

L-Rhamnulose-1-phosphate Aldolase from *Thermotoga maritima* in Organic Synthesis: One-Pot Multistep Reactions for the Preparation of Imino- and Nitrocyclitols

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Received: February 13, 2015; Revised: April 14, 2015; Published online: May 18, 2015



Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/adsc.201500187>.

Abstract: Rhamnulose-1-phosphate aldolase from *Thermotoga maritima* (Rhu1PAT_m) has been recently cloned and characterised. This hyperthermophilic enzyme offers intriguing possibilities for practical catalysis. This is due to its high stability under extreme reaction conditions, such as high temperature or the presence of organic co-solvents. The Rhu1PAT_m potentiality in organic synthesis has been explored focusing on (i) the reaction stereocontrol, (ii) the possibility of combining it with other mesophilic enzymes in multienzyme systems and (iii) its application to the synthesis of imino- and nitrocyclitols. In our study, the diastereoselectivity of Rhu1PAT_m was similar to the one reported for Rhu1PA from *Escherichia coli* (Rhu1PA_{Ec}). However, we observed significant differences for some aldehyde acceptors.

Indeed, the diastereoselectivity control was not complete, since mixtures of *L*-threo (2*R*,3*S*; natural stereopreference) and *D*-erythro (2*R*,3*R*) diastereoisomers were obtained as described for Rhu1PA_{Ec}. Rhu1PAT_m was able to catalyse aldol reactions using nitroaldehydes and *N*-Cbz-aminoaldehydes. Conversion of the selected nitroaldehydes was complete, leading to mixtures of *L*-threo and *D*-erythro diastereoisomers, in 95:5 and 75:25 ratios. When *N*-Cbz-aminoaldehydes were used, aldol reaction yields were lower and the stereoselectivity *L*-threo:*D*-erythro ranged between 32:68 to >95:5.

Keywords: aldol reaction; azasugars; biocatalysis; cascade reactions; lyases; thermophilic enzymes

Introduction

Carbon-carbon (C–C) bond formation is one of the cornerstone reactions in synthetic organic chemistry.^[1] In this sense, the catalytic asymmetric C–C bond formation by the aldol addition reaction has long been recognized as one of the most useful tools for the construction of new C–C bonds, since the formation of one or two new stereocentres is concomitant with the bond forming process.^[2] The use of enzymes as catalysts is a good alternative to non-enzymatic synthesis. This is because they provide a number of advantages,

such as their ability to carry out the reactions under moderate conditions of temperature, pH and pressure, high efficiency and regio-, chemo- and stereoselectivity.^[3] Enzymes also represent an environmentally attractive option because they can catalyse reactions in water and are biodegradable.^[4] The high stereoselectivity of aldolases in C–C bond formation gives them a wide range of synthetic applications. Among the different families of aldolases, dihydroxyacetone phosphate (DHAP)-dependent aldolases have particularly attracted the attention of chemists. Furthermore, they have been extensively used in chemoenzymatic syn-

theses.^[5] DHAP-dependent aldolases lead to the formation of two new stereocentres with a high degree of stereochemical control. Despite this well proven synthetic utility of aldolases, there are still few processes that have been developed at an industrial scale and none of them – to the best of our knowledge – used DHAP-dependent aldolases.^[6] This is unfortunate, considering the recent advances in our understanding of the mechanism of aldolase catalysis. This has allowed the successful application of many different engineering approaches to alter the catalytic properties of aldolases such as stability, substrate specificity and stereoselectivity.^[7]

