Facile Enzymatic Aldol Reactions with Dihydroxyacetone in the Presence of Arsenate

Rob Schoevaart, Fred van Rantwijk, and Roger A. Sheldon*

Laboratory of Organic Chemistry and Catalysis, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands

r.a.sheldon@tnw.tudelft.nl

Received December 18, 2000

Aldol reactions of in situ formed dihydroxyacetone arsenate with different aldehydes were catalyzed by bacterial D-fructose-1,6-bisphosphate aldolase (FruA). Aldolases from bacteria were found to be much more stable and active than FruA from rabbit muscle. Arsenate acts as a phosphate mimic and can, in principle, be used in catalytic amounts. The use of inorganic arsenate and dihydroxyacetone afforded high yields with hydrophobic aldehydes. Cosolvents increased the solubility of hydrophobic aldehydes and afforded higher reaction rates and enzyme stability. Insight is given, for the first time, in the influence of arsenate on the stereoselectivity of the aldol reaction.

Introduction

Enzyme-catalyzed aldol addition is a powerful methodology for building carbohydrate derivatives.[1,2] The group of aldolases that depends on dihydroxyacetone phosphate (DHAP) as the donor reactant is a potentially powerful synthetic tool because total control over the newly created stereogenic centers is obtained.[1] They are hampered, nevertheless, in their practical application by a very strict dependence on the expensive and labile DHAP. We have demonstrated a potential solution to the latter problem by transforming glycerol, via in situ formed DHAP, into nonnatural carbohydrates in the presence of a multi-enzyme system.[3]

Alternatively, the use of DHAP can be circumvented altogether by utilizing the propensity of arsenate esters to mimic phosphate esters in phosphate-dependent enzymatic reactions.[4,5] Arsenate esters are formed spontaneously and reversibly (e.g., from dihydroxyacetone (DHA) and arsenate) in aqueous solutions, in contrast with organic phosphates, which require kinases and ATP and ATP regeneration systems for their synthesis. Hence, only catalytic amounts of inorganic arsenate are required, in principle.

The replacement of DHAP by in situ generated dihydroxyacetone arsenate (DHAAs) in the preparation of uncommon sugars catalyzed by DHAP-dependent aldolases (see Figure 1) has previously been demonstrated with D-fructose-1,6-bisphosphate aldolase (FruA) from rabbit muscle (RAMA) as well as the microbial L-rhamnulose-1-phosphate (RhuA) and L-fuculose-1-phosphate (FcuA) aldolases.[4,5]

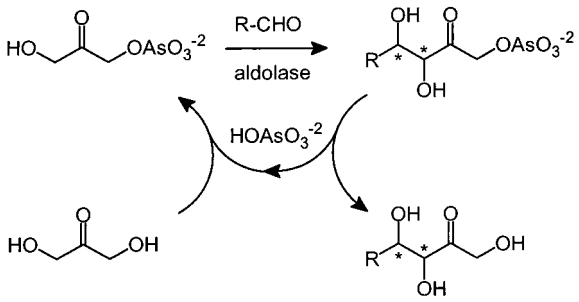

Figure 1. Arsenate-catalyzed aldol reaction with DHAP aldolases.

We now report the scope and limitations of aldol reactions of DHAAs catalyzed by FruA from *Staphylococcus carnosus*, which is much more stable than RAMA. Our study encompasses a wide range of unnatural acceptor substrates, the use of cosolvents, and the effects of arsenate on the stereoselectivity of the aldol reaction.

Results and Discussion

Reaction Monitoring. The consumption of DHAP in enzymatic aldol reactions can rapidly and reliably be monitored by measuring its reduction by NADH in the presence of glycerolphosphate dehydrogenase (GDH). Although the GDH/NADH system can also be used to monitor DHAAs, the reaction is much slower because of a much higher K_m as compared to DHAP[5] and is unsuitable for rapid routine analysis. Consequently, we opted for monitoring the consumption of aldehyde by yeast alcohol dehydrogenase (ADH)-catalyzed reduction with NADH. The method is fast and reliable but is restricted to the substrate spectrum of ADH. To study the scope of DHAAs as a substrate analogue, propanal was chosen as an example acceptor substrate since it dissolves readily in water and is a good substrate for both FruA and ADH.

