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Influence of *N*-amino protecting group on aldolase-catalyzed aldol additions of dihydroxyacetone phosphate to amino aldehydes

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Dedicated to Professor Francisco Camps on the occasion of his 70th birthday.

Abstract—This work examines the influence of *N*-protecting groups on the conversion and stereoselectivity of dihydroxyacetone phosphate (DHAP) dependent aldolase-catalyzed aldol additions of DHAP to *N*-protected-3-aminopropanal. Phenylacetyl-(PhAc-), *tert*-butyloxycarbonyl- (^{t}Boc-) and fluoren-9-ylmethoxycarbonyl- (Fmoc-)-3-aminopropanal were evaluated as substrates for D-fructose 1,6-bisphosphate aldolase from rabbit muscle (RAMA), and L-rhamnulose-1-phosphate aldolase (RhuA) and L-fuculose-1-phosphate aldolase (FucA), both from *Escherichia coli*. Using PhAc and ^{t}Boc ca. 70% conversions to the aldol adduct were achieved, whereas Fmoc gave maximum conversions of ca. 25%. The stereoselectivity of the DHAP-aldolases did not depend on the *N*-protected-3-aminopropanal derivative. Moreover, inversion of FucA stereoselectivity relative to that obtained with the natural L-lactaldehyde was observed. Both *N*-PhAc and ^{t}Boc adduct product derivatives were successfully deprotected by penicillin G acylase (PGA)-catalyzed hydrolysis at pH 7 and by treatment with aqueous TFA (6% v/v), respectively. However, the corresponding cyclic imine sugars could not be isolated, presumable due to the presence of a highly reactive primary amine and a keto group in the molecule, which lead to a number of unexpected reactions.

1. Introduction

Aldolases are a class of lyases that catalyze stereoselective aldol additions of aldehydes and ketones,[1,2] constituting powerful tools in the asymmetric synthesis of both conventional and uncommon carbohydrates as well as other complex hydroxylated products.[1–4]

We recently reported aldol additions of DHAP to *N*-benzyloxycarbonyl (Cbz) amino aldehydes catalyzed by D-fructose-1,6-diphosphate aldolase from rabbit muscle (RAMA), and L-rhamnulose-1-phosphate aldolase (RhuA) and L-fuculose-1-phosphate aldolase (FucA), both from *Escherichia coli*.[5,6] These enzymatic reactions afforded, after cleavage of the phosphate group, the corresponding 2-keto-*N*-Cbz-amino-3,4-diols, which can be converted into iminocyclitols, potent inhibitors of glycoprocessing enzymes, by reductive amination.

In the course of our ongoing project on the chemoenzymatic synthesis of iminocyclitols we investigated whether other *N*-blocking groups of the amino aldehyde may also be suitable for the synthesis of these compounds. Furthermore, alternative *N*-protecting groups to suit any further synthetic strategies upon the *N*-protected amino-2-keto-3,4-diols was also pursued. To this end, three *N*-protecting groups for the model aldehyde 3-aminopropanal were selected: phenylacetyl (PhAc), *tert*-butyloxycarbonyl (^{t}Boc) and fluoren-9-ylmetoxycarbonyl (Fmoc). PhAc is structurally similar to Cbz, and it can be cleaved by penicillin amidase (PGA)-catalyzed hydrolysis under mild and selective conditions.[7] Removal of ^{t}Boc requires acidic conditions but less strenuous than simple amides like acetyl.[8] Fmoc group can be eliminated in the presence of secondary amines, such as piperidine, by base induced β-elimination.

Herein, we report on the reactivity and stereoselectivity of RAMA, RhuA and FucA DHAP aldolases as catalysts for aldol additions of DHAP to aldehydes: *N*-(PhAc)-(**1**), *N*-(^{t}Boc)- (**2**) and *N*-(Fmoc)-3-aminopropanal (**3**) (Scheme 1). In this study, we focused on three aspects. First, the influence of two reactions media, namely emulsion

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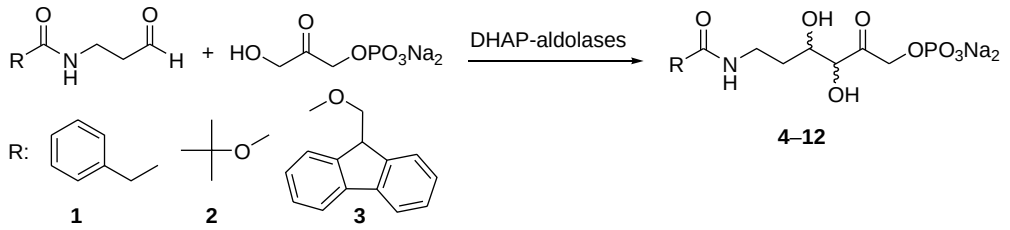


Scheme 1. RAMA, RhuA and FucA DHAP aldolases catalyzed synthesis of products **4–12**.

systems developed and assayed in previous works[9] and cosolvent DMF/H_2O 1:4 mixtures, on the reaction conversion to aldol adducts was investigated. Second, to assess the stereoselectivity of the aldolases towards the three *N*-protected-3-aminopropanal derivatives, the aldol adducts were prepared under the best reaction conditions and their structure and stereochemistry determined. Third, the deprotection reactions of *N*-blocked-2-keto-aminodiol adducts obtained in higher yield were studied.

