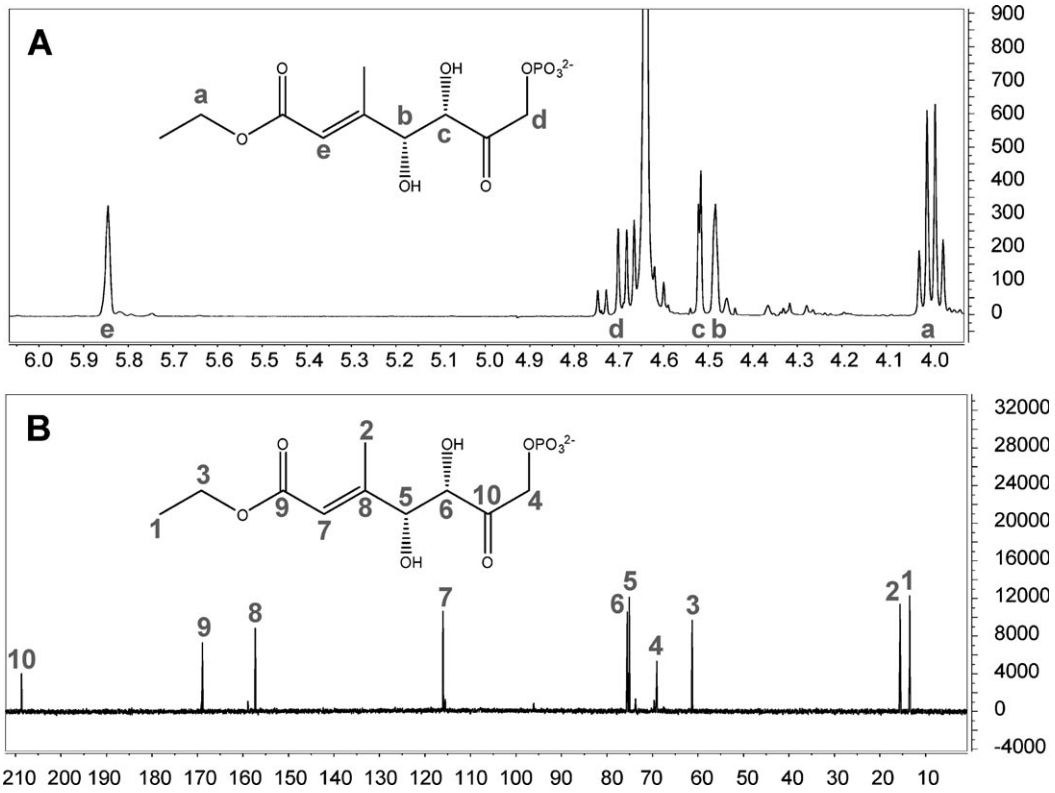

Figure 1. ^{1}H (**A**) and ^{13}C (**B**) NMR spectra of aldol **4l**.

Those reactions that gave a percentage of aldol formation exceeding 25%, were repeated in a 0.3 mmol of DHA scale, the aldol products were purified by precipitation with barium salt[12] and their identity established by NMR analysis. Thus, we could validate that the α,β-unsaturated aldehyde **3l** was accepted as substrate by RAMA in the context of this multienzyme system yielding the aldol product **4l**.

α,β-Unsaturated Aldehyde 3l as Substrate of RAMA

In order to prove the synthetic utility of this approach, we repeated the reaction with aldehyde **3l** in a 3 mmol of DHA scale. In a first attempt, we used proportionally, a lower amount of RAMA than in the reaction at the 0.3 mmol scale. In these conditions we obtained 51.4% of aldol **4l** after 26 h of reaction. Since some degree of RAMA precipitation was observed, it was necessary to add more enzyme along the reaction time. This precipitation was due to the presence of **3l**, since in its absence protein precipitation was not observed. In subsequent reactions, we increased the amount of RAMA and the reaction was completed in 8 h with 60% of aldol product formation. The phosphorylated aldol adduct **4l** was purified by HPLC with 54% overall yield. ^{1}H and ^{13}C NMR of the purified aldol showed signals almost exclusively for one diastereoisomer, presumably the one corresponding to the *syn* configuration of the hydroxy groups in the new stereogenic centres (Figure 1).

To distinguish between the two *syn* enantiomers, we applied the enzymatic assay described by Sheldon and co-workers based on the reversibility of the aldol reaction.[13] Aldol **4l** was submitted to retro-aldol reaction catalysed by RAMA, Rha-1PA and Fuc-1PA (Figure 2).

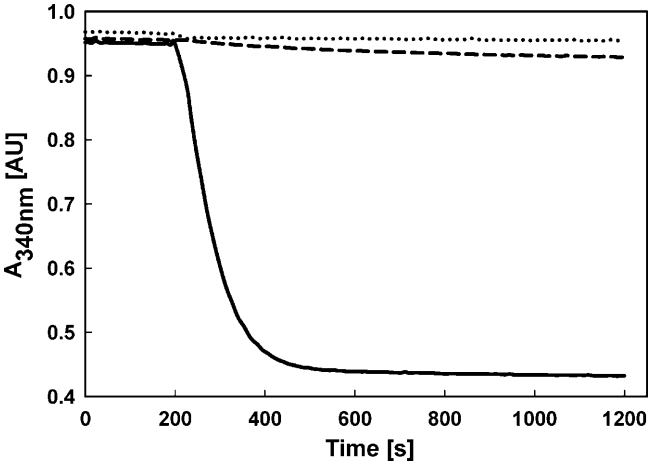

Figure 2. Enzymatic determination of the stereoisomers presents in aldol **4l**. Retro-aldol reaction catalysed by RAMA (—); Rha-1PA (- - -) and Fuc-1PA (····).

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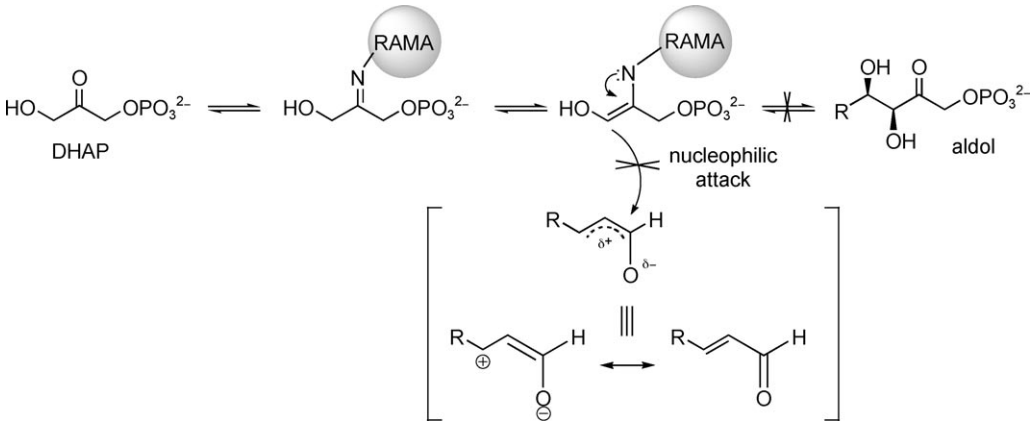
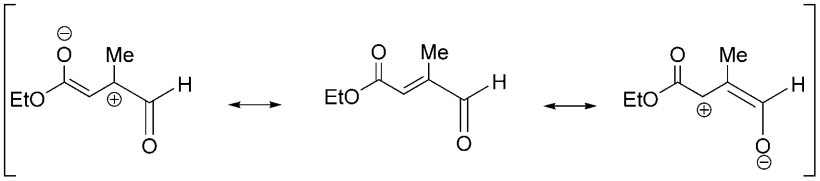


Scheme 2.