One of the main difficulties in applying these processes at an industrial scale is the lack of stability under the reaction conditions. An approach to overcome this issue is the use of enzymes from extremophile microorganisms.^[8] From a biocatalytical point of view, thermophilic and hyperthermophilic microorganisms have attracted particular attention, since they are sources of thermostable enzymes. Thermozymes display outstanding stability against high temperature, as well as against other denaturalisation conditions, such as the presence of organic solvents, detergents or extreme pH values.^[9] In this sense, we have recently described the heterologous expression of the rhamnulose-1-phosphate aldolase from *Thermotoga maritima*, its functional characterisation and preliminary synthetic properties.^[10] This enzyme presented an optimum temperature at 95°C and displayed a great stability to extreme reaction conditions, such as high temperatures (i.e., half-life of more than 3 h at 115°C) and in the presence of different organic solvents. Furthermore, Rhu1PAT_m showed activity in a wide range of pH, with an optimum activity at pH at 8.0. A multienzyme system^[11b] previously developed by the García-Junceda group, coupling the *in situ* phosphorylation of DHA with the aldolase catalysed aldol reaction, was successfully assayed with Rhu1PAT_m.^[11] Rhu1PAT_m was assayed using the one-pot/two-steps protocol with four different aldehydes: L-lactaldehyde, benzyloxyacetaldehyde, phenylacetaldehyde and ethyl 3-methyl-4-oxocrotonate. Rhu1PAT_m was able to react with these four aldehydes at 45°C and 80°C in combination with other mesophilic enzymes, in a one-pot/two-steps approach. The reactions were significantly faster at the highest temperature.^[10]

Herein, we describe a study of the synthetic utility of the Rhu1PAT_m, focusing on the stereocontrol of the aldol reaction, its ability to integrate with other mesophilic enzymes into multienzyme systems and, finally, its application to the synthesis of imino- and nitrocyclitols.

Results and Discussion

Study of the Stereochemical Outcome of Rhu1PAT_m-Catalysed Reactions

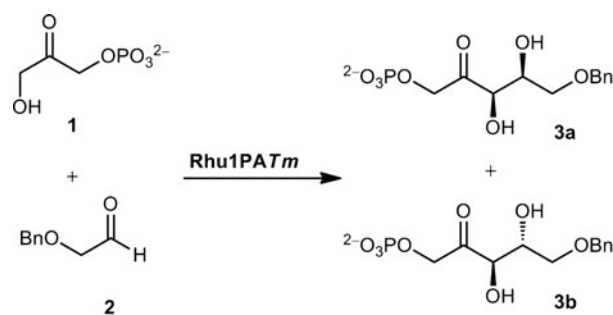
We first performed a study on the stereochemical outcome of the reaction catalysed by Rhu1PAT_m as a function of the temperature. To this end, the percentage of each diastereoisomer formed in the aldol addition reaction of DHAP to benzyloxyacetaldehyde **2** was measured at different times (Scheme 1). Reactions were conducted at 4°C, 25°C, 45°C and 80°C.

As expected, the diastereoisomer *L-threo* (3*R*,4*S*), which is the one of the natural preference of the enzyme, was synthesised faster (i.e., kinetic product) than the *D-erythro* counterpart. In particular, this difference was highlighted at 4°C due to the slowing down of the reaction rate (Figure 1A). In all temperatures assayed, the ratio *threo:erythro* decreased with the progress of the reaction, until reaching a plateau at a 2:1 ratio (Figure 1).

However, thermodynamic equilibrium was reached faster with the increase of temperature. A similar result was obtained when Rhu1PAE_c was used with (*R*)-*N*-Cbz-2-aminohexanal as substrate. In this case, the diastereoisomer ratio obtained did not vary at different reaction temperatures. However, the *L-threo*:*D-erythro* ratio changed with the temperature when (*R*)-*N*-Cbz-2-aminopentanal or *N*-Cbz-2-amino-4-methylpentanal were used as acceptor substrates.^[12] Thus, it cannot be excluded that the Rhu1PAT_m stereoselectivity could change with the temperature when using other aldehydes as substrates.

Synthesis of Nitrocyclitols Mediated by Rhu1PAT_m

Nitrocyclitols belong to the large family of cyclitols and are essentially found as synthetic precursors of aminocyclitols.^[13] These amines are present in many pharmacologically significant compounds exhibiting antitumour, antibacterial, antifungal, antimalarial, and



Scheme 1. Aldol addition reaction of DHAP (**1**) – *in situ* obtained by enzymatic phosphorylation of DHA – to benzyloxyacetaldehyde **2** catalysed by Rhu1PAT_m.

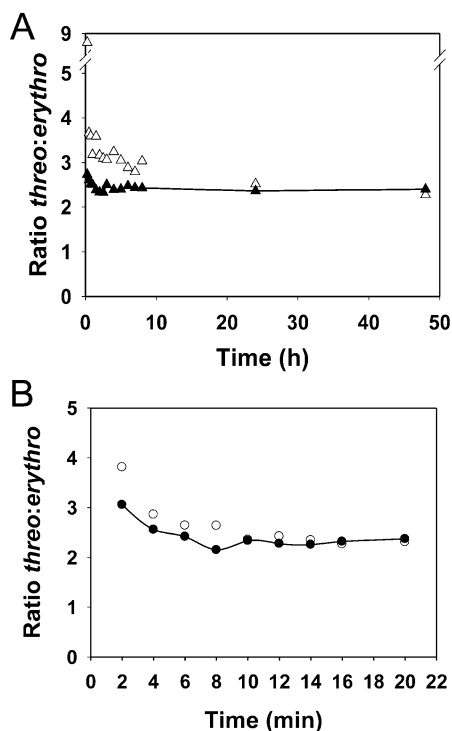


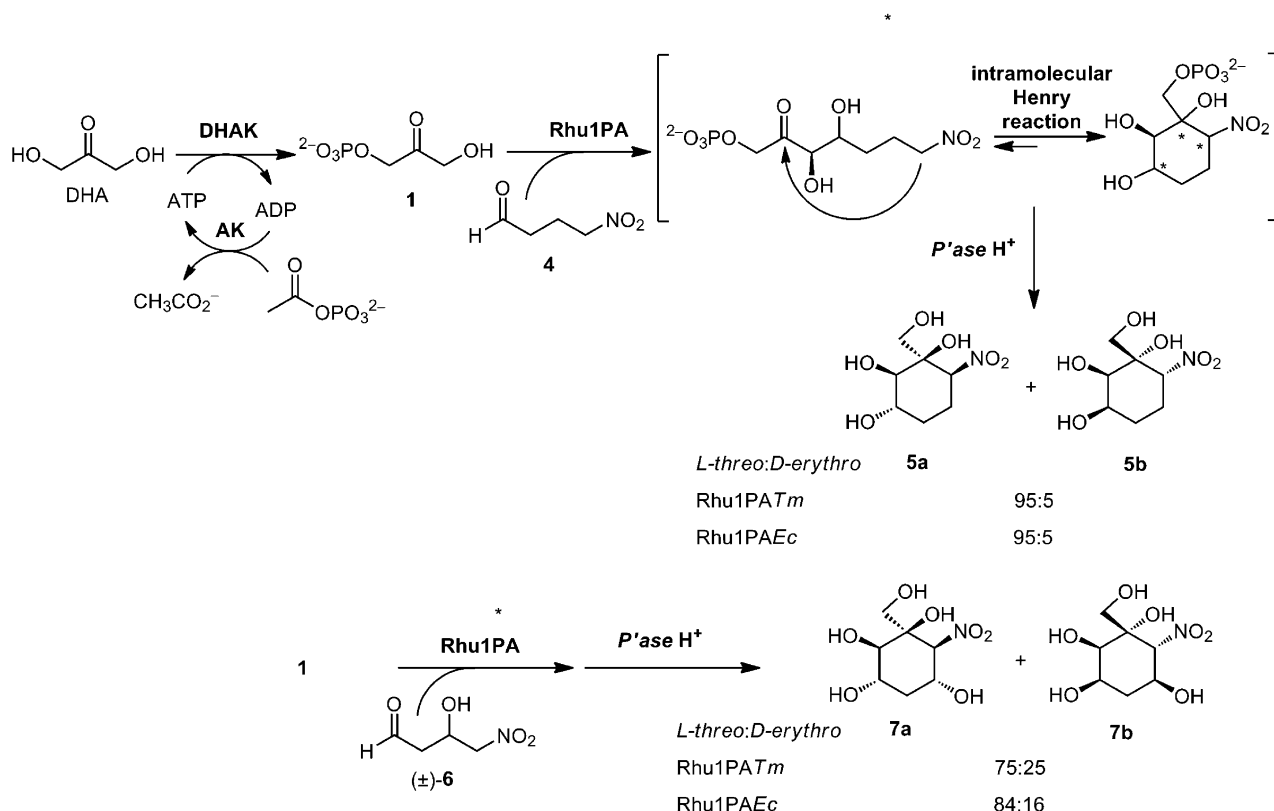
Figure 1. Diastereoisomer *L*-threo:*D*-erythro ratio obtained in the aldol reaction of DHAP to **2** catalysed by Rhu1PATm at 4°C (A; Δ), 25°C (A; ▲), 45°C (B; ○) and 80°C (B; ●).

antiviral activities.^[14] Consequently, in the last decade there has been an increasing demand for the development of appropriate synthetic procedures to prepare cyclitols as well as analogues with enhanced or more selective biological profiles.^[15]

Lemaire's group reported a straightforward chemo-enzymatic cascade to access nitro- and aminocyclitols. This process involved two stereoselective C–C bond formations. One was catalysed by an aldolase and the other was a spontaneous intramolecular Henry reaction.^[16] Herein, we have studied the application of the Rhu1PATm in the context of one-pot/two-steps chemoenzymatic cascade to access to nitrocyclitols (Scheme 2).

Aldehydes 4-nitrobutanal (**4**) and (±)-3-hydroxy-4-nitrobutanal [(±)-**6**] were used as acceptor substrates in the aldol addition to DHAP catalysed by Rhu1PATm. When the DHAP concentration reached its maximum, the aldolase and aldehydes **4** or (±)-**6** were added.

In the first attempt, the aldol addition of DHAP to **4** at a 0.1-mmol scale, with a DHAP/nitroaldehyde ratio of 2:1 at 45°C failed. Unfortunately, nitroaldehyde **4** was labile at this temperature and induced DHAP degradation. Thus, reactions were carried out



Scheme 2. Cascade reactions for the synthesis of nitrocyclitols. First step: phosphorylation of DHA catalysed by recombinant ATP-dependent DHAK from *Citrobacter freundii* CECT 4626 (AK=acetate kinase);^[18] second step: aldol reaction catalysed by Rhu1PA and *in situ* intramolecular Henry reaction; third step: hydrolysis of the phosphate group using acid phosphatase. Data for Rhu1PAEc, taken from ref.^[17]

Table 1. Percentage of aldol adducts (**11**, **14a–d**, and **23a, b**) formed in analytical scale reactions catalysed by Rhu1PATm.

Aldehyde	Aldol product [%] ^[a]	Optimum reaction time [min] ^[b]
10	11 (51)	15
13a	14a (61)	20
13b	14b (74)	30
13c	14c (39)	60
13d	14d (49)	10
22a	23a (37)	10
22b	23b (38)	30

^[a] Percentage of aldol adduct formed determined by HPLC using an external standard method.

^[b] Time at which maximum accumulation of aldol adduct was reached.

at 25°C. In spite of its hyperthermophile nature, Rhu1PATm retains between 5 and 10% of its maximum activity at 25°C.^[10] In these conditions, aldehyde **4** led to nitrocyclitols **5a** and **5b** and aldehyde (\pm)-**6** led to nitrocyclitols **7a** and **7b** (Scheme 2). These compounds were isolated after purification in 70% and 71% yields for each couple of isomers, respectively. These compounds are identical to those previously described.^[17] Their configurations were established on the basis of the 3*R* stereoselectivity of the enzyme and ¹H NMR analysis.

The stereoselectivity shown by the aldolase depended on the aldehyde acceptor (Table 1). Using the aldehyde **4** the major diastereoisomer obtained was the *L*-threo, which corresponds to the expected stereochemical preference for the Rhu1PA (Table 1).

The data presented in this work were consistent with the ones previously described using Rhu1PAEc as catalyst.^[17] Using (\pm)-**6** the actual stereoselectivity of Rhu1PATm could not be disclosed. The reaction produced **7a** and **7b** and the enzymatic aldol addition appeared to virtually produce exclusively the *L*-threo diastereoisomer from (*R*)-**6** and the *D*-erythro from (*S*)-**6** instead of the four possible diastereoisomers (Supporting Information, Scheme S3). This may be

explained because an all-equatorial arrangement of substituents (e.g., **7a** and almost all equatorial in **7b**) corresponds to the more stable product. The remaining mixture of diastereoisomers can then be re-subjected to further retroaldol-retro Henry equilibration and products **7a** and **7b** were accumulated.^[19]

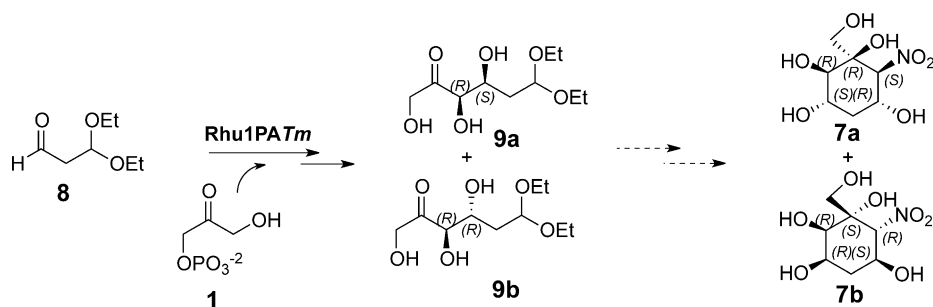
This study was continued using the 3,3-diethoxypropanal (**8**) as an acceptor (Scheme 3). The aldol reaction led to the linear compounds **9a** and **9b** with an isolated yield of 67%. Remarkably, both diastereoisomers *L*-threo and *D*-erythro were obtained in a 70:30 ratio for the first time in this work using a rhamnulose-1-phosphate aldolase.

Products **9a** and **9b** were previously synthesized using the *L*-fuculose-1-phosphate aldolase from *E. coli* (Fuc1PAEc).^[20] As expected, the percentage obtained from *D*-erythro diastereoisomer was higher in the reactions catalysed by Fuc1PAEc (*L*-threo:*D*-erythro ratio 3:97) than when the catalyst was the Rhu1PATm. These compounds could be considered as nitrocyclitol precursors. Indeed, deprotection of the aldehyde followed by a Henry reaction with nitromethane would lead to nitrocyclitols **7a** and **7b**.

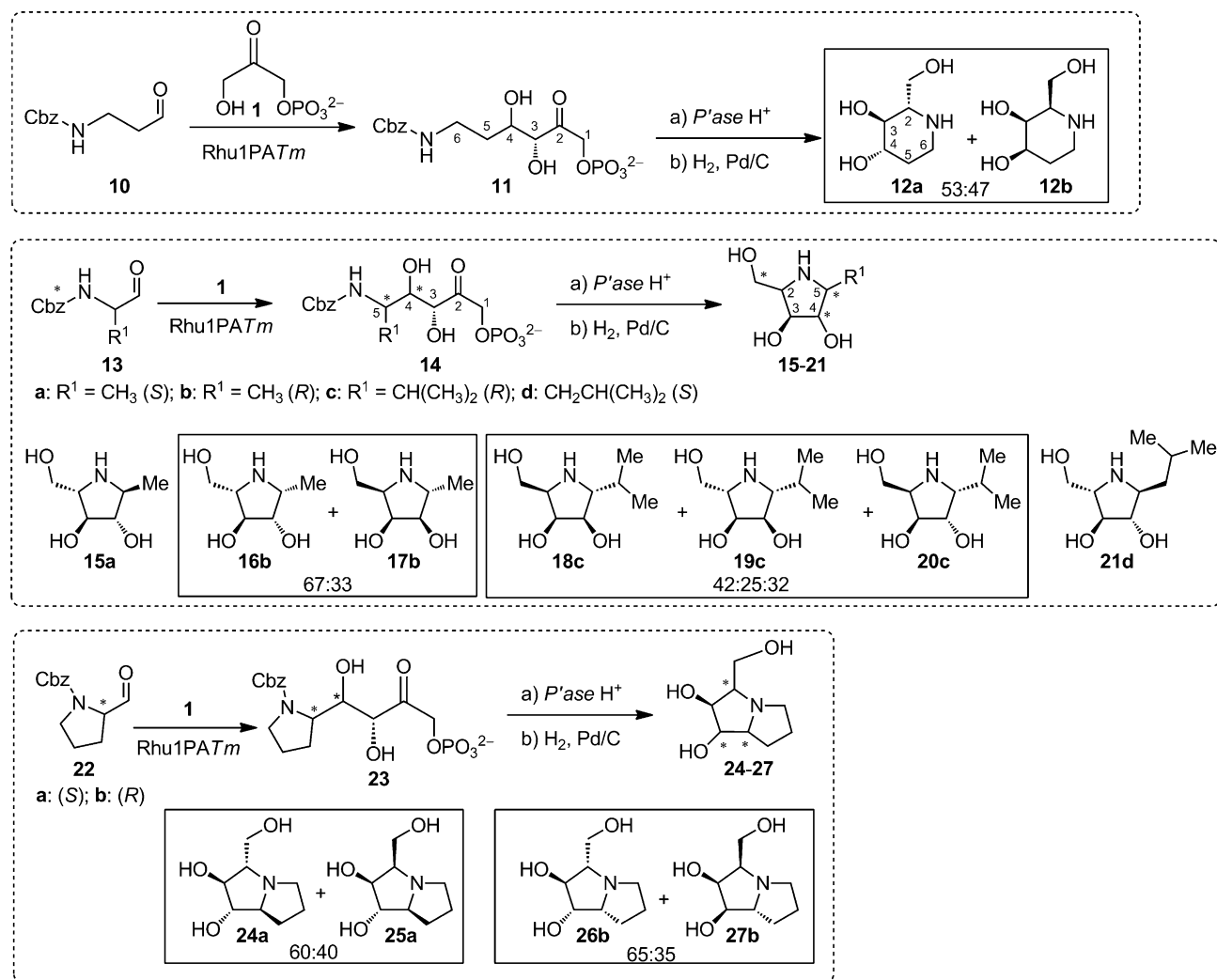
Synthesis of Iminocyclitols Mediated by Rhu1PATm

The second cascade reaction of the Rhu1PATm, with the DHAP-mediated the DHAP formation was the one developed by Clapés's group for obtaining iminocyclitols (Scheme 4). The strategy consists of the aldol addition of DHAP to either (*R*)- or (*S*)-*N*-Cbz-aminoaldehydes. This is followed by an acid phosphatase-catalysed hydrolysis of the phosphate group and subsequent intramolecular reductive amination by treatment with H₂ in the presence of Pd/C. This strategy allows the formation of three new stereogenic centres for each aldehyde tested (Scheme 4).^[12a,21] two of them controlled by the aldolase and one generated during the reductive amination.

First, the capability of the Rhu1PATm to catalyse the aldol addition reaction of DHAP to *N*-Cbz-aminoaldehydes was tested on an analytical scale using



Scheme 3. Rhu1PATm-catalysed synthesis of aldol products **9a** and **9b**, which are putative precursors of nitrocyclitols **7a** and **7b**.



Scheme 4. Cascade reaction for the synthesis of iminocyclitols employing Rhu1PATm.

an *N*-Cbz-aminoaldehyde:DHAP ratio of 1:1.7. DMF (30% v/v) was added to the reaction medium to improve the solubility of the aldehydes. Reaction temperature was set up at 45°C to retain a good activity of the aldolase, while preventing a high degradation rate of the DHAP.^[10]

Reactions were monitored by HPLC and stopped when the formation of aldol adduct was maximal (Table 1), thus preventing the spontaneous retroaldol reaction as has been reported for similar reactions.^[21a] In these conditions, all the *N*-Cbz-aminoaldehydes tested were accepted as substrates by Rhu1PATm, which was able to use aldehydes with both linear and branched substituent at C α (Table 1).

Good conversions (61–74%) were obtained with *N*-Cbz-aminoaldehydes with small C α substituents (e.g., **10**, **13a** and **13b**) (Table 2) and moderate (37–49%) with C α branched substituents including prolinol derivatives. Using the same aldehyde acceptors, Rhu1PAEc at 25°C gave similar yields for **10** and **13a**, **13b**

but required longer reaction times (4–5 h).^[21a] For **13c**, **13d**, **22a** and **22b** Rhu1PAEc gave superior conversions (58–69% conversion).^[12a,21c]

While this ability to accept a wide variety of substituents on the C α is shared with its mesophilic counterpart from *E. coli*, studies with other DHAP-dependent aldolases demonstrated that not all of them exhibit the same degree of tolerance towards structurally diverse *N*-Cbz-aminoaldehydes.^[12a,22,23]

After these preliminary studies, reactions for obtaining the corresponding iminocyclitols were scaled up to the mmol scale (i.e., 2 mmol DHAP; 1.2 mmol aldehyde). Once the reaction reached the maximum aldol adduct formation, the unreacted aldