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PAPER

Highly efficient aldol additions of DHA and DHAP to *N*-Cbz-amino aldehydes catalyzed by L-rhamnulose-1-phosphate and L-fuculose-1-phosphate aldolases in aqueous borate buffer†

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Aldol addition reactions of dihydroxyacetone (DHA) to *N*-Cbz-amino aldehydes catalyzed by L-rhamnulose-1-phosphate aldolase (RhuA) in the presence of borate buffer are reported. High yields of aldol adduct (*e.g.* 70–90%) were achieved with excellent (>98 : 2 *syn*/*anti*) stereoselectivity for most *S* or *R* configured acceptors, which compares favorably to the reactions performed with DHAP. The stereochemical outcome was different and depended on the *N*-Cbz-amino aldehyde enantiomer: the *S* acceptors gave the *syn* (3*R*,4*S*) aldol adduct whereas the *R* ones gave the *anti* (3*R*,4*R*) diastereomer. Moreover, the tactical use of Cbz protecting group allows simple and efficient elimination of borate and excess of DHA by reverse phase column chromatography or even by simple extraction. This, in addition to the use of unphosphorylated donor nucleophile, makes a useful and expedient methodology for the synthesis of structurally diverse iminocyclitols. The performance of aldol additions of dihydroxyacetone phosphate (DHAP) to *N*-Cbz-amino aldehydes using RhuA and L-fuculose-1-phosphate aldolase (FucA) catalyst in borate buffer was also evaluated. For FucA catalysts, including FucA F131A, the initial velocity of the aldol addition reactions using DHAP were between 2 and 10 times faster and the yields between 1.5 and 4 times higher than those in triethanolamine buffer. In this case, the retroaldol velocities measured for some aldol adducts were lower than those without borate buffer indicating some trapping effect that could explain the improvement of yields.

Introduction

The catalytic asymmetric carbon–carbon bond formation by the aldol addition reaction is one of the simplest and most powerful strategies for the synthesis of polyoxygenated molecules with many stereogenic centers, such as carbohydrates and analogues.[1–4] Dihydroxyacetone (DHA) constitutes an important C3 building block for the construction of these compounds through aldol addition.[5–12] In this regard, a great deal of progress has been achieved in biocatalytic aldol additions of dihydroxyacetone phosphate (DHAP) or DHA to aldehydes.[13–18]

During our ongoing project on the chemo-enzymatic synthesis of iminocyclitols, the enzymatic aldol addition of DHAP or DHA to *N*-Cbz-amino aldehydes is the key step for the preparation of these compounds.[10,12,19–23] Therefore, improving the efficiency of this step by increasing the catalytic activity of the aldolases, the yield of the process and the atom economy by using non-phosphorylated donor substrates is of paramount importance from scientific and industrial viewpoints. Although the preparation and synthetic applications of DHAP and other phosphorylated analogues have reached a high degree of sophistication and efficiency[24] the preferred choice for the preparation of unphosphorylated targets is DHA.

Recently, it was uncovered that L-rhamnulose-1-phosphate aldolase (RhuA) can accept DHA as donor when the reactions were carried out in the presence of borate,[25] similarly to what was suggested in pioneering studies with arsenate salts.[26,27] In this context, we reported that RhuA can catalyze the aldol addition of DHA to *N*-Cbz-amino aldehydes without borate.[23] Interestingly, when sodium borate was added the rates of aldol formation improved between 35- and 100-fold.[23] The tolerance of unphosphorylated DHA by a Class II DHAP-dependent aldolase appears to be an exclusive property of RhuA, since the stereocomplementary L-fuculose-1-phosphate aldolase from *E. coli* (FucA) had no detectable activity with DHA either with or without borate added.[23] This is a remarkable difference with the arsenate esters, which were accepted by both RhuA and FucA aldolases.[26]

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Table 1 L-Rhamnulose-1-phosphate aldolase catalyzed aldol addition reactions of DHA-borate to *N*-Cbz-amino aldehydes (**1**)[a]

Entry	Aldehyde	%[b]	*syn* : *anti*[c]
1	**1a**	91	**94 : 6**
2	(*S*)-**1b**	99	**>98 : 2**
3	(*R*)-**1b**	95	45 : 55
4	(*S*)-**1c**	94	**>98 : 2**
5	(*R*)-**1c**	99	76 : 24
6	(*S*)-**1d**	94	**>98 : 2**
7	(*R*)-**1d**	91	**>2 : 98**
8	(*S*)-**1e**	62	**>98 : 2**
9	(*R*)-**1e**	64	**>2 : 98**
10	(*S*)-**1f**	93	**>98 : 2**
11	(*R*)-**1f**	93	**>2 : 98**
12	(*S*)-**1g**	89	**>98 : 2**
13	(*S*)-**1h**	42	**>98 : 2**
14	(*R*)-**1h**	52	**>2 : 98**
15	**1i**	99	**>98 : 2**
16	**1j**	94	81 : 19
17	(*S*)-**1k**	95	**>98 : 2**
18	(*R*)-**1k**	nr	nr

[a] Reaction conditions: [Aldehyde] = 65 mM; [DHA] = 100 mM; RhuA = 0.6 U; volume of the mixture 300 µL; 200 mM sodium borate pH 7.5. Percentage of aldol adduct measured after 24 h. [b] Percentage of aldol adduct **2** formed after 24 h respect to the limiting substrate, *i.e.* acceptor aldehyde. The amount of product was measured by HPLC from the peak integration using an external standard method. [c] The stereochemistry of the aldol adducts of the aldol additions of DHA to the aldehydes **1a**, (*S*)-**1b**, (*S*)- (*R*)-**1c**, (*S*)- (*R*)-**1d**, (*S*)- (*R*)-**1e**, (*R*)-**1f**, (*R*)-**1h**, **1i**, and **1j**, was inferred from the corresponding iminocyclitols obtained after reductive amination of the corresponding aldol adduct, after elimination of the excess of DHA and borate (see experimental procedures and ESI†). The stereochemical outcome of the aldol additions of DHA to aldehydes (*R*)-**1b**, (*S*)-**1f**, (*S*)-**1g**, (*S*)-**1h** and (*S*)-**1k**, were determined by HPLC (see experimental procedures and ESI†); *syn* and *anti* adducts refer to those with (3*R*,4*S*) and (3*R*,4*R*) configuration, respectively. nr: no reaction.

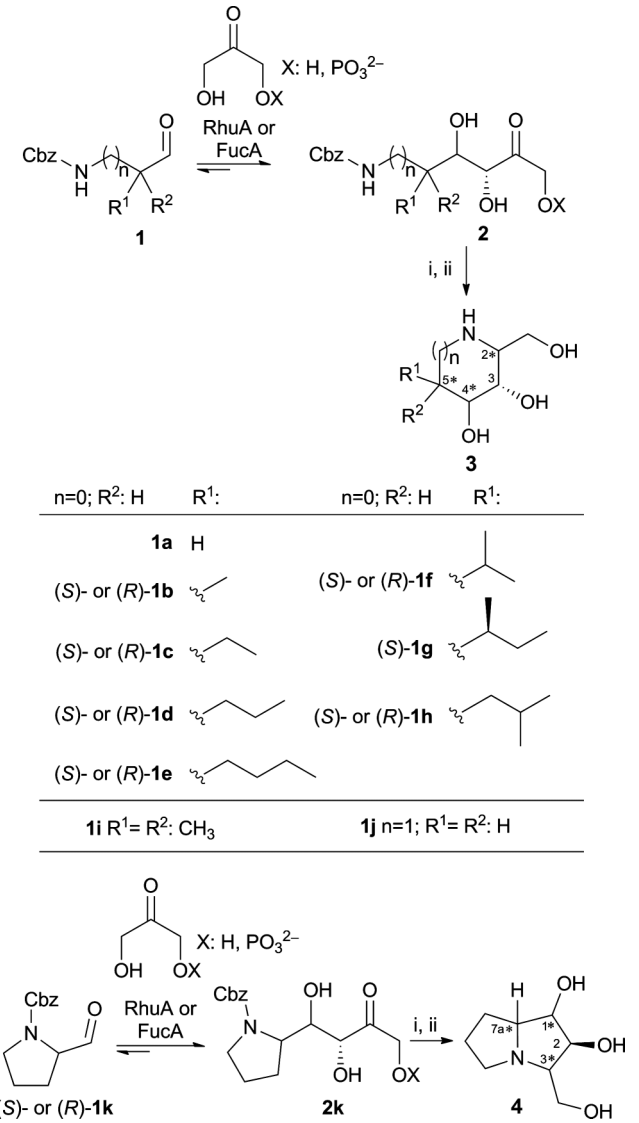
Besides the intrinsic tolerance of RhuA for DHA, the measured retroaldol rates for some aldol adducts in the presence of borate were low or negligible as compared with the synthetic ones, making the process virtually irreversible.[25] Therefore, it was further suggested that the aldol adduct may be trapped by formation of borate complexes which would be less active substrates for the aldolase.[25]

In this paper, we investigated the exploitation of the aldol additions of DHA-borate to a structural variety of *N*-Cbz-amino aldehydes catalyzed by RhuA for the two-step synthesis of iminosugars. Since the acceptance of DHA seems to be an exclusive property of RhuA, we also investigated whether the addition of sodium borate may improve the performance of enzymatic aldol addition reactions of DHAP to *N*-Cbz-amino aldehydes catalyzed by RhuA and FucA aldolases.

Results and discussion

Enzymatic aldol addition catalyzed by L-rhamnulose-1-phosphate aldolase

Aldol additions of DHA to Cbz-*N*-amino aldehydes **1** in the presence of 200 mM sodium borate pH 7.5 (Scheme 1)[28] at 25 °C furnished high conversions (Table 1), which are comparable to those achieved under different optimized conditions using DHAP as donor (see Table S1, ESI†).[19,21,29]



Scheme 1 RhuA- and FucA-catalyzed aldol addition reactions of DHA and DHAP to selected *N*-Cbz-amino aldehydes (**1**). i) when X = PO_3^{2-}, two steps: a) dephosphorylation by acid phosphatase, b) reductive amination, H_2/Pd; ii) when X = H, one step: a) reductive amination, H_2/Pd. Stereocenter control (compounds **3** and **4**, respectively): 4* (**3**) and 1* (**4**) by the aldolase, 2* (**3**) and 3* (**4**) by reductive amination; 5* (**3**) and 7a* (**4**) are selected from the starting *N*-Cbz-amino aldehyde. The absolute configuration of 3* (**3**) and 2* (**4**) depended on the aldolase and it is conserved upon the reaction with electrophiles.

The full equivalence of the stereochemical outcome observed for the DHA and DHAP nucleophiles, indicates the unbiased orientation of both donors in the active site of RhuA catalyst.[19,21,29] Differences in the *syn*/*anti* ratio between the *S* and *R* enantiomers of *N*-Cbz-amino aldehydes were also reported using DHAP donor.[21] Notably, the additions of DHA-borate to (*R*)-**1d**, (*R*)-**1e**, (*R*)-**1f** and (*R*)-**1h** (Table 1, entries 7, 9, 11 and 14) furnished exclusively the *anti* adducts, whereas the (*S*)-aldehydes yielded always the *syn* ones. This high stereoselectivity towards the *R* enantiomers of *N*-Cbz-amino aldehydes at 25 °C using DHA donor with borate contrasted with the aldol addition reactions

with DHAP catalyzed by RhuA where different mixtures of *syn*/*anti* aldol adduct were obtained.[21]

The possibility that the borate may lead to the formation of borate-ester complexes with the aldol adduct was also investigated. In previous works, we have observed that the aldol adducts formed from the aldol addition of DHAP, DHA or glycolaldehyde to *N*-Cbz-aminoaldehydes, consist of a mixture of an acyclic and cyclic hemiaminal compounds in equilibrium.[12,19,20,30,31] As an example, the ^{1}H NMR spectra at 263 K of pure aldol adduct (3*R*,4*S*,5*S*)-**2b** (Fig. 1), which was obtained from the addition of DHA to (*S*)-**2b** (Table 1, entry 2), confirmed a major acyclic compound and two hemiaminal conformers (I) and (II) (Fig. 1) (*i.e.* rotamers from the Cbz group) with the *syn* configuration (3*R*,4*S*), as ascertained by NMR (see ESI†).[32]

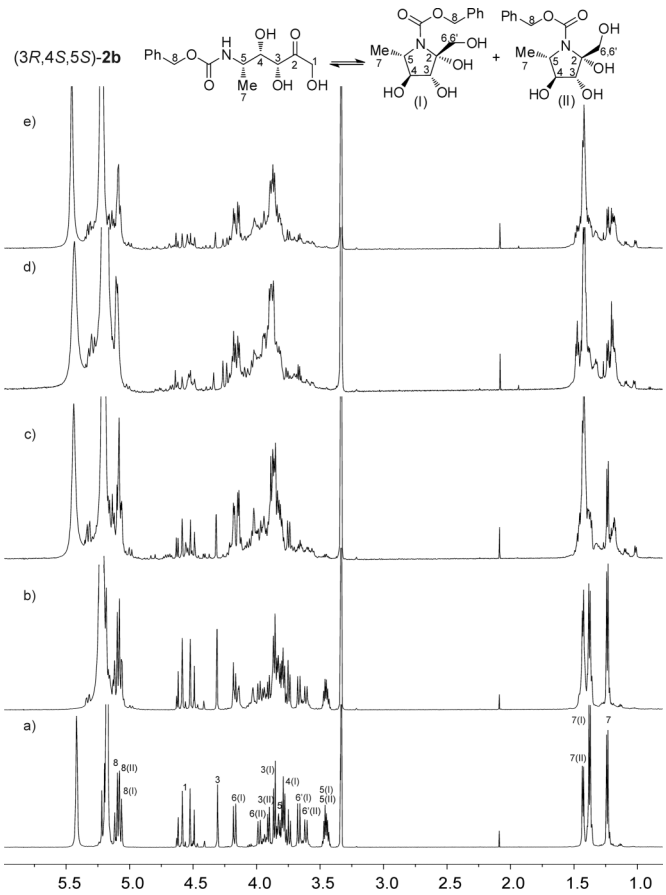


Fig. 1 ^{1}H NMR spectra of pure (3*R*,4*S*,5*S*)-**2b** at 263 K (a) and records at different millimolar ratios of (3*R*,4*S*,5*S*)-**2b**/sodium borate: b) 114 : 42; c) 106 : 136; d) 100 : 209; e) 89 : 357.

On increasing the concentration of borate the ^{1}H NMR signals corresponding to the acyclic species decreased while the ones assigned to the cyclic hemiaminals became broad (see signals corresponding to the methyl group 7, and those of the hydroxymethyl moiety 1 of the acyclic compound, Fig. 1). This result suggests that different borate complexes are formed which interconvert in a slow equilibrium (Fig. 1). Unfortunately, we were unable to resolve the NMR signals, even by decreasing the temperature below 263 K and, therefore, none of the possible borate-hemiaminal structures could be elucidated. Analysis of the same samples by ^{11}B NMR (Fig. 2) revealed the formation of different borate complexes