Scheme 3. Resonance structures of the aldehyde ethyl 3-methyl-4-oxocrotonate (**3l**).

More than 95% of the aldol adduct was cleaved by RAMA (D-*threo* configuration) and only about 4% was cleaved by Rha-1PA (L-*threo* configuration). No reaction was observed with Fuc-1PA (D-*erythro* configuration) and, althougth we could not perform the reaction with tagatose bisphosphate aldolase (L-*erythro* configuration), the presence of a stereoisomer with the corresponding configuration was discarded since the the sum of the products with D- and L-*threo* configurations represented the 100% of aldol **4l**. These results clearly showed that the main compound obtained was a D-*threo* configured aldol and that the reaction proceeded with high enantioselectivity.

α,β-Unsaturated aldehydes are not substrates of RAMA because the electron delocalisation through the double bond reduces the positive charge in the carbonyl carbon precluding the nucleophilic attack of the enamine intermediate formed from the ketone substrate and the enzyme (Scheme 2).

In the case of the aldehyde **3l**, the presence of the ester group introduces an additional resonance structure with a negative charge in the carbonyl oxygen of the ester and a positive charge in alpha position of carbonyl of the aldehyde (Scheme 3).

Thus, the presence of an electron-withdrawing substituent must activate the aldehyde carbonyl group increasing its reactivity. This effect is in agreement with the general observation by Whiteside and co-workers[10] in which electron-withdrawing groups activated the carbonyl group against the nucleophilic attack increasing, therefore, its reactivity.

Synthesis of the L-*threo* and D-*erythro* Enantiomers of 4l *via* Rha-1PA- and Fuc-1PA-Catalysed Reactions

Since the aldol adduct **4l** is a highly functionalised molecule and, therefore, of interest in organic synthesis, we decided to obtain the enantiomers L-*threo* and D-*erythro* accessible *via* Rha-1PA- and Fuc-1PA-catalysed reactions, respectively. Reaction conditions were similar to those described for **4l**. The highest yield achieved for aldol adduct L-*threo*-**4l**, was 60% after 6 h of reaction. Aldehyde **3l** was a poor substrate for Fuc-1PA. In this case the maximum yield achieved was only 33% after 20 h of reaction. ^{1}H and ^{13}C NMR of the isolated aldols L-*threo*-**4l** and D-*erythro*-**4l** clearly showed that the products were mixtures of two diastereoisomers (Figure 3).

One group of the signals was identical to that previously assigned to diastereoisomer *syn*, therefore the other group of signals must correspond to the *anti* diastereoisomer. As expected, in the reaction catalysed by Rha-1PA the major compound is the *syn* diastereoisomer with a *syn*:*anti* ratio of 76:24. In the Fuc-1PA-catalysed reaction the *syn*:*anti* ratio is reversed (32:68) and the *anti* diastereoisomer is the major compound. Retro-aldol analysis of compounds L-*threo*-**4l** and D-*erythro*-**4l**, showed that only the enantiomers L-*threo* (major product in Figure 2 **A**) and D-*erythro* (major product in Figure 2 **B**) were present in the samples. Although these results are consistent with the natural stereochemistry of these enzymes, they also show that in these cases the aldol reaction