Optimum Reaction Conditions. Two factors that are considered to have a major influence on the enzymatic aldol reaction of DHAAs were first examined:

* Author to whom correspondence should be addressed. Tel: +31 15 278 2675; fax: +31 15 278 1415.

(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) Schoevaart, R.; Van Rantwijk, F.; Sheldon, R. A. *J. Org. Chem.* **2000**, *65*, 6940−6943.
(4) Durrwachter, J. R.; Drueckhammer, D. G.; Nozaki, K.; Sweers, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1986**, *108*, 7812−7818.
(5) Drueckhammer, D. G.; Durrwachter, J. R.; Pederson, R. L.; Crans, D. C.; Daniels, L.; Wong, C.-H. *J. Org. Chem.* **1989**, *54*, 70−77.

10.1021/jo001766k CCC: $20.00
Published on Web 05/22/2001

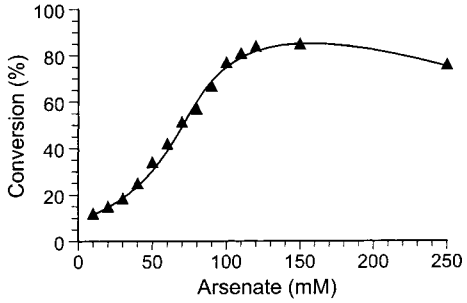

Figure 2. Effect of the arsenate concentration on the aldol reaction of dihydroxyacetone (20 mM) and propanal (50 mM) in the presence of D-fructose-1,6-bisphosphate aldolase (1 U) at pH 7.6; conversion after 24 h.

the pH and the arsenate concentration. The enzymatic aldol reaction took place between pH 6 and pH 9 with an optimum at pH 7.6. At values above pH 9 or below pH 6, no aldol reaction was observed. No aldol reaction of DHA was detected, within this pH window, in the absence of aldolase (data not shown). In contrast, DHAP undergoes a slow (0.0047 mM min^{-1}) uncatalyzed aldol reaction even at neutral pH.

Although the reaction is, in principle, catalytic[5] in arsenate, a large excess is needed in practice to compensate for the reduced binding of the substrate by the enzyme: DHAAs reacted with a 5× higher K_m and an 8× lower V_{max}[5] than DHAP when FruA was used as the catalyst. Aldol reactions were observed at arsenate/substrate ratios as low as 0.01−0.05, but with 20 mM DHA, the reaction rate reached a maximum at about 120 mM arsenate, a 6-fold excess (Figure 2). Practically speaking, this means that arsenate can be used to buffer the reaction medium. Higher arsenate concentrations gradually slowed the aldolase, presumably due to reversible inhibition. The reduced activity could, in principle, be compensated for by increasing the amount of enzyme. For example, the use of DHA and arsenate in concentrations of up to 0.5 M has been reported,[5] but this required a 50-fold increase in the amount of aldolase. Because FruA is inactivated by propanal at concentrations above 200 mM, which in effect increases with less hydrophilic aldehydes, the aldehyde concentration should be kept as low as practical. As a compromise, we performed the reaction at 20 mM DHA, 120 mM arsenate, and 50 mM aldehyde.

Although a large excess of arsenate is required to achieve acceptable reaction rates, DAAs is synthesized in situ by direct reaction with DHA, in contrast to DHAP. Moreover, since the arsenate is not consumed, it can, in principle, be easily recycled. Nonetheless, since the toxicity of arsenate is also an issue, we attempted to replace it with tungstate. However, in the presence of tungstate a slow aldol reaction was observed in the absence of aldolase.[6]

Use of Cosolvents. The more hydrophobic aldehydes were only sparingly soluble in water resulting in an inhomogeneous reaction system, which caused deactivation of the biocatalyst and hampered representative sampling. Hence, we studied the use of organic cosolvents.

Table 1. Residual Activity and Relative Reaction Rate of D-Fructose-1,6-bisphosphate Aldolases from Different Sources

	residual activity after 24 h[a]			relative rate[b]
cosolvent (25%, v/v)	*S. carnosus*	*S. aureus*	RAMA	*S. carnosus*
water	100	100	96	100
DMSO	151	146	66	104
DMF	112	139	106	118
acetonitrile	98	91	0	92
ethanol	116	99	92	50
dioxane	114	123	60	87
1,2-dimethoxyethane	134	125	65	107
tert-butyl alcohol	81	86	18	121

[a] Residual activity, after 24 h incubation, in the cleavage of D-fructose-1,6-bisphosphate in aqueous buffer; the activity at $t = 0$ has been taken as 100%. [b] Initial reaction rate of propanal. Reaction conditions: 20 mM DHA, 50 mM propanal, and 1 U of FruA in 0.5 mL of the appropriate solvent containing 120 mM arsenate buffer with pH 7.6 at rt for 2 h.

Possible irreversible effects of water-miscible organic solvents on the activity of FruA from *S. carnosus*, *Staphylococcus aureus*, and RAMA were assessed by incubating the aldolases in aqueous/organic mixtures (75:25, v/v) for 24 h. Subsequently, the residual activity in a standard FruA retro-aldol assay was measured in aqueous medium (Table 1). RAMA lost a major part of its activity when treated in this way, except with DMF or ethanol. In contrast, the bacterial aldolases from *S. carnosus* and *S. aureus* were often activated, particularly after treatment with DMSO, DMF, or 1,2-dimethoxyethane. Only *tert*-butyl alcohol caused a considerable loss of activity. FruA from *S. carnosus* was remarkably stable;[7] when it was added to pure DMSO, shaken for 1 h, and centrifuged, the pellet contained 85% of the original activity while the remaining 15% was found in the supernatant.

The aldol reaction of DHA and propanal in the presence of FruA from *S. carnosus* and arsenate was conducted in the same range of aqueous/organic reaction media (Table 1) to assess the effect of the medium on the reaction rate. The reactions were monitored by measuring the consumption of the aldehyde via an ADH-based assay (see Experimental Section). Ethanol conspicuously reduced the reaction rate to 50% of that in aqueous medium. This effect is most likely caused by the formation of ethyl arsenate, which would lower the effective concentration of arsenate. DMF and *tert*-butyl alcohol exerted a significant activating effect. *tert*-Butyl alcohol apparently does not form arsenate esters, presumably because of steric hindrance. The increased enzyme activity presumably could result from structural changes due to removal of water from the protein,[8] but "salting in" effects on arsenate could also play a role. The activating effect exerted by DMSO, DMF, and 1,2-dimethoxyethane could render the use of these solvents an attractive option while maintaining long-term stability, even with aldehydes that readily dissolve in water.

The effect of DMSO was studied in more detail, using propanal as the acceptor. This aldehyde does not require any cosolvent, but its use allowed us to study the effect

(6) Reaction of butanal and DHA (0.25 M) in the presence of tungstate (0.25 equiv) for 48 h at pH 7 yielded 13% aldol adduct after column chromatography (identified by NMR).

(7) Residual activity: 73% after 9 d in 90% (v/v) DMSO; 69% after 1 d in 90% DMF; and 52% after 7 d in 70% 1,2-dimethoxyethane.

(8) Almarsson, Ö.; Klibanov, A. M. *Biotechnol. Bioeng.* **1996**, *49*, 87−92.

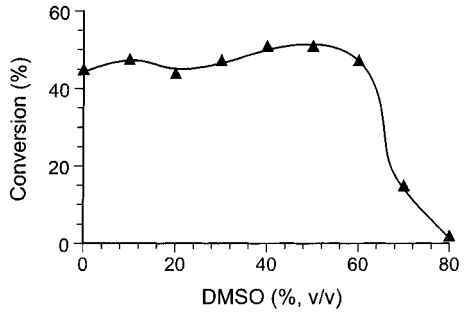

Figure 3. Aldol reaction of dihydroxyacetone (20 mM) and propanal (50 mM) in the presence of arsenate (120 mM, pH 7) and D-fructose-1,6-bisphosphate aldolase (1 U) in aqueous DMSO; conversion after 3.5 h.

Table 2. FruA-Catalyzed Aldol Reactions of DHAAs and DHAP[a]

aldehyde RCHO	DHAAs		DHAP[b]	
R	conversion (%)	time (h)[c]	conversion (%)	time (h)[c]
H	67[d]	48	81[e]	2
CH_3	78	48	84[e]	2.5
CH_3CH_2	79	24	67[e]	4
$CH_3(CH_2)_2$	86	24	78[e]	4
$CH_3(CH_2)_3$	95	24	64[e]	3
$CH_3(CH_2)_4$[f]	76	24	62[e]	5
$CH_3(CH_2)_5$[f]	54	24	30	16
$(CH_3)_2CH$[f]	83[d]	48	78	16
$(CH_3)_3C$[f]	67[d]	48	85	16
$PhCH_2$	77[d]	48	66[e]	2
$Ph(CH_2)_2$[f]	83	24	65	16
OHC	83[d]	48	98[e]	5
CH_3CO	73[d]	48	86[e]	6
$ClCH_2$	63	24	76[e]	1.5
$HOCH_2$	83	24	96[e]	0.5

[a] Reaction conditions: 20 mM DHA, 50 mM propanal, and 1 U of FruA in 0.5 mL of 120 mM arsenate buffer with pH 7.6 at rt. [b] Reaction conditions as described in ref 9. [c] Time to reach maximum conversion. [d] The conversion was calculated via phosphorylation of DHA and subsequent assay of DHAP (error ±10%). [e] Data taken from ref 9. [f] Reaction in water/DMSO (75:25, v/v).

of the cosolvent without interference by heterogeneity issues. The conversion of propanal and DHAAs in the presence of FruA from *S. carnosus* increased slightly up to 50% (v/v) of DMSO and then dropped sharply above 60% (Figure 3).

Acceptor Specificity with DHAAs. FruA-catalyzed aldol reactions of DHAAs were performed with a variety of aldehydes (see Table 2) that were already known[9] to react with DHAP in the presence of FruA. Formaldehyde, phenylacetaldehyde, 2-methylpropanal, 2,2-dimethylpropanal, glyoxal, and methylglyoxal were not amenable to the above-mentioned ADH assay; hence, these reactions were monitored by (partially) phosphorylating the unconverted DHA with ATP in the presence of glycerol kinase[10] and assaying DHAP. The maximum conversions are given in Table 2; the results of the corresponding reactions with DHAP have been included for comparison. The hydrophobic acceptors hexanal, heptanal, 2-methylpropanal, 2,2-dimethylpropanal, and 3-phenylpropanal required the addition of 25% (v/v) DMSO as a cosolvent for reliable sampling.

All of the aldehydes reacted with DHAAs in the presence of FruA. The reactions were much slower, but the acceptor specificity was similar to that of DHAP. With this latter donor, aldehydes with an α-hydroxyl group as well as activated aldehydes are good substrates.[9,11] However, DHAAs gave higher product yields with hydrophobic aldehydes than with hydrophilic aldehydes, the opposite trend to that observed with DHAP. This is a potential advantage of the use of arsenate, although it entails a penalty with regard to reaction rate, leading to an increase in reaction time in all cases. Under optimized conditions and with the same amount of enzyme, hydrophilic aldehydes reacted approximately 6× slower and hydrophobic aldehydes reacted 20× slower with DHAAs than with DHAP.

Under the established optimized conditions, a 20-fold scale-up of the aldol reaction with butanal was performed with 25% DMF as cosolvent. The product was easily separated from the reaction mixture by extraction with diethyl ether to afford the aldol adduct in 86% yield, as predicted from the result in Table 2.

Effect of Arsenate on the Stereoselectivity. We previously described a coupled enzymatic stereochemical assay for aldol adducts of DHAP.[12] This method involves a retro-aldol reaction, catalyzed by the appropriate DHAP-dependent aldolase,[13] and is combined with enzymatic detection of the liberated DHAP. The concentration of each single stereoisomer can be detected in this way, giving full insight into the stereoselectivity of the aldol reaction. We wished to determine the stereoselectivity of the aldol adducts of DHAAs in a similar way. Using arsenate as a phosphate mimic in the assay proved to be impractical because the retro-aldol reaction of the aldol arsenate esters is too slow and the enzymatic assay of DHAAs is inefficient. A solution was found by phosphorylating the aldol adducts, prior to the retro-aldol assay, with ATP in the presence of a glycerol kinase. The phosphorylation could not be carried out to completion since the aldol adducts were even worse substrates for glycerol kinases than DHA. Since glycerol kinase did not discriminate between the different stereoisomers—which was demonstrated by conducting an aldol reaction in the presence of RhuA instead of FruA—this method is, nevertheless, suited for determining the ratios of the stereoisomers. D-Tagatose-1,6-bisphosphate aldolase was not available; hence, the fraction of the products with the tagatose configuration could not be measured; neither could it be calculated[12] since phosphorylation was incomplete.

When comparing the steric course of the FruA mediated aldol reactions of DHAAs and DHAP, the high stereospecificity of the aldolase in both cases is evident (Table 3). The fraction of products with D-fructose configuration (**1**, see Figure 4) is invariably lower when DHAAs is the donor however. Moreover, the products with the L-rhamnulose configuration (**2**), which were never detected in aldol adducts of DHAP,[9,12] were found with acetaldehyde and to a lower extent also with propanal as the acceptor substrate. Furthermore, the

(9) Schoevaart, R.; Van Rantwijk, F.; Sheldon, R. A. *Tetrahedron: Asymmetry* **1999**, *10*, 705−711.
(10) Wong, C.-H.; Whitesides, C. M. *J. Org. Chem.* **1983**, *48*, 3493−3497.
(11) Bednarski, M. D.; Simon, E. S.; Bischofberger, N.; Fessner, W.-D.; Kim, M.-J.; Lees, W.; Saito, T.; Waldmann, H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627−635.
(12) Schoevaart, R.; Van Rantwijk, F.; Sheldon, R. A. *Biotechnol. Bioeng.* **2000**, *70*, 349−352.
(13) All aldolases were free of contaminating aldolase activity; see ref 12.

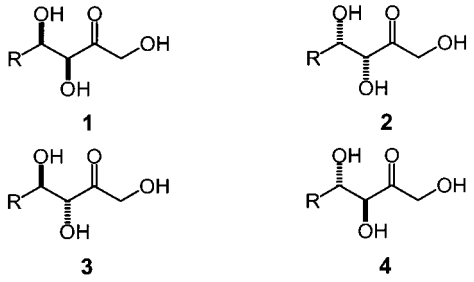

Figure 4. Four possible stereoisomeric aldol adducts of dihydroxyacetone. The configurations are as follows: D-fructose (**1**), L-rhamnulose (**2**), L-fuculose (**3**), and D-tagatose (**4**).

Table 3. Ratio of Stereoisomeric Aldol Adducts of DHAAs and DHAP

RCHO	DHAAs			DHAP		
R	**1**	**2**	**3**	**1**	**2**	**3**
CH_3	90	10	0	96	0	4
CH_3CH_2	93	5	2	100	0	0
$CH_3(CH_2)_2$	95	0	5	97	0	3
$(CH_3)_2CH$	86	0	14	100	0	0

fraction of products with the L-fuculose configuration (**3**) in the aldol adducts of DHAAs increased with the hydrophobicity of the acceptor, whereas such an effect was not observed with DHAP. In conclusion, the use of DHAAs instead of DHAP somewhat depreciates the stereoselectivity of the reaction.

Conclusion

In situ formed dihydroxyacetone arsenate (DHAAs) can advantageously replace the traditionally used DHAP in D-fructose-1,6-bisphosphate aldolase catalyzed aldol reactions. The procedure is simple and straightforward, it obviates the elaborate preparation of the donor, and it gives improved yields with hydrophobic acceptor aldehydes. The use of DHAAs somewhat reduces the stereoselectivity of the aldol reaction, but the stereoselectivity ultimately depends on the nature of the acceptor. Cosolvents can be used with a triple advantage: increased solubility of hydrophobic aldehydes, faster reaction, and stabilization of the enzyme.

Experimental Section

General. UV spectroscopy was performed with a Varian Cary 3 Bio equipped with a Cary temperature controller. Bacterial aldolases were obtained from Roche Diagnostics as a gift. DHAP was prepared from its ethyl hemiacetal dimer barium salt (Fluka). All other enzymes and chemicals were purchased from Sigma.

FruA Activity Assay. To 1.95 mL of a 50 mM Tris buffer with pH 7.6 containing 0.16 mM NADH in a 2-mL cuvette were added 20 μL of a 190 mM D-fructose-1,6-bisphosphate as well as 20 μL of a mixture containing 1.25 U of D-glycerol-3-phosphate dehydrogenase and 12.5 U of triose-1-phosphate isomerase. Then, a FruA sample was added. The reduction of the reaction products DHAP and GAP by NADH was monitored at 25 °C. One unit of FruA converts 1 μmol of D-fructose-1,6-bisphosphate/min.