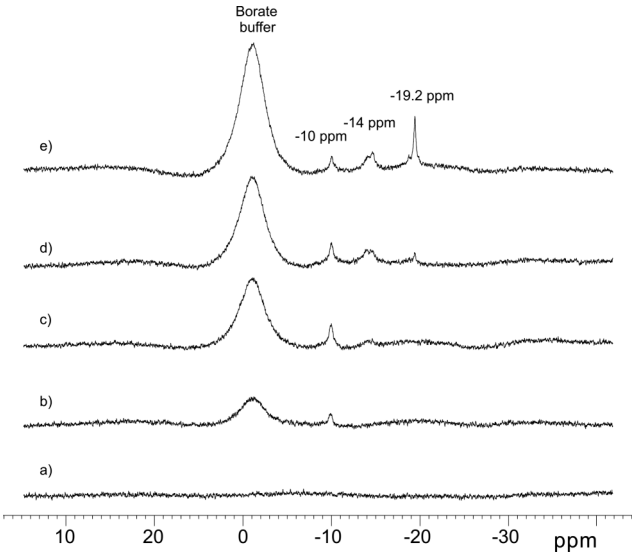


Fig. 2 Observed ^{11}B NMR of a sample of (3*R*,4*S*,5*S*)-**2b** (a) without borate added and at (3*R*,4*S*,5*S*)-**2b** : sodium borate millimolar ratios of (b) 114 : 42, (c) 106 : 136, (d) 100 : 209, and (e) 89 : 357.

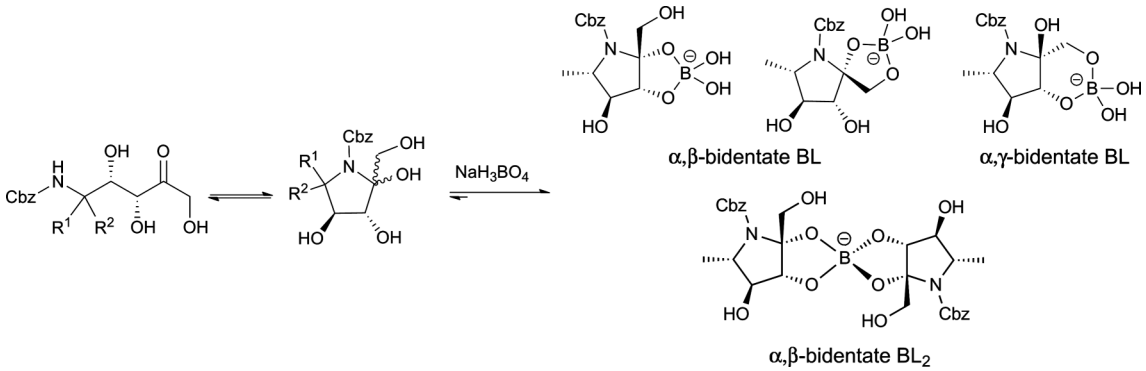
at different molar ratios of (3*R*,4*S*,5*S*)-**2b** : borate. Thus, when [(3*R*,4*S*,5*S*)-**2b**] ≥ [borate] the ^{11}B NMR spectrum showed the appearance of a signal (Fig. 2b,c) at $\delta \sim -10$ ppm, while at higher borate concentrations (Fig. 2d,e) signals at $\delta \sim -14$ ppm and ~ -19 ppm also appeared. Taking into consideration the structure of the (3*R*,4*S*,5*S*)-**2b** hemiaminal and by comparison with literature data on borate-sugars complexes,[33–35] it could be reasoned that the signal at ~ −10 ppm could arise from the formation of an α,β-bidentate BL_2 complex, those at ~ −14 ppm from α,β-bidentate BL complexes and that at ~ −19 ppm from α,γ-bidentate BL or BL_2 complexes.[33,36]

Although carrying exhaustive modelization studies on the potential borate esters that could arise from the (3*R*,4*S*,5*S*)-**2b** hemiaminal was beyond the scope of this work, DFT calculations at the B3LYP/6-31G** level (see ESI†) allowed to propose the complexes shown in Scheme 2 as plausible structures responsible for our observations.

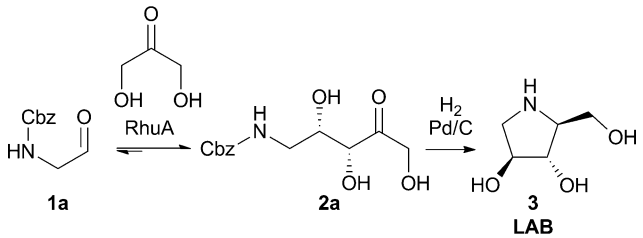
The formation of borate ester complexes with the aldol adduct appears to be plausible, although we cannot conclude that this could be the only explanation for the high yields obtained. Indeed, among the examples shown in Table 1, the behavior of prolinal derivatives is remarkable (Table 1, entries 17 and 18): the aldol addition of DHA-borate to the (*S*)-**1k** furnished almost quantitative conversions to (3*R*,4*S*,5*S*)-**2k**, whereas no reaction was detected with the enantiomer (*R*)-**1k**. Both acceptors were well tolerated by RhuA with DHAP donor.[22,29] Moreover, it can be anticipated that the protected secondary amine of (3*R*,4*S*,5*S*)-**2k** and (3*R*,4*S*,5*R*)-**2k** aldol adducts precludes the formation of a cyclic hemiaminal.

As a proof of concept of this methodology, the synthesis of the potent glycosidase inhibitor 1,4-dideoxy-1,4-*imino*-L-arabinitol (LAB, **3**) was conducted (Scheme 3).[37–40]

The aldol adduct intermediate **2a** was obtained in a 73% isolated yield and, after reductive amination, 600 mg of LAB (see experimental and ESI†) were obtained. This result compares much more favorably to that obtained using the corresponding



Scheme 2 Formation of potential hemiaminal-borate complexes derived from aldol adduct (3*R*,4*S*,5*S*)-**2b**.



Scheme 3 Chemo-enzymatic synthesis of 1,4-dideoxy-1,4-*imino*-L-arabinitol (LAB, **3**) mediated by L-rhamnulose-1-phosphate aldolase.

azido aldehyde as acceptor for the synthesis of LAB (4% isolated yield).[25] The Cbz group was tactically essential to effectively purify the aldol adducts intermediates by simple column chromatography onto a hydrophobic Amberlite® XAD16. Once the reaction crude was loaded onto the column, the excess of DHA, borate and enzyme were eliminated by washing with aqueous HCl (10 mM) and plain water. The aldol adduct was eluted with an ethanol : water mixture.

Effect of borate buffer on the yield of aldol addition reactions of DHAP to N-Cbz-amino aldehydes catalyzed by RhuA and FucA aldolases

As mentioned in the introduction, the presence of borate decreased the retroaldol activity of RhuA catalyst for some aldol adducts, while the aldol addition of DHA to aldehydes in borate is efficiently catalyzed by RhuA.[25] In this way, the process might be virtually irreversible. Moreover, it was demonstrated that cyclic aldol adducts form stable complexes with borate and that might help to shift the reaction towards the synthetic direction. Therefore, it was considered both interesting and significant to investigate if those borate effects can also be found in the aldol addition reactions of DHAP to *N*-Cbz-amino aldehydes catalyzed by L-fuculose-1-phosphate aldolase (FucA) wild type and FucA F131A mutant, which were the most active ones towards sterically demanding α-substituted aldehydes and conformationally restricted prolinal derivatives.[22] For the sake of comparison, RhuA catalyst was included in the set of experiments.

First, the influence of the buffer composition, *i.e.*, borate or triethanolamine, on the initial rate, v_o, of the aldol addition of DHAP to (*R*)-**1h**, as model reaction, was investigated. Working with RhuA, variations on the buffer type and strength (up to 200 mM) did not affect the initial reaction rate.[41] On the other hand, using FucA catalysts under the same reaction conditions the

Table 2 Initial reaction rates (v_o, μmol aldol adduct formed h^{-1} mg^{-1}) of the aldol addition reaction of DHAP to (*R*)-**1h** catalyzed by L-fuculose-1-phosphate aldolase at 25 °C

				v_o (μmol h^{-1} mg^{-1})[a]	
Entry	Aldehyde acceptor	FucA catalyst	pH	Borate[a]	TEA[a]
1	(*R*)-**1h**	F131A	7.0	66	22(15)[b]
2	(*R*)-**1h**	Wild-type	7.0	14	1.5
3	(*R*)-**1h**	F131A	6.6	42	12
4	(*R*)-**1h**	F131A	7.4	82	38

[a] Reactions were conducted at 25 °C using 200 mM sodium borate, 50 mM TEA adjusted to pH 6.6, 7.0 or 7.4. [DHAP] = 50 mM; [(*R*)-**1h**] = 65 mM, volume = 360 μL, FucA wild-type = 2.3 U (8 U mL^{-1}), FucA F131A = 0.024 U (0.08 U mL^{-1}). Values are the mean of three determinations with a standard error between 11–13%. [b] The DHAP solution was adjusted to pH 6.9–7.0 and the reaction was conducted without any buffer solution added.

v_o values were significantly different when using 200 mM sodium borate, 50 mM TEA at pH 7 or the DHAP solution adjusted to pH 7 without buffer added (Table 2).

Indeed, when borate was used, the v_o for FucA F131A was between 2.1- to 4-fold higher than that with TEA or when no buffer solution was used (Table 2, entry 1) and up to 10-fold higher for FucA wild type (Table 2, entry 2). The pH also affected significantly the v_o of the aldol additions catalyzed by FucA catalysts: values >7 were the most favorable (Table 2, entries 3 and 4), albeit higher pH must be avoided since the rate of DHAP degradation increases and aldehydes may undergo side reactions.[42,43] A similar behavior was found when using the (*S*)-**1h** as acceptor and either using FucA as an ammonium sulphate suspension or in solution (see ESI† for the preparation of FucA in solution and the results).

For preparative purposes, we found that the aldol additions of DHAP to *N*-Cbz-amino aldehydes at 4 °C provided the best yields in FucA catalysis.[21,22,43] Therefore, to better compare with previously published data[22] and conduct the reactions under optimum conditions to avoid DHAP degradation, the screening of the reactions using 200 mM sodium borate was performed at 4 °C.

Working with RhuA catalyst, the aldol adduct yields using DHA in the presence of borate were similar to those with DHAP (see Table S2 in ESI†) and therefore there is no general positive effect of borate in this case. On the other hand, the presence of borate has a positive effect on the aldol adduct yield for FucA

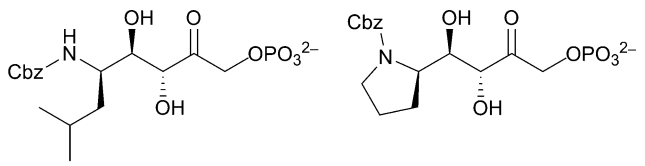
Table 3 L-Fuculose-1-phosphate aldolase catalyzed aldol addition reactions of DHAP to *N*-Cbz-amino aldehyde derivatives in the presence of borate

		%[a]	
Entry	Aldehyde	FucA wild-type[b]	FucA F131A[b]
1	**1a**	63[c](52[d])	50(59[d])
2	(*S*)-**1b**	65[c](68[d])	64[c](64[d])
3	(*R*)-**1b**	85[c](72[d])	83[c](76[d])
4	(*S*)-**1f**	48[c](16[d])	60[c](38[d])
5	(*R*)-**1f**	65[c](16[d])	76[c](56[d])
6	(*S*)-**1g**	60[c](22[d])	60[c](36[d])
7	(*S*)-**1h**	62[c](29[d])	65[c](68[d])
8	(*R*)-**1h**	75[c](27[d])	78[c](50[d])
9	(*S*)-**1k**	47[c](20[d])	71[c](39[d])
10	(*R*)-**1k**	7[c](2[d])	70[c](60[d])

[a] Percentage of aldol adduct **2** formed respect to the limiting substrate, DHAP. The amount of aldol adduct formed was determined by HPLC using an external standard method. [b] The stereochemical outcome of the aldol addition of DHAP to *N*-Cbz-amino aldehydes catalyzed by FucA wild-type and FucA F131A in borate buffer was identical to the ones previously published for the same reactions conducted with DHAP adjusted to pH 6.9–7.0 without any buffer solution added (see ref. 22). [c] Reactions were conducted at 4 °C in 200 mM sodium borate pH 7.0. [DHAP] = 40 mM; [aldehyde] = 68 mM, volume = 360 µL, FucA wild-type = 2.3 U (8 U mL^{-1}), FucA F131A = 0.024 U (0.08 U mL^{-1}); conversions measured after 24 h. [d] Experiments conducted with DHAP adjusted to pH 6.9–7.0 without any buffer solution added. Reactions were conducted at 4 °C. The rest of the conditions were identical to the experiments performed in borate. Conversions measured after 24 h. Data from ref. 22

wild-type and FucA F131A mutant catalysis (Table 3, entries 4–6 and 8–9). Interestingly, this was more significant for aldehydes **1f**, **1g** and **1h** (Table 3 entries 4–8), which had previously been determined to be poor acceptor substrates particularly with FucA wild-type,[22] and limited for good substrates such as **1a** and **1b** (Table 3, entries 1–3). It is noteworthy that, under these conditions and with the exception of the prolinal derivatives, the conversions achieved with FucA wild-type for the most sterically demanding α-substituted aldehydes were comparable to those achieved by FucA F131A mutant, the most active biocatalyst found towards those substrates.[22]

Measurements of the initial retroaldol velocities (v_o^{retro}) for the adducts (3*R*,4*R*,5*R*)-**2h** and (3*R*,4*R*,5*R*)-**2k** (Fig. 3) catalyzed by FucA F131A mutant at 25 °C in borate, showed a significant 33- and 2-fold reduction, respectively, as compared to those with TEA buffer: from 20 to 0.6 µmol h^{-1} mg^{-1} for (3*R*,4*R*,5*R*)-**2h** and from 571 to 260 µmol h^{-1} mg^{-1} for (3*R*,4*R*,5*R*)-**2k**, without and with 200 mM sodium borate, respectively. This indicates that some phosphorylated aldol adducts may be weak substrates for the aldolase in borate buffer, thus favoring the synthetic reaction as observed with some examples with RhuA catalysts (*vide supra*). Interestingly, the retroaldolization of (3*R*,4*R*,5*R*)-**2h**, which forms cyclic hemiaminals, is more strongly inhibited than that of (3*R*,4*R*,5*R*)-**2k**, whose tertiary amine prevents the formation of that cyclic species.



(3*R*,4*R*,5*R*)-**2h** (3*R*,4*R*,5*R*)-**2k**

Fig. 3 Aldol adducts used for the initial retroaldol velocities.

Conclusions

The aldol additions of DHA to a variety of *N*-Cbz-amino aldehydes catalyzed by RhuA in the presence of borate provide high conversions with excellent stereochemistry, which compares favorably to the reactions performed with DHAP. In addition to that, the tactical use of Cbz protecting group allows simple and efficient elimination of borate and excess of DHA by reverse phase column chromatography or even by simple extraction. Moreover, the use of DHA as donor for the preparation of unphosphorylated targets simplifies the synthetic strategy avoiding the manipulation of the phosphate moiety and fostering the methodology to industrial application. To sum up, this is a useful and expedient methodology for the preparation of structurally diverse iminocyclitols.

The effect of borate on the performance of the aldol additions of DHAP to *N*-Cbz-amino aldehydes was different for RhuA and FucA aldolases. In general, it was positive for the yield of aldol adduct but depended on each particular acceptor. Interestingly, for FucA catalysis, the rate of the aldol addition reaction of DHAP to *N*-Cbz-amino aldehydes in borate were between 2 to 10 times faster than that in TEA buffer. Moreover, the yields of aldol adduct improved between 1.5 to 4-fold for both FucA wild-type and the F131A mutant. In this case, it appears that the borate inhibits the retroaldol reaction favoring the aldol adduct formation.

Experimental

Materials

Dihydroxyacetone phosphate (DHAP) was obtained from the cyclic dimer precursor 2,5-diethoxy-*p*-dioxane-2,5-dimethanol-*O*-2^1-*O*-5^1-bisphosphate, which was synthesized in our lab using a procedure described by Jung *et al.*[44] with slight modifications. DHAP is liberated from the precursor by mild acidic hydrolysis at 65 °C. *N*-Cbz-amino aldehydes used in these studies were synthesized in our lab using procedures published in previous works.[22] L-Rhamnulose-1-phosphate aldolase [Co^{II}] (3.8 U mg^{-1} protein) (1 U catalyzes the cleavage of 1 µmol of L-rhamnulose-1-phosphate per minute at 25 °C and pH 7.5 (100 mM Tris·HCl + 150 mM KCl));[23,45] L-fuculose-1-phosphate aldolase wild-type 9.8 U mg^{-1} protein and FucA F131A mutant 0.1 U mg^{-1} were obtained as previously described (1 U cleaves 1 µmol of L-fuculose-1-phosphate per minute at 25 °C and pH 7.5 (100 mM Tris·HCl + 150 mM KCl)).[22,46] Aqueous sodium borate solutions were prepared by adjusting the desired pH of a solution of boric acid with NaOH (2 M). Deionized water was used for preparative HPLC and Milli-Q-grade water for analytical HPLC. All other solvents used were of analytical grade.

HPLC analyses

HPLC analyses were performed on a RP-HPLC XBridge® C18, 5 µm, 4.6 × 250 mm column (Waters). The solvent system used was: solvent (A): 0.1% (v/v) trifluoroacetic acid (TFA) in H_2O and solvent (B): 0.095% (v/v) TFA in CH_3CN/H_2O 4 : 1; gradient

elution from 10% to 70% B over 30 min to monitor reactions with aldehydes **1a**, **1b**, **1c**, **1i**, **1j**; or 30% to 90% B over 30 min to monitor those with **1d**, **1e**, **1f**, **1g**, **1h**, **1k**, flow rate was 1 mL min^{-1}, detection was at 215 nm and column temperature at 30 °C. The amount of aldol adduct produced was quantified from the peak areas using an external standard methodology.

Reactions with DHA as donor in borate buffer

Analytical scale reactions (300 µL total volume) were conducted in 2 mL test tubes with screw cap stirred with a vortex mixer (VIBRAX VXR basic, Ika) at 1000 rpm and 25 °C. Aldehydes **1** (19.5 µmol) were dissolved in dimethylformamide (60 µL) and these solutions were mixed with a DHA solution (90 µL, 30.0 µmol) and buffer (60 µL of 1.0 M sodium borate, pH 7.5). RhuA (90 µL, 0.16 mg of protein, 0.6 U, corresponding to 2 U mL^{-1}) was then added to start the aldol reaction. Conversions were measured at 24 h reaction time. Reaction monitoring was as follows: samples (25 µL) were withdrawn at several reaction times, diluted with methanol (975 µL) and analyzed by HPLC under the conditions described above.

Enzymatic aldol reactions with DHAP as donor

Analytical scale reactions (360 µL total volume) were conducted in 2 mL test tubes with screw cap stirred with a vortex mixer (VIBRAX VXR basic, Ika) at 1000 rpm at 4 °C. Aldehydes **1** (24.5 µmol) were dissolved in dimethylformamide (72 µL). Each of the aldehydes were mixed with water (47.2 µL) or sodium borate (47.2 µL of 1.52 M, pH 7.0 solution), a freshly neutralized (pH 6.9–7.0) DHAP solution (141 µL, 14.4 µmol), and the aldolase (for RhuA catalyzed reactions: 100 µL, 0.19 mg of protein, 0.72 U corresponding to 2 U·mL^{-1} reaction; for reactions with FucA wild-type or FucA mutants: 100 µL, 0.29 mg of protein, (corresponding to 2.9 U FucA wild-type (8 U mL^{-1}), and 0.029 U FucA F131A (0.08 U mL^{-1})) was added to start the aldol reaction. Conversions were measured at 24 h of reaction time. Reaction monitoring was as follows: samples (25 µL) were withdrawn at several reaction times, diluted with methanol (975 µL) and analyzed by HPLC under the conditions described above. In parallel, samples (25 µL) were mixed with a solution (25 µL) of acid phosphatase (5.3 U mL^{-1} in 400 mM sodium citrate pH 4.5) and incubated for 24 h. After dilution with methanol (950 µL), samples were analyzed by HPLC.

Enzymatic aldol reactions with DHAP as donor: initial velocities (v_o) of aldol adduct formation with the acceptor (*R*)-1h

The initial velocities of aldol formation (v_o) were determined by measuring the amount of aldol adduct produced by HPLC at the initial reaction times. The amount of aldol adduct produced was quantified by an external standard method. Reactions were conducted as described above for analytical reactions using 360 µL reaction volume: 47.2 µL of 380 mM or 1.52 M triethanolamine-HCl (TEA) pH 6.6, 7.0 or 7.4, or 47.2 µL of 1.52 M sodium borate pH 6.6, 7.0 or 7.4, final concentration 50–200 mM TEA and 200 mM sodium borate. RhuA = 0.72 U (2 U mL^{-1}), FucA wild-type = 2.9 U (8 U mL^{-1}), FucA F131A = 0.029 U (0.08 U mL^{-1}). The amount of aldehyde, dimethylformamide, and freshly neutralized DHAP solution used for these reactions was identical to that for the analytical reactions. Samples were withdrawn at 5, 10, 20, 30 and 45 min and analyzed by HPLC as described above. Linear correlations with the reaction time were found for aldol adduct production lower than 15% with respect to the limiting reactant, DHAP. The estimated standard error for three determinations was between 11–13%.

Initial retroaldol velocity (v_o) with phosphorylated aldol adducts

Retroaldol assays with aldol adducts were carried out as follows: A solution of NADH (0.9 mL, 2 mM) in Tris-HCl (110 mM, pH 7.5) or in sodium borate (220 mM, pH 7.5), containing KCl (160 mM), the aldolase (variable amount depending on the activity) and glycerol-3-phosphate dehydrogenase (1.7 U mL^{-1}) was incubated at 25 °C for 5 min. To this solution the aldol adduct (3*R*,4*R*,5*R*)-**2h** or (3*R*,4*R*,5*R*)-**2k** (100 µL, 100 mM) was added up to a total final volume of 1 mL. The decrease of absorbance of NADH at 340 nm was measured. The estimated standard error for three determinations was between 5–10%.

Addition of DHA to aldehydes in borate buffer. Laboratory scale reactions (70 µmol scale). General procedure

To determine the stereochemistry of the aldol adducts obtained with RhuA using DHA and aldehydes **1a**, (*S*)-**1b**, (*S*)-**1c**, (*R*)-**1c**, (*S*)-**1d**, (*R*)-**1d**, (*S*)-**1e**, (*R*)-**1e**, (*R*)-**1f**, (*R*)-**1h**, **1i**, and **1j** in the presence of sodium borate, reactions (~70 µmol scale) were conducted in 25 mL screw capped test tubes (2.5 mL, total reaction volume). Each aldehyde (0.07 mmol) was dissolved in dimethylformamide (0.5 mL) and this solution was mixed with a DHA solution (1.9 mL, 0.09 mmol) in 263 mM sodium borate, pH 7.5. Stock solution of RhuA (0.1 mL, 5 U) in Tris-HCl 100 mM, pH 7.5 was added to start the reaction. The stock solution of RhuA was prepared as follows: the ammonium sulphate suspension of RhuA (1.76 mL, 113 U mL^{-1}) was centrifuged and the pellet resuspended in Tris-HCl 100 mM pH 7.5 (400 µL). After 48 h, MeOH (5 mL) was added to the reaction mixture to stop the enzymatic reaction and precipitate the enzyme. MeOH was removed in a rotary evaporator. Then, the mixture was filtered through a 0.45 mm cellulose membrane filter. The filtrate was adjusted to pH 3.0 and loaded onto a semi-preparative column X-Terra® (19 × 250 mm). Products were eluted using CH_3CN gradient from 0% to 52% CH_3CN in 30 min. The flow rate was 10 mL min^{-1} and the products were detected at 215 nm. Analysis of the fractions was accomplished under gradient conditions (10 to 70% of solvent B in 30 min) on the analytical HPLC. Pure fractions were pooled and lyophilized. The solid thus obtained was dissolved in H_2O/MeOH 1 : 1 and treated with H_2 (50 psi) in the presence of 10% Pd/C (50 mg) at room temperature during 24 h. After removal of the catalyst by filtration through a 0.45 µm nylon membrane filter, the filtrate was adjusted to pH 6.4 with formic acid and lyophilized obtaining a solid material. These experiments were intended to determine the stereochemical outcome of the reactions, therefore the purification procedures were not optimized and quantities around 5–10 mg of each iminocyclitol were obtained. The ^1H and ^{13}C NMR matched those previously described and, from the corresponding ^1H spectra, the diastereomeric excess for each reaction was inferred similarly as previously reported (see ESI†).[19–22] The stereochemical outcome

of the aldol additions of DHA to aldehydes (*R*)-**1b**, (*S*)-**1f**, (*S*)-**1g**, (*S*)-**1h** and (*S*)-**1k**, were determined by HPLC. The corresponding diastereomeric mixtures of unphosphorylated aldol adducts were separated under the HPLC conditions and compared to those obtained in a previous work (see ESI†).[22]

Preparative synthesis of 4-dideoxy-1,4-*imino*-L-arabinitol (LAB)

As a proof of synthetic utility of these methodology, the aldol addition of DHA with aldehyde **1a** in the presence of borate was scaled up to 200 mL total volume at 12 mmol scale: **1a** (2.3 g, 12 mmol) was dissolved in dimethylformamide (40 mL). To this solution, DHA (1.4 g, 15 mmol) in water (120 mL) and 1.0 M sodium borate, pH 7.5 (40 mL, 200 mM final concentration) was added. RhuA (400 U, 32 mL of an ammonium sulphate suspension of 12.5 U mL^{-1} were centrifuged and the pellet dissolved in 32 mL of 200 mM sodium borate, pH 7.5). After 48 h the reaction mixture was loaded onto a chromatography column (5 × 15 cm, 295 mL) filled with Amberlite XAD 16. First, the main impurities were removed by washing with aq HCl 10 mM and water (4 column volume, 1178 mL), then the product was eluted with ethanol : water 3 : 2 (2 column volume, 589 mL). Pure fractions were collected and evaporated to dryness (2.5 g, 73% yield). Following the procedure described above, treatment of the aldol adduct with H_2 in the presence of Pd/C, furnished the 1,4-dideoxy-1,4-*imino*-L-arabinitol (LAB) (600 mg), which was not further purified (see ESI†). Physical and spectral data matched those obtained in a previous work.[19]

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