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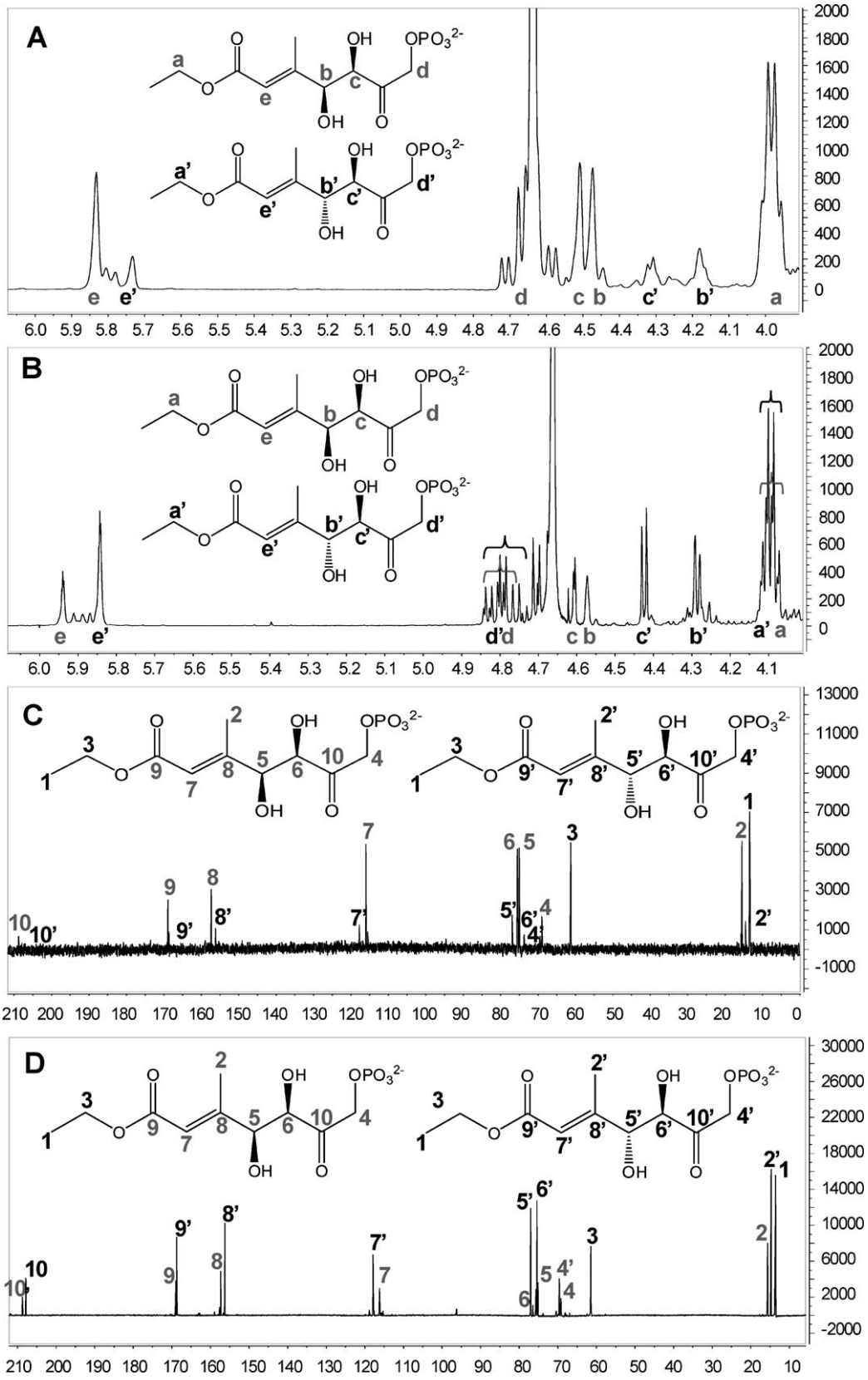

Figure 3. ^{1}H (**A**, **B**) and ^{13}C (**C**, **D**) NMR spectra of isolated products from Rha-1PA (**A**, **C**) and Fuc-1PA (**B**, **D**) catalysed reactions.

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proceeds with lower enantioselectivity control that in the RAMA-catalysed reaction.

Conclusions

In conclusion, the described multienzyme system is robust enough to be used with the three synthetically useful DHAP-dependent aldolases and with a great variety of aldehydes. The possibility to apply one-step or two-steps strategies, makes the system sufficiently flexible to be able to work under different reaction conditions such as the presence of co-solvents. Interestingly, within the frame of our studies of this multienzyme system we have found that an α,β-unsaturated aldehyde bearing an electron-withdrawing group in the β position to the double bond with a *trans* configuration is a good acceptor for RAMA. Aldol addition catalysed by this enzyme proceeds with excellent D-*threo* enantioselectivity and good conversion. The L-*threo* and D-*erythro* enantiomers are also accessible in good yield from Rha-1PA- and in moderate yield from Fuc-1PA-catalysed, reactions respectively. In both cases, the reaction proceeds with lower enantioselectivity than that of the RAMA-catalysed reaction and a mixture of L-*threo* and D-*erythro* enantiomers was obtained, in each case the major one being that corresponding to the natural stereochemical preference of each enzyme. The aldol products are functionalised compounds that can be useful intermediates in organic synthesis.

Experimental Section

General Remarks

^{1}H and ^{13}C NMR spectra, using D_2O as solvent, were recorded on a Varian System 500 spectrometer equipped with a 5 mm HCN cold probe with field z-gradient, operating at 500.13 and 125.76 MHz for ^{1}H and ^{13}C, respectively. The sample temperature was maintained constant at 298 K. One-dimensional NMR experiments were performed using standard Varian pulse sequences. Two-dimensional [^{1}H, ^{1}H] NMR experiments (gCOSY and TOCSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 3000 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 256 time increments, and linear prediction to 512. The data were zero-filled to 4096 × 4096 real points. Two-dimensional [^{1}H-^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^{1}H spectral window, a ^{13}C spectral windows of 15,000 Hz, 1 s of relaxation delay, 1024 data points, and 256 time increments, with a linear prediction to 512. The data were zero-filled to 4096 × 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively. UV/Visible spectra were recorded on a SpectraMax Plus 384 spectrophotometer at 25 °C. TLC was performed on silica-gel plates (GF254 Merck) with fluorescent indicator and detection was carry out by charring with Ce_2MoO_4. HPLC analyses were carried out on a chromatograph JASCO, Dual Gradient Pump, with UV/VIS detector. Optical rotations were determined on a Perkin–Elmer 241 MC polarimeter for D_2O solutions and are given in 10^{-1} deg·cm^2 g^{-1}. Aldehydes **3a**–**3l** were purchased from Sigma–Aldrich or Fluka. Fructose 1,6-bisphosphate aldolase from rabbit muscle (RAMA), acetate kinase (AK) and α-glycerophosphate dehydrogenase/triose phosphate isomerase (α-GDH/TIM) were purchased from Sigma–Aldrich. Rha-1PA, Fuc-1PA and DHAK were cloned in our laboratory.[9,14] All other chemicals were purchased from commercial sources as reagent grade.

General Protocol for One-Pot/One-Step Reactions

The one-pot/one-step reactions were carried out in HEPES buffer (3 mL, 20 mM pH 7.5) containing DHA (0.1 mmol), aldehyde (0.15 mmol), acetyl phosphate (0.2 mmol) and $MgSO_4$ (25 μmol), DHAK (1 U), AK (6 U) and RAMA (11.5 U). After 3 h of reaction an additional amount of RAMA (12 U) was added due to the instability of the aldolase. To reactions with aldehydes **3g** and **3j** 10% and 5% of DMSO was added, respectively, to improve their solubility. Aldehyde **3f** was supplied in a 50% solution in benzyl alcohol that afforded 3.3% of benzyl alcohol in the reaction mixture. Reactions were initiated upon addition of ATP (6.8 μmol) and their progress was followed spectrophotometrically. A continuous pH adjustment to 7.5 during the reaction course was needed to keep the ATP regeneration system working.

General Protocol for One-Pot/Two-Step Reactions

The one-pot/two-steps reaction were carried out in HEPES buffer (2 mL, 20 mM, pH 7.5) containing DHA (0.1 mmol), acetyl phosphate (0.2 mmol), $MgSO_4$ (25 μmol), DHAK (6.5 U) and AK (6 U). The reactions were initiated upon addition of ATP (6.8 μmol). When DHAP accumulation was higher than 95%, the pH was adjusted to 6.8 and water (1 mL) containing the corresponding aldehyde (0.15 mmol) and RAMA (11.5 U) was added. As before, after 3 h of reaction more RAMA (12 U) was added. For aldehydes **3g** and **3j** 10% and 5% of DMSO, respectively, was added. The reaction progress was followed spectrophotometrically. For reactions at the 0.3 mmol DHA scale, the concentrations of reactives were kept constant. To initiate the aldol reaction RAMA (51.6 U) was added. Additional aliquots of RAMA (12.3 U) were added after 1 h and 3 h of reaction.

Tracking of Reaction Progress

The amount of DHAP formed was quantified by an enzymatic assay based in the reduction of DHAP, catalysed by the α-GDH with concomitant oxidation of NADH to NAD$^+$ monitoring the decrease of absorbance at 340 nm (ε^{340}_{NADH} = 6220 cm^{-1}M^{-1}).[11] The assays were run at room temperature, measuring the absorbance at 340 nm for 15 min in a reaction mixture (1 mL) containing the reaction aliquot, Tris-HCl (40 mM, pH 8.0), NADH (0.2 μmol) and α-GDH/TIM (2 U). For the measurement of the DHA consumption, DHAK (0.375 U), ATP (5 μmol) and $MgSO_4$ (3.75 μmol) were added to the reaction mixture. The accumulation of

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the aldol product was measured using the retro-aldol activity of the aldolase (0.05 U).

5-Deoxy-D-*threo*-pent-2-ulose 1-phosphate (4a): 2 isomers (3:1) – *Major isomer:* ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.54 (d, 1H, J = 5.9 Hz, H-1$_A$), 4.46 (d, 1H, J = J = 6.2 Hz, H-1$_B$); 4.18 (d, 1H, J = 2.3 Hz, H-3); 4.10 (dq, 1H, J = 6.6, 2.3 Hz, H-4), 1.07 (d, 3H, J = 6.6 Hz, CH_3-5); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 211.6 (C-2), 78.5 (C-3), 67.9 (C-4), 67.7 (C-1), 18.4 (C-5). *Minor isomer:* ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.50 (d, 1H, J = 6.2 Hz, H-1$_A$), 4.42(d, 1H, J = J = 6.2 Hz, H-1$_B$); 4.24 (d, 1H, J = 4.3 Hz, H-3); 4.0–3.9 (m, 1H, 2.3 Hz, H-4), 0.96 (d, 3H, J = 6.6 Hz, CH_3-5).

5-Deoxy-5-ethyl-D-*threo*-pent-2-ulose 1-phosphate (4c): 2 isomers (10:1) – *Major isomer:* ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.55 (dd, 1H, J = 18.7, 6.1 Hz, H-1$_A$), 4.44 (dd, 1H, J = 18.7, 6.1 Hz, H-1$_B$); 4.22 (d, 1H, J = 2.1 Hz, H-3); 3.87 (dt, 1H, J = 7.0, 2.1 Hz, H-4), 1.4–1.3 (m, 2H, CH_2-5), 1.3–1.1 (m, 2H, CH_2-6), 0.71 (t, 3H, J = 7.4 Hz, CH_3-7); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 212.2 (C-2), 77.6 (C-3), 71.5 (C-4), 68.1 (C-1), 34.5 (C-5), 18.5 (C-6), 13.4 (C-7).

5-Deoxy-5-propyl-D-*threo*-pent-2-ulose 1-phosphate (4d): ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.60 (dd, 1H, J = 18.8, 5.9 Hz, H-1$_A$), 4.50 (dd, 1H, J = 18.8, 5.9 Hz, H-1$_B$); 4.27 (s, 1H, H-3); 3.90 (t, 1H, J = 7.1 Hz, H-4), 1.5–1.4 (m, 2H, CH_2-5), 1.3–1.1 (m, 4H, CH_2-6, CH_2-7), 0.72 (t, 3H, J = 6.9 Hz, CH_3-8); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 212.1 (C-2), 77.6 (C-3), 71.9 (C-4), 68.1 (C-1), 32.1 (C-5), 27.4 (C-6), 22.1 (C-7), 13.6 (C-8).

5-Deoxy-5-phenyl-D-*threo*-pent-2-ulose 1-phosphate (4f): 2 isomers (8:1) – *Major isomer:* ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 7.2–7.1 (m, 5H, Ar), 4.53 (d, 1H, J = 6.3 Hz, H-1$_A$), 4.49 (d, 1H, J = 6.3 Hz, H-1$_B$), 4.2–4.1 (m, 2H, H-3, H-4), 2.9–2.7 (m, 2H, CH_2-5); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 211.9 (C-2), 138.2 (Cq-Ar), 129.3–128.4 (C-Ar), 77.1 (C-3), 73.1 (C-4), 70.8 (C-1), 39.8–38.2 (C-5).

5-*O*-Benzyl-D-*threo*-pent-2-ulose 1-phosphate (4g): ^{1}H NMR (500 MHz, DO, 298 K): δ = 7.2–7.1 (m, 5H, Ar), 4.52 (dd, 1H, J = 18.5, 5.8 Hz, H-1$_A$), 4.40 (dd, 1H, J = 18.5, 5.8 Hz, H-1$_B$); 4.4–4.3 (m, 2H, CH_2Ph), 4.1–4.0 (m, 1H, H-3), 3.5–3.4 (m, 1H, H-4), 3.5–3.4 (m, 1H, H-4),3.27 (d, 2H, J = 4.9 Hz); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 212.3 (C-2), 129.5 (Ar), 129.3 (Ar), 128.9 (Ar), 128.6 (Ar), 75.5 (C-1), 73.1 (C-5), 72.4 (CH_2Ph), 70.5 (C-4); 70.2 (C-3).

5-Deoxy-5-(methylthio)methyl-D-*threo*-pent-2-ulose 1-phosphate (4h): ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.55 (dd, 1H, J = 18.8, 6.6 Hz, H-1$_A$), 4.45 (dd, 1H, J = J = 18.8, 6.6 Hz, H-1$_B$); 4.28 (d, 1H, J = 2.2 Hz, H-3); 4.05 (ddd, 1H, J = 14.4, 11.5, 9.2 Hz, H-4), 2.5–2.4 (m, 2H, H-6), 1.95 (s, 3H, Me), 1.8–1.7 (m, 2H, H-5); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 211.5 (C-2), 77.7 (C-3), 70.4 (C-4), 68.1 (C-1), 29.6 (C-6), 23.5 (C-5), 14.3 (Me).

5-*O*-Methyl-5-methoxy-D-*threo*-pent-2-ulose 1-phosphate (4k): ^{1}H NMR (500 MHz, D_2O, 298 K): δ = 4.54 (dd, 1H, J = 18.8, 6.1 Hz, H-1$_A$), 4.48 (dd, 1H, J = 18.8, 6.1, H-1$_B$), 4.42 (m, 1H, H-3), 4.34 (d, 1H, J = 7.5 Hz, H-5), 3.9–3.8 (m, 1H, H-4), 3.30 (s, 3H, OMe), 3.28 (s, 3H, OMe); ^{13}C NMR (125 MHz, D_2O, 298 K): δ = 211.3 (C-2), 105.1 (C-5), 75.1 (C-3), 70.9 (C-4), 68.2 (C-1), 56.1 (OMe), 54.8 (OMe).

(*E*)-5,6-Dideoxy-6-(ethoxycarbonyl)-5-methyl-D-*threo*-pent-5-en-2-ulose 1-phosphate (4l): Enzymatic synthesis of **4l** was carried out by the one pot/two steps approach as described before but in a 3 mmol of DHA scale. Thus, the reaction mixture (60 mL) contained: HEPES pH 7.5 (20 mM), DHA (3 mmol), MgSO4 (0.75 mmol), acetyl-P (6 mmol), DHAK (66 U) and AK (180 U). The reaction was initiated upon addition of ATP (0.2 mmol). When DHAP accumulation was higher than 95%, the pH was adjusted to 6.8, the aldehyde **3l** (4.5 mmol) and RAMA (246 U) were added. After 6 h of reaction another 123 U of RAMA were added. Compound **4l** was purified by HPLC (see the Supporting Information); $[\alpha]_D^{25}$: −1.67 (*c* 0,9, D_2O); ^{1}H NMR (400 MHz, D_2O, 298 K): δ = 5.85 (s, 1H, H-7), 4.72 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_A$), 4.63 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_B$), 4.52 (d, 1H, J = 2.2 Hz, H-3), 4.48 (s, 1H, H-4), 4.00 (c, 2H, J = 7.1 Hz, H-9), 1.91 (s, 3H, CH_3-6), 1.08 (t, 3H, J = 7.1 Hz, CH_3-10); ^{13}C NMR (100 MHz, D_2O, 298 K): δ = 208.7 (C-2), 168.9 (C-8), 157.3 (C-5), 116.0 (C-7), 75.5 (C-3), 75.1 (C-4), 69.1 (C-1), 61.3 (C-9), 15.5 (C-6), 13.5 (C-10); EM (IES-MFE): m/z = 311.0536 $[M]^+$; anal. calcd. for $C_{10}H_{15}O_9P^{2-}$ (310.0465): C 38.47, H 5.49; found: C, 38.61; H, 5.75.

L-*threo*-4l: The procedure was similar to that described for **4l**. 42 U of Rha-1PA were added at the beginning of the reaction. After 3 h of reaction other 30 U of Rha-1PA were added; ^{1}H NMR (400 MHz, D_2O, 298 K): δ = 5.73 (s, 1H, H-7), 4.71 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_A$), 4.62 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_B$), 4.31 (d, 1H, J = 5.9 Hz, H-3), 4.18 (d, 1H, J = 6.1 Hz, H-4), 3.90 (c, 2H, J = 7.1 Hz, H-9), 1.99 (s, 3H, CH_3-6), 1.08 (t, 3H, J = 7.1 Hz, CH_3-10); ^{13}C NMR (100 MHz, D_2O, 298 K): δ = 208.7 (C-2), 168.6 (C-8), 156.2 (C-5), 117.7 (C-7), 76.9 (C-4), 75.2 (C-3), 69.4 (C-1), 61.3 (C-9), 15.7 (C-6), 14.5 (C-10); EM (IES-EM): m/z = 311.0 $[M]^+$, 623.2 (2 × $[M]^+$).

D-*erythro*-4l: The procedure was similar to that described for **4l**. 110 U of Fuc-1PA were added at the beginning of the reaction. After 6 h of reaction other 40 U of Fuc-1PA were added; ^{1}H NMR (400 MHz, D_2O, 298 K): δ = 5.84 (s, 1H, H-7), 4.72 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_A$), 4.63 (dd, 1H, J = 18.3, 7.5 Hz, H-1$_B$), 4.51 (d, 1H, J = 2.2 Hz, H-3), 4.48 (s, 1H, H-4), 4.00 (c, 2H, J = 7.1 Hz, H-9), 1.91 (s, 3H, CH_3-6), 1.08 (t, 3H, J = 7.1 Hz, CH_3-10); ^{13}C NMR (100 MHz, D_2O, 298 K): δ = 208.7 (C-2), 168.9 (C-8), 157.3 (C-5), 116.0 (C-7), 75.5 (C-3), 75.1 (C-4), 69.1 (C-1), 61.3 (C-9), 15.5 (C-6), 13.5 (C-10). EM (IES-EM): m/z = 311.0 $[M]^+$, 623.2 (2 × $[M]^+$).

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