Assay of Aldehydes with Alcohol Dehydrogenase. The aldehydes were assayed with ADH from yeast. To 1.97 mL of a 50 mM Tris buffer with pH 7.6 containing 0.16 mM NADH in a 2-mL cuvette, a 10 μL solution containing 5 U of ADH was added. Then, a 20-μL sample of 10-fold diluted reaction mixture was added, and the absorption was monitored at 25 °C. The molar absorption coefficient taken was 6.22 L $mmol^{-1}$ cm^{-1}.

Assay of DHA. After maximum conversion, the reaction mixture was centrifuged in an eppendorf centrifuge, transferred into a Microcon centrifugal filter (cutoff, 10 kDa), and again centrifuged for 10 min. The filtrate contained no aldolase activity. To 1 mL of this aldolase-free mixture containing a maximum amount of 4 mM DHA, 5.5 mg of ATP (10 mM) and 10 U of glycerol kinase from *Pseudomonas* species were added. After being shaken for 6 h at pH 7.5, the mixture was assayed for DHAP.

DHAP was assayed with a coupled enzyme system. To 1.95 mL of 50 mM Tris buffer with pH 7.6 containing 0.16 mM NADH in a 2-mL cuvette, 10 μL of a mixture containing 1.25 U of d-glycerol-3-phosphate dehydrogenase and 12.5 U of triose-1-phosphate isomerase were added. Then, 40 μL of the phosphorylation mixture was added, and the absorption was monitored at 25 °C.

FruA Stability Assay. 1 U of FruA was incubated in 1 mL of the appropriate solvent mixture. A 50-μL sample was withdrawn periodically and assayed for FruA activity as described above.

Aldol Reactions with Dihydroxyacetone Arsenate. In a gastight bottle, DHA (1.8 mg, 20 mM) and aldehyde (50 mM) were added to 1 mL of a 120 mM arsenate buffer with pH 7.6. Then, 0.5 mL was taken out to monitor the background reaction. To the other half, 1 U of fructose-1,6-bisphosphate aldolase from *S. carnosus* was added. Samples were withdrawn and assayed for unconverted aldehyde, using the DHA assay or unconverted DHA as described above.

Initial Rate Measurement in Aqueous Solvent Mixtures. Aldol reactions of propanal and DHA in the appropriate solvent mixture were performed as described above; a reaction in aqueous medium was performed simultaneously for comparison. After being incubated at rt for 2 h, the amount of propanal converted in both reaction mixtures was assayed with ADH.

1,3,4-Tri-*O*-acetyl-5-deoxy-5-ethyl-D-xylulose. In a flask, DHA (36 mg, 20 mM) and 88 μL of butanal (50 mM) were added to 20 mL of 120 mM arsenate buffer with pH 7.6 containing 25% DMF. To initiate the reaction, 40 U of fructose-1,6-bisphosphate aldolase from *S. carnosus* was added. After being stirred for 24 h, the conversion was 86% (assay), and the mixture was extracted with diethyl ether. Drying and concentrating the extract in vacuo afforded 100 mg of crude product. Acetylation in 2 mL of pyridine and 1 mL of acetic anhydride yielded 1,3,4-tri-*O*-acetyl-5-deoxy-5-ethyl-D-xylulose (analyzed using ^{1}H NMR spectra of authentic samples).

Stereoisomer Assay. The reaction mixture containing the aldol adducts and DHA was deproteinated and partially phosphorylated as described above. A total of 20−40 μL of this reaction mixture was then added to a quartz cuvette containing 1.97 mL of a 50 mM Tris buffer with pH 7.6, 0.16 mM NADH, 1.25 U of GDH, and 12.5 U of triose-1-phosphate isomerase. The absorption was monitored at 340 nm at 25 °C. After complete conversion of residual DHAP, the retro-aldol reaction was initiated by adding 10 μL of aldolase (5 U). The amount of DHAP released from this reaction is equal to the amount of adduct with the configuration corresponding with the used aldolase. For each of the three aldolases, a new sample was taken from the deproteinated solution.

Acknowledgment. Generous gifts of enzymes by Roche Diagnostics (Penzberg, Germany) are gratefully acknowledged. This work was financially supported by the Innovation Oriented Program Catalysis (IOP-catalysis, IKA 94010).

JO001766K