2. Results and discussion

2.1. Aldolase-catalyzed reactions

Aldol additions of DHAP to aldehydes **1–3** catalyzed by RAMA, RhuA and FucA were investigated in two reaction systems namely high water content emulsions and DMF–H_2O (1/4) cosolvent mixture (Table 1). Three emulsion formulations were employed: $H_2O/C_{14}E_4$/tetradecane, $H_2O/C_{14}E_4$/hexadecane and $H_2O/C_{14}E_4$/squalane always in 90/4/6 wt%, where $C_{14}E_4$ is a technical grade tetra(ethyleneglycol)tetradecyl ether surfactant ($C_{14}H_{29}(OCH_2CH_2)_4OH$), with an average of 4 mol of ethylene oxide per surfactant molecule.[5,9] Both PhAc and the sterically more demanding ^{t}Boc derivatives **1** and **2** were good substrates (Table 1, entries 1, 2, 4, 5, 7 and 8), the conversions to aldol adduct being similar to those achieved with benzyloxycarbonyl (Cbz) *N*-protecting group.[5,6] The most hydrophobic and bulky Fmoc derivative **3** was also tolerated as substrate, although it gave the lowest conversions with the three aldolases (Table 1, entries 3, 6 and 9).

Diastereomeric ratio of aldol adducts **4–12** were assessed by NMR spectroscopy and are summarized in Table 1, last column. The absolute configuration of the newly formed stereogenic centers was assigned assuming that the stereochemistry at the C-3 position depended exclusively on the DHAP aldolase and was conserved upon reaction with any electrophile.[10–16] Hence, epimeric products at C-4 arose from attack on the inverted face of the *N*-protected-3-aminopropanal carbonyl group relative to that on the natural aldehyde. RAMA catalyst was the most stereoselective towards the *N*-protected-3-aminopropanal derivatives, both ^{t}Boc and Fmoc giving the highest diastereomeric excesses (de $>$ 80%). The stereoselectivity of RhuA enzyme was lower (de 40–60%) than that of RAMA, the de with both PhAc and Fmoc being similar to that obtained with the corresponding Cbz derivative.[5] The NMR spectra of the aldol adducts obtained with FucA catalyst were indistinguishable from those observed with RhuA enzyme. Similarly to *N*-Cbz-3-aminopropanal,[6] an inversion of FucA stereoselectivity towards the *N*-protected amino aldehydes **1–3** was observed.

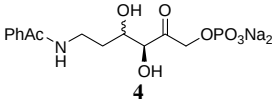
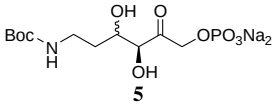
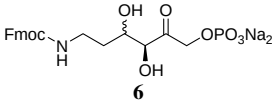
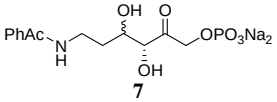
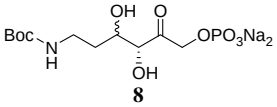
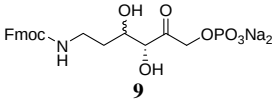
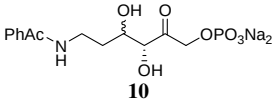
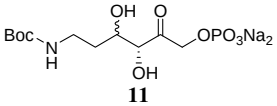
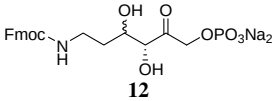
The conversions and diastereomeric ratios shown in Table 1 remained constant up to 24 h of reaction, therefore it was assumed that they reflect the final equilibrium compositions. In a previous paper,[6] we suggested that the stereochemical outcome of the aldol addition of DHAP to Cbz-3-aminopropanal catalyzed by FucA was thermodynamically controlled. To assess if the diastereomeric ratios of the aldol adducts generated from DHAP and PhAc-, ^{t}Boc- and Fmoc-3-aminopropanal correlate with the thermodynamic stability of the corresponding diastereoisomers, an extensive exploration of the conformational space available to adducts **4–12** was carried out. Only the linear forms of these products were considered, since the relative abundance by NMR of the corresponding cyclic forms was always low. The results of these calculations showed that the lowest energy minima conformers of the (3*S*,4*R*), or (3*R*,4*S*), diastereoisomers of adducts **4–12** were always more stable, by approximately 1.5 kcal/mol, than the corresponding (3*S*,4*S*), or (3*R*,4*R*), isomers independently of the protecting group present. Assuming that the entropic contributions to ΔG cancel out, this energetic difference suggests a predicted (3*S*,4*R*):(3*S*,4*S*) or (3*R*,4*S*):(3*R*,4*R*) ratio close to 93:7 at 25 °C, in general good agreement with the ratios shown in Table 1, particularly for the reactions catalyzed by RAMA. The maximal deviation (67:33) was observed for the FucA catalyzed reactions with substrates **1** and **2**.

Altogether, these results suggest that, under our reactions conditions, the major products of the aldolic condensation of aldehydes **1–3** with DHAP catalyzed by RAMA, RhuA and FucA are those thermodynamically favoured, similarly to what was previously observed for the condensation of different *N*-Cbz-aminoaldehydes catalyzed by FucA.[6] Thus, while for RAMA and RhuA catalysts the stereofacial selectivity observed with the natural substrates (glyceraldehyde-3-phosphate and L-lactaldehyde, respectively) was conserved, the contrary was true for FucA and the main adducts formed are those from the 'wrong' face attack (i.e., relative to that with the natural substrate, L-lactaldehyde).

Docking simulations carried out with the different diastereoisomers of products **4–12** bound into the active centre of RAMA and RhuA[†] suggest that in all cases the bulky *N*-protecting group cannot get into the catalytic site of the

[†] We did not attempt to model the FucA complexes because of the difficulty to predict the conformation of the flexible C-terminal tail of the protein.[17]

Table 1. DHAP-dependent aldolase-catalyzed aldol addition of DHAP to *N*-protected-3-aminopropanal derivatives **1–3**

Entry	Acceptor aldehyde	Aldolase (U mL^{-1})	DHAP concn[a] (mM)	Conversion,[b] % (Time, h)		Product	Diastereomeric ratio (C-4) *R*:*S*
				A[c]	B[d]		
1	**1**	RAMA 20	86	66 (3)	65 (2)	 **4**	89:11
2	**2**	RAMA 20	97	66 (4)	70 (6)	 **5**	93:7
3	**3**	RAMA 20	53	25 (1)	19 (2)	 **6**	92:8
4	**1**	RhuA 0.4	100	47 (2)	28 (4)	 **7**	19:81
5	**2**	RhuA 0.4	91	63 (7)	45 (7)	 **8**	30:70
6	**3**	RhuA 0.4	53	15 (6)	15 (6)	 **9**	23:77
7	**1**	FucA 8	45	72 (3)	71 (4)	 **10**	33:67
8	**2**	FucA 8	45	70 (3)	70 (4)	 **11**	33:67
9	**3**	FucA 8	53	20 (2)	18 (2)	 **12**	21:79

[a] Acceptor aldehyde (1.8 equiv mol^{-1}); reaction volume 5 mL. $T=25$ °C.
[b] Molar percent conversion to the aldol adduct (**4**–**12**) with respect to the starting DHAP concentration, determined by HPLC from the crude reaction mixture using purified standards.
[c] High water content emulsions. Reaction conversions to the corresponding aldol adduct in emulsions were similar regardless of the formulation used, therefore, the mean values obtained in the three emulsion systems, $H_2O/C_{14}E_4$/tetradecane, $H_2O/C_{14}E_4$/hexadecane and $H_2O/C_{14}E_4$/squalane 90/4/6 wt%, are always given.
[d] DMF/H_2O 1:4 v/v.

protein and probably remains at the entrance of the cavity, partially exposed to the solvent. This is shown in Figure 1 for the major 3*S*,4*R*-isomers of adducts bearing *N*-PhAc, ^{t}Boc and Fmoc bound in the active centre of RAMA and for the corresponding 3*R*,4*S*- enantiomers in the active center of RhuA. That would explain the little effect of the *N*-protecting groups with different size and shape on the stereochemical outcome of the reactions, since those groups would remain far from the reactive atoms. In addition, it also suggests that the lower yields observed for the Fmoc containing adducts (**6**, **9**, **12**) could be due to the steric hindrance arising from the bulkiest Fmoc moiety, which could difficult the approach of the aldehyde group to the reactive enzyme-bound DHAP.

2.2. Deprotection of the phenylacetyl and *tert*-butyloxycarbonyl groups

The results obtained showed that both PhAc and ^{t}Boc, provided the highest reaction conversions with similar stereoselectivities. At this point, it was also important to establish proper reaction conditions for the *N*-protecting group removal. To this end *N*-PhAc and *N*-^{t}Boc aminopolyols **4** and **5**, and their corresponding unphosphated derivatives, were treated with penicillin acylase at pH 7 and aqueous trifluoroacetic acid, respectively.

Removal of PhAc and ^{t}Boc was achieved quantitatively under the aforementioned conditions (see Section 4). The formation of the six-membered imine sugar, in equilibrium

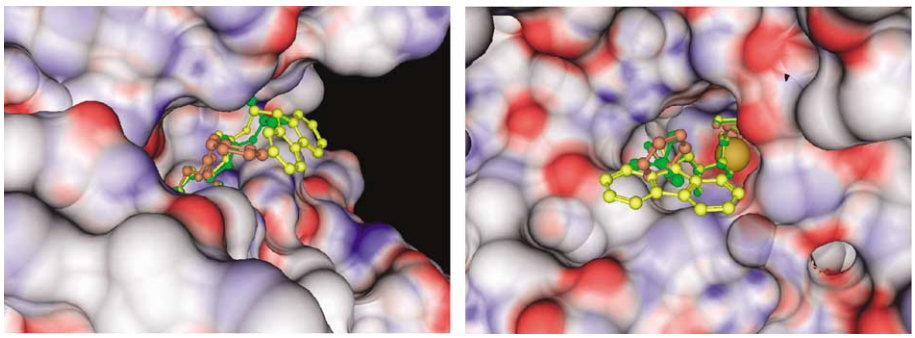


Figure 1. Structures of aldol adducts bearing PhAc (brown), ^{t}Boc (green) and Fmoc (yellow) protecting groups docked into the active center of RAMA (left panel) and RhuA (right panel). Structures on the left panel correspond to the (3*S*,4*R*) diastereoisomers, while those on the right panel correspond to the (3*R*,4*S*) diastereoisomers.

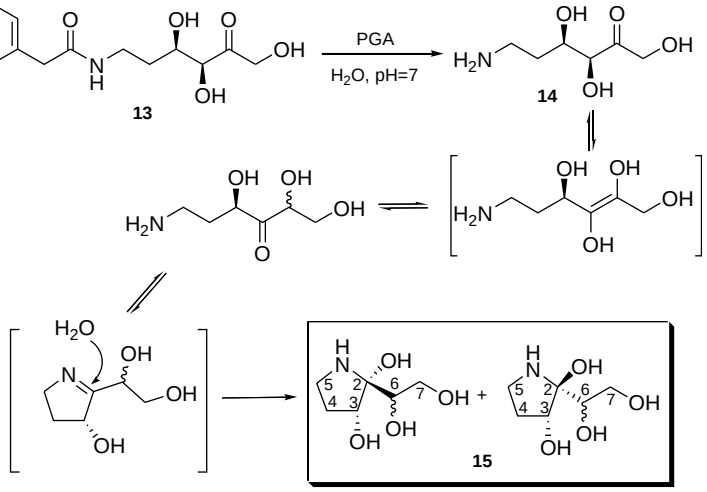
with the corresponding unprotected aminoketopolyol, was the product expected after the deprotection reaction. However, the cyclic imine sugar could not be isolated in any reaction condition. Hence, the NMR spectrum of the residue obtained after PhAc removal of **4** and work up was complex with signals that presumably belong to a number of decomposition products since they can hardly be assigned to any single structure. To avoid any possible influence of the work up on the stability of the final product, the deprotection reaction was performed into an NMR tube and monitored continuously. This experiment confirmed the previous observation: the signals corresponding to the product disappeared while no major product was formed with the exception of phenylacetic acid. On the other hand, the deprotection of unphosphated derivative **13** gave the hemiaminal mixture **15** as the major products. A possible mechanism for the formation of **15** is outlined in Scheme 2. The key step was the enolization of **14** and subsequent shift of the ketone to position 3.

Deprotection of ^{t}Boc blocking group was accomplished by aqueous TFA. It has been reported that treatment of *N*-formyl-aminopolyol with aqueous acid at pH 1 efficiently cleaves the amide, and that at pH 3 the same compound is stable.[8] On this basis and being aware of the sensitivity of these compounds to acids, we decided to perform the deprotection at the lowest possible TFA concentration. We surveyed different TFA concentrations from 1 to 6% and we found that 6% aqueous TFA lead to complete Boc cleavage in 24 h. The reaction was monitored by NMR and, in agreement with the expectations, the spectra revealed the presence of the linear compound **16** in equilibrium with the cyclic imine **17** (Scheme 3).

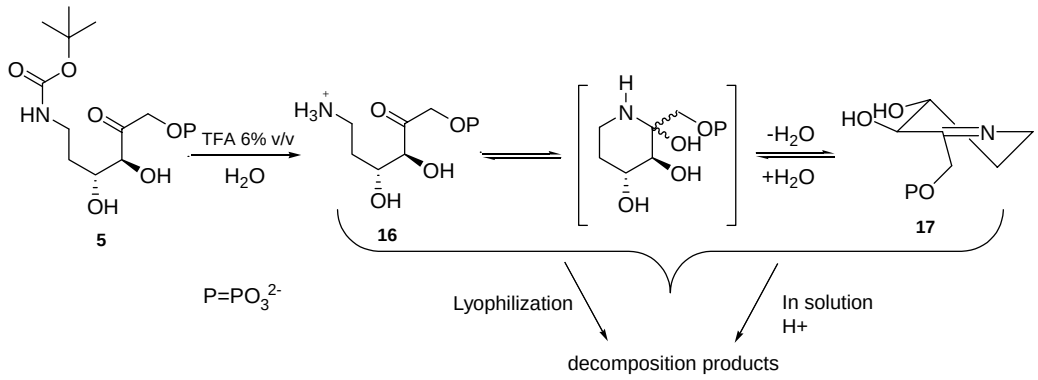
Nevertheless, the resulting imine could not be isolated and after lyophilization, the NMR spectrum revealed a mixture of decomposition products. Besides, when the reaction mixture was left for more than 10 days, the solution turned dark and no single compound could be assigned by NMR. A similar result was also found for the ^{t}Boc deprotection of unphosphated derivative of **5**.

3. Conclusions

The results obtained demonstrated that DHAP-aldolases tolerate a variety of *N*-protecting groups for the 3-amino-propanal. The outcome of the reaction performance, however, depended on the protecting group. Thus, ca. 70% conversion to aldol adduct were achieved with PhAc



Scheme 2. Deprotection of **13** by penicillin G acylase.



Scheme 3. Deprotection of **5** by aqueous TFA (6%).

and ^{t}Boc, similar to those obtained before with Cbz, whereas Fmoc gave ca. 20% conversion. Modifications on the protecting group structure, however, did not affect the stereoselectivity of the aldolases to a significant extent, nor the inversion of FucA stereoselectivity towards the *N*-protected derivatives of 3-aminopropanal. *N*-PhAc and ^{t}Boc adduct product derivatives were successfully deprotected by PGA-catalyzed hydrolysis at pH 7 and with aqueous TFA (6% v/v) respectively. However, the corresponding six-membered imine sugar could not be isolated, even though under the mild reactions conditions used with the PGA. When, *N*-PhAc was deprotected from the unphosphated derivatives a five-membered iminocyclytol was identified. When both phosphated or Boc derivatives were deprotected a complex NMR spectra were recorded after lyophilization with signals that presumably belong to a number of decomposition products. This behaviour may be due to the presence of a highly reactive primary amine and a keto group in the molecule, which lead to a number of unexpected reactions. The situation was different with the previously reported benzyloxycarbonyl group (Cbz).[5,6] In this case, the hydrogenolysis of the Cbz and the reductive amination took place in one pot reaction, safely catching the imine intermediate being less prone to side reactions.

Protecting groups such as PhAc, ^{t}Boc and, Cbz provide also a range of removal conditions to fulfill most of the required orthogonalities for functional group manipulation on the 2-ketoaminodiols.

4. Experimental

4.1. Materials

Fructose-1,6-diphosphate aldolase from rabbit muscle (RAMA; EC 4.1.2.13, crystallized, lyophilized powder, 19.5 U mg^{-1}) was from Fluka (Buchs, Switzerland). Rhamnulose 1-phosphate aldolase (RhuA; EC 4.1.2.19, suspension 100 U mL^{-1}) was kindly donated by Boehringer Mannhein (Mannhein, Germany). L-Fuculose-1-phosphate aldolase (FucA, EC 4.1.2.17, lyophilized 500–800 U g^{-1}) was from Departament d'Enginyeria Química of the Universitat Autònoma de Barcelona, produced from a recombinant *E. coli* (ATCC no. 86984) and purified by affinity chromatography. Acid phosphatase (PA, EC 3.1.3.2, 5.3 U mg^{-1}) was from Sigma (St. Louis, USA). Penicillin Amidase, immobilized on Eupergit® C from *E. coli* (EC 3.5.1.11, 100 U g^{-1} immobilized preparation) was from Fluka. Non-ionic polyoxyethylene ether surfactant with an average of 4 mol of ethylene oxide per surfactant molecule ($C_{14}E_4$) was from Albright and Wilson (Barcelona, Spain). The precursor of dihydroxyacetone phosphate (DHAP), dihydroxyacetone phosphate dimer bis (ethyl ketal), was synthesized in our lab using a procedure described by Jung et al.[18] with slight modifications.

Molecular modelling. Molecular simulations were conducted with the programs MOE (v. 2004.03, Chemical Computing Group, Montreal). The conformational space of all the possible diastereoisomers of adducts **4–6** was exhaustively searched using the systematic conformational search algorithm implemented in MOE, and the conformations generated were minimized and ranked according to their energy, as previously decribed.[5] These energy calculations were carried out using the implemented MMFF94x force field with its standard atomic charges and parameters,[19] and the Born continuum solvation model[20–22] without cut-offs. Geometries were optimized up to an RMS gradient <0.01.

Docked structures of the stereoisomers of products **4–6** in the active centres of RAMA and RhuA were determined by sampling the conformational space of the products in the enzyme environment. The methodology used was similar to that previously described.[5] However, in this case we used the above mentioned MMFF94x force field and the Born continuum salvation model, with a smoothed cut-off between 14 and 15 Å to model the nonbonded interactions.

4.2. General methods

HPLC analyses. HPLC analyses were performed on a RP-HPLC cartridge, 250×4 mm filled with Lichrosphere® 100, RP-18, 5 μm from Merck (Darmstadt, Germany). Samples (50 mg) were withdrawn from the reaction medium, dissolved with methanol to stop any enzymatic reaction, and analyzed subsequently by HPLC. The solvent system was the following: solvent A: 0.1% v/v trifluoroacetic acid (TFA) in H_2O, solvent B: 0.095% v/v TFA in H_2O/CH_3CN 1:4. Elution conditions for *N*-PhAc and *N*-^{t}Boc derivatives: isocratic 10% B during 2 min followed by a gradient from 10 to 33% B over 18 min; elution conditions for *N*-Fmoc derivatives: gradient from 30 to 90% B over 30 min, always

at a flow rate of 1 mL min^{-1} and detection at 215 nm. Retention factors (k') for the acceptor aldehydes and condensation products are given below.

NMR analysis. High field ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses were carried out at the Servei de Ressonància Magnètica Nuclear, Universitat Autònoma de Barcelona using an AVANCE 500 BRUKER spectrometer for D_2O solutions. Full characterization of the described compounds was performed using typical gradient-enhanced 2D experiments: COSY, NOESY, HSQC and HMBC, recorded under routine conditions. When possible, NOE data was obtained from selective 1D NOESY versions using a single pulsed-field-gradient echo as a selective excitation method and a mixing time of 500 ms. When necessary, proton and NOESY experiments were recorded at different temperatures in order to study the different behaviour of the exchange phenomena to avoid the presence of false NOE cross-peaks that difficult both structural and dynamic studies. ^1H (300 MHz) and ^{13}C NMR (75 MHz) spectra were carried out at the Instituto de Investigaciones Químicas y Ambientales-CSIC.

Elemental analyses. Elemental analyses were performed by the Servei de Microanàlisi Elemental IIQAB-CSIC.

4.3. Synthesis of *N*-protected amino aldehydes

The synthesis of *N*-protected amino aldehydes was carried out in two steps. First, the *N*-protected 3-aminopropanol was obtained and second the oxidation of the alcohol group to aldehyde was performed.

4.4. Synthesis of *N*-protected amino alcohols

4.4.1. *N*-(3-Hydroxypropyl)-2-phenylacetamide. To a cooled (−20 °C) solution of 3-amino-1-propanol (9.2 mL, 122.5 mmol) in CH_2Cl_2 (40 mL) was added dropwise phenylacetyl chloride (8 mL, 60.1 mmol) in CH_2Cl_2 (4 mL) and, simultaneously, an aqueous solution of NaOH (2.4 g, 60 mmol) in water (8 mL) under vigorous stirring. After the addition was complete, the reaction was kept at −20 °C for 2 h and then allowed to warm to room temperature under stirring overnight. Then, the crude reaction mixture was evaporated under vacuum to dryness, the residue was dissolved with ethyl acetate and washed successively with citric acid 5% w/v (3×50 mL), $NaHCO_3$ 10% w/v (3×50 mL) and brine (3×50 mL). After being dried over Na_2SO_4, the organic layer was evaporated under reduced pressure to yield **3** as a white solid (8.1 g, 70%, 99% pure by HPLC). The ^1H and ^{13}C NMR spectra were consistent with those reported in the literature.[23]

4.4.2. *tert*-Butyl-3-hydroxypropylcarbamate. To a solution of 3-amino-1-propanol (4.3 mL, 57.1 mmol) in CH_2Cl_2 (3 mL) at 25 °C was added dropwise a solution of $(Boc)_2O$ (12.5 g, 57.1 mmol) in CH_2Cl_2 (7 mL). After 12 h, the mixture was worked up as described above to yield **4** as a colourless oil (6.4 g, 64%, 99% pure by HPLC), whose ^1H and ^{13}C NMR spectra matched those reported.[24]

4.4.3. Fluoren-9-yl-3-hydroxypropylcarbamate. The synthesis of the title compound was performed by a procedure described previously in our lab using Fmoc-OSu as acylating agent.[5] The compound was obtained as a white solid (3.6 g, 90%, 99% pure by HPLC). The ^1H NMR spectrum matched that reported.[25] ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 157.3 (CONH), 143.7, 141.2, 127.6, 126.9, 124.9 (Fmoc), 66.5 (OCH_2), 59.3 (CH_2OH), 47.2 ($NHCH_2$), 37.4 (*C*H), 32.5 (CH_2CH_2OH).

4.5. Synthesis of *N*-protected aminoaldehydes

The synthesis of *N*-protected amino aldehydes was achieved by 2-iodoxobenzoic acid (IBX) oxidation method.[26,27] Caution! IBX has been reported to detonate upon heavy impact and/or heating over 200 °C. To a solution of the *N*-protected amino aldehyde (10–19 mmol) in DMSO (60–120 mL), IBX (24–48 mmol) was added. The reaction was monitored by HPLC until no alcohol was detected. At this point, the reaction mixture was diluted with water (30–60 mL) and the mixture was extracted with ethyl acetate (3×75–100 mL). The organic layers were pooled, washed with $NaHCO_3$ 5% (w/w) (3×100 mL) and brine (3×100 mL), dried over Na_2SO_4 and evaporated under reduced pressure.

4.5.1. *N*-(3-Oxopropyl)-2-phenylacetamide (PhAc-aminopropanal) (1). The title compound (1.04 g) was obtained as a white solid in 52% yield by using the above general procedure. HPLC $k'=7.05$. ^1H NMR (300 MHz, $CDCl_3$, ppm): $\delta=9.7$ (1H, s, CHO), 3.5 (2H, s, CH_2CO), 3.4 (2H, q, $NHCH_2$), 2.6 (2H, t, CH_2CHO). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 201.0 (CHO), 171.1 (CONH), 43.6 ($NHCH_2$), 33.0 (CH_2CHO).

4.5.2. *tert*-Butyl-3-oxoethylcarbamate (^{t}Boc-aminopropanal) (2). The title compound (1.7 g) was obtained as a pale yellow oil in 90% yield by using the above general procedure. HPLC $k'=9.17$. ^1H NMR[28] (300 MHz, $CDCl_3$, ppm): 9.7 (1H, s, CHO), 5.0 (1H, br, NH), 3.3 (2H, q, $NHCH_2$), 2.6 (2H, t, CH_2CHO), 1.3 (9H, s, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 201 (CHO), 155.7 (CONH), 79.3 ($OC(CH_3)_3$), 44.2 ($NHCH_2$), 33.9 (CH_2CHO), 28.3 (CH_3).

4.5.3. Fluoren-9-yl-3-oxoethylcarbamate (Fmoc-aminopropanal) (3). The title compound (1.7 g) was obtained as a pale yellow solid in 98% yield by using the above general procedure. HPLC $k'=9.42$. The ^1H NMR spectrum matched that reported.[29] ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 201.2 (CHO), 157.0 (CONH), 143.7, 141.2, 127.5, 126.9, 124.9 (Fmoc), 66.6 (OCH_2), 47.1 (CH_2CHO), 43.9 (*C*H), 34.3 ($NHCH_2$).

4.6. Enzymatic aldol condensations

Enzymatic aldol condensations in emulsions. Reactions were carried out in 10 mL test tubes with screw caps. The aldehyde (0.23–0.90 mmol), the oil (6% w/w) and the surfactant (4% w/w) were mixed vigorously. Then, the DHAP solution (0.13–0.50 mmol) at pH 6.9, freshly prepared as described by Effenberger et al.,[30] was added dropwise while stirring at 25 °C with a vortex mixer. The final reaction volume was 5 mL. Finally, RAMA (100 U), RhuA (2 U) or FucA (40 U) was added and mixed again.

The test tubes were placed on a horizontal shaking bath (100 rpm) at constant temperature (25 °C). The reactions were followed by HPLC until the peak of the product reached a maximum. The enzymatic reactions were stopped by addition of MeOH. Then, the methanol was evaporated and the aqueous solution washed with ethyl acetate to remove the unreacted *N*-protected amino-aldehyde. The aqueous layer was collected and lyophilized. The residue was dissolved in water, adjusted to pH 3 with trifluroacetic acid (TFA) and purified by reversed phase HPLC on a Perkin-Elmer semipreparative 250×25 mm column, filled with C18, 10 μm type stationary phase and eluted using a CH_3CN gradient (8–56% in 30 min; 24–72% in 30 min for the Fmoc derivative) in 0.10% (v/v) aqueous TFA. The best fractions were pooled, diluted, re-loaded onto the column and eluted with a CH_3CN gradient (0% 10 min and then 0–56% in 30 min) in plain water to eliminate the TFA. The pure fractions were pooled and lyophilized.

Enzymatic aldol condensations in mixtures water/dimethylformamide 4:1. Reactions were carried out in 10 mL test tubes with screw caps. The aldehyde (0.4–0.9 mmol) was dissolved in DMF 20% (v/v). Then, the DHAP solution (0.23–0.50 mmol), prepared as described above, was added dropwise while mixing. The rest of the experimental procedure was identical to that described for the reaction in emulsions.

The yields of the compounds **4**–**12** correspond to the amounts from of the aldol enzymatic reactions at semipreparative level. Most of them contained salts from the purification process. The purification procedures were not optimized.

4.6.1. (3*S*,4*R*)-5,6-Dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt and (3*S*,4*S*)-5,6-dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt (4). The title compounds were obtained as a mixture in a proportion of 89:11, respectively, following the general methodology described above. 219 mg, 35%, 99.5% purity by HPLC ($k'=2.79$). $[\alpha]_D^{20}$ +6.7 (*c* 1 in H_2O/MeOH 5:95) and $[\alpha]_D^{20}$ +12.9 (*c* 1 in H_2O/MeOH 1:1). ^1H NMR (500 MHz, D_2O, ppm): δ 7.24 (5H, m, Ph), 4.55 (2H, dd, $J=6.3$, 18.8 Hz, CH_2OP), 4.27 (1H, d, $J=1.7$ Hz, C*H*OH), 4.00 (1H, br t, $J=5.9$ Hz, C*H*(*R*)OH), 3.47 (2H, s, PhCH_2), 3.18 (2H, t, $J=6.9$ Hz, NHCH_2), 1.66 (2H, m, NHCH$_2$CH_2C(*R*)HOH); minor signals corresponding to the diastereomer 3*S*,4*S*: δ 3.79 (1H, m, C*H*(*S*)OH), 1.56 (2H, m, NHCH$_2$CH_2CH(*S*)OH). ^{13}C NMR (125 MHz, D_2O, ppm): δ 211.1 (CO), 174.5 (OCONH), 134.9 (C ar), 128.9 (CH ar), 128.7 (CH ar), 127.1 (CH ar), 77.4 (CHOH), 69.0 (CHOH), 67.8 (CH_2OP), 42.2 (CH_2), 36.1 (CH_2), 31.6 (CH_2). (Found: C, 38.22; H, 4.62; N, 3.22. $C_{14}H_{18}NO_8Na_2P\cdot 1/2H_2O\cdot 1/2NaCl$ requires: C, 37.92; H, 4.32; N, 3.16%).

4.6.2. (3*S*,4*R*)-5,6-Dideoxy-{[(*tert*-butyloxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*S*,4*S*)-5,6-dideoxy-{[(*tert*-butyloxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt (5). The title compounds were obtained as a mixture in a proportion of 93:7, respectively, following the general methodology described above. 203 mg, 47%, 99.5% purity by HPLC ($k'=3.81$). $[\alpha]_D^{20}$ +18.8 (*c* 1 in H_2O/MeOH 5:95). ^1H NMR (500 MHz, D_2O, ppm): δ 4.56 (2H, dd, $J=5.9$, 6.3, 18.9 Hz, CH_2OP), 4.21 (1H, d, $J=1.4$ Hz, C*H*OH), 4.03 (1H, br t, $J=7.7$ Hz, C*H*(*R*)OH), 3.06 (2H, m, NHCH_2), 1.64 (2H, br m, CH_2), 1.30 (9H, s, CH_3); minor signals corresponding to the diastereoisomer 3*S*,4*S*: δ 4.3 (1H, s, C*H*(*S*)OH), 3.84 (1H, m, C*H*OH), 1.53 (2H, m, CH_2). ^{13}C NMR (125 MHz, D_2O, ppm): δ 210.1 (CO), 158.0 (OCONH), 80.6 (C), 77.5 (CHOH), 68.9 (CHOH), 67.70 (CH_2OP), 36.6 (CH_2), 32.2 (CH_2), 27.5 (CH_3); minor signals corresponding to the diastereoisomer 3*S*,4*S*: 77.6 (CHOH), 69.4 (CHOH), 68.4 (CH_2OP). (Found: C, 31.83; H, 5.62; N, 3.34. $C_{11}H_{20}NNa_2O_9P\cdot 3/2H_2O$ requires: C, 31.89; H, 5.60; N, 3.38%).

4.6.3. (3*S*,4*R*)-5,6-Dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*S*,4*S*)-5,6-dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]-amino}-1-*O*-phosphonohex-2-ulose sodium salt (6). The title compounds were obtained as a mixture in a proportion of 92:8, respectively, following the general methodology described above. 31 mg, 23%, 99.5% purity by HPLC ($k'=$ 7.48 broad peak). Due to signal overlapping and for the sake of simplicity, the NMR spectra were recorded for the unphosphated derivatives of **6**. ^1H NMR (500 MHz, D_2O, ppm): δ 7.84 (2H, d, $J=7.5$ Hz, Ph), 7.66 (2H, d, $J=$ 7.4 Hz, Ph), 7.41 (2H, t, $J=7.4$ Hz, Ph), 7.33 (2H, t, $J=$ 7.4 Hz, Ph), 4.50 (2H, dd, $J=19.3$, 46.6 Hz, CH_2OP), 4.37 (2H, m, CH_2O), 4.22 (H, t, $J=6.8$ Hz, C*H*–CH_2O), 4.14 (H, d, $J=2.1$ Hz, C*H*(R)OH), 4.01–3.98 (1H, m, C*H*OH), 3.29–3.22 (2H, m, NHCH_2), 1.82–1.72 (2H, m, NHCH$_2$-CH_2C(*R*)HOH). Signals corresponding to the diastereomer (4*S*): 4.10 (H, d, $J=5.7$ Hz, C*H*(R)OH), 3.87 (1H, br, C*H*OH). ^{13}C NMR (125 MHz, D_2O, ppm): δ 215.8 (CO), 161.4 (OCONH), 147.8 (C), 145.0 (C) 131.2, 130.6, 128.6, 123.4 (arom), 81.9 (*C*H(OH)CO), 73.8 (*C*H(OH)-CH(OH)CO), 70.3 (*C*H$_2$–O–CO), 70.1 (*C*H$_2$OH), 48.22 (*C*H–CH$_2$–O–), 41.0 (CH_2), 36.8 (CH_2). (Found: C, 22.36; H, 1.15; N, 0.48. $C_{21}H_{22}NNa_2O_9P\cdot 7NaCl\cdot 7CF_3COONa$ requires: C, 22.47; H, 1.19; N, 0.75%). Unphosphated derivative: (Found: C, 62.28; H, 6.44; N, 3.64. $C_{21}H_{23}NO_6\cdot H_2O$ requires: C, 62.52; H, 6.27; N, 3.45%).

4.6.4. (3*R*,4*S*)-5,6-Dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*R*)-5,6-dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt (7). The title compounds were obtained as a mixture in a proportion of 81:19, respectively, following the general methodology described above. 376 mg, 49%, 99.5% purity by HPLC ($k'=2.79$). $[\alpha]_D^{20}$ −12.0 (*c* 1 in H_2O/MeOH 5:95). NMR spectra were undistinguishable from those obtained for the corresponding diastereoisomers **4**. (Found: C, 34.31; H, 3.68; N, 2.47 $C_{14}H_{18}NNa_2O_8P\cdot CF_3COONa$ NaCl requires: C, 34.36; H, 3.60; N, 2.50%).

4.6.5. (3*R*,4*S*)-5,6-Dideoxy-{[(*tert*-butyloxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*R*)-5,6-dideoxy-{[(*tert*-butyloxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt (8). The title compounds were obtained as a mixture in a proportion of 70:30, respectively, following the general methodology

described above. 115 mg, 37%, 99.5% purity by HPLC ($k'=3.81$). $[\alpha]_D^{20}$ −11.8 (*c* 1 in H_2O/MeOH 5:95). NMR spectra were undistinguishable from those obtained for the corresponding diastereoisomers **5**. (Found: C, 30.53; H, 5.40; N, 3.14. $C_{11}H_{20}NNa_2O_9P\cdot 1/2H_2O\cdot NaCl$ requires: C, 30.53; H, 5.12; N, 3.24%).

4.6.6. (3*R*,4*S*)-5,6-Dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*R*)-5,6-dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]-amino}-1-*O*-phosphonohex-2-ulose sodium salt (9). The title compounds were obtained as a mixture in a proportion of 77:23, respectively, following the general methodology described above. 12 mg, 4%, 99.5% purity by HPLC ($k'=$ 7.48 broad peak). Due to signal overlapping and for the sake of simplicity, the NMR spectra were recorded for the unphosphated derivatives and were undistinguishable from those obtained for the corresponding diastereoisomers **6**. (Found: C, 34.97; H, 4.23; N, 2.15. $C_{21}H_{22}NNa_2O_9P\cdot 5H_2O\cdot 2NaCl$ requires: C, 35.21; H, 4.50; N, 1.96%); Unphosphated derivative: (Found: C, 59.21; H, 6.36; N, 3.07. $C_{21}H_{23}NO_6\cdot 9/4H_2O$ requires: C, 59.22; H, 6.51; N, 3.29%).

4.6.7. (3*R*,4*R*)-5,6-Dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*S*)-5,6-dideoxy-[(phenylacetyl)amino]-1-*O*-phosphonohex-2-ulose sodium salt (10). The title compounds were obtained as a mixture in a proportion of 33:67, respectively, following the general methodology described above. 255 mg, 35%, 99.5% purity by HPLC ($k'=2.79$). $[\alpha]_D^{20}$ −11.4 (*c* 1 in H_2O/MeOH 5:95). NMR spectra were undistinguishable from those obtained for the corresponding diastereoisomers **4**. (Found: C, 34.52; H, 4.32; N, 3.14. $C_{14}H_{18}NNa_2O_8P\cdot 3/2H_2O$ NaCl requires: C, 34.27; H, 4.31; N, 2.85%).

4.6.8. (3*R*,4*R*)-5,6-Dideoxy-{[(*tert*-butyloxy)carbonyl]-amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*S*)-5,6-dideoxy-{[(*tert*-butyloxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt (11). The title compounds were obtained as a mixture in a proportion of 33:67, respectively, following the general methodology described above. 143 mg, 22%, 99.5% purity by HPLC ($k'=3.81$). $[\alpha]_D^{20}$ −10.4 (*c* 1 in H_2O/MeOH 5:95). NMR spectra were undistinguishable from those obtained for the corresponding diastereoisomers **5**. (Found: C, 30.58; H, 5.63; N, 3.30. $C_{11}H_{20}NNa_2O_9P\cdot 5/2H_2O$ requires: C, 30.56; H, 5.83; N, 3.24%).

4.6.9. (3*R*,4*R*)-5,6-Dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]amino}-1-*O*-phosphonohex-2-ulose sodium salt and (3*R*,4*S*)-5,6-dideoxy-{[(fluoren-9-ylmetoxy)carbonyl]-amino}-1-*O*-phosphonohex-2-ulose sodium salt (12). The title compounds were obtained as a mixture in a proportion of 21:79, respectively, following the general methodology described above. 41 mg, 22% (contained some salts), 99.5% purity by HPLC ($k'=7.48$ broad peak). Due to signal overlapping and for the sake of simplicity, the NMR spectra were recorded for the unphosphated derivatives and were undistinguishable from those obtained for the corresponding diastereoisomers **6**. (Found: C, 21.70; H, 1.75; N, 0.82. $C_{21}H_{22}NNa_2O_9P\cdot H_2O\cdot 10NaCl\cdot 2CF_3COONa$ requires: C, 21.70; H, 1.72; N, 1.01%). Unphosphated derivative: (Found: C, 61.12; H, 6.65; N, 3.13. $C_{21}H_{23}NO_6\cdot 3/2H_2O$ requires: C, 61.16; H, 6.35; N, 3.40%).

4.7. Removal of protecting groups

4.7.1. Removal of phosphate group. The phosphate group of compounds **4**–**12** was removed by hydrolysis catalyzed by acid phosphatase following the procedure described by Bednarski et al.[31] The reaction was followed by HPLC until no starting material was detected. Then the crude was desalted by HPLC and lyophilized.

4.7.2. Removal of *N*-phenylacetyl (PhAc) protecting group by penicillin G acylase. Compound **4**, or the corresponding unphosphated analogue (0.320 mmol, 90 mg) was dissolved in plain water (5 mL). To this solution was added penicillin amidase immobilized on Eupergit® (100 mg). The pH was controlled by a pH meter and maintained between 6.5 and 7 by additions of NaOH 0.1 M. Samples were withdrawn every 45 min and analyzed by HPLC, a peak corresponding to the phenylacetic acid appeared. When no signal of the starting material was detected, the immobilized enzyme was filtered off. Then, the phenylacetic acid was eliminated by anion exchange chromatography on a Macroprep High-Q support eluting with plain water. Finally, the product, which was not retained under the elution conditions, was lyophilized.

4.7.3. Removal of *N*-*tert*-butyloxycarbonyl (^{t}Boc) protecting group by trifluoroacetic acid. Compound **5**, or the corresponding unphosphated analogue, (0.162 mmol) was dissolved in plain water (9 mL). To this solution was added an aqueous solution of TFA (1.2 mL, 1:1 TFA/H_2O). Samples were withdrawn every 45 min and analyzed by HPLC. After 14 h, no signal of the starting material was detected. Then the crude was lyophilized.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2005.12.031. ^{1}H NMR and ^{13}C spectra of **4**–**12**, ^{1}H NMR spectrum of the residue after work up and lyophilization of the penicillin acylase-catalyzed removal of phenylacetyl amino protecting group of **5**; ^{1}H NMR reaction monitoring of the deprotection reaction using penicillin acylase and ^{1}H NMR spectrum of **15**–**17** are supplied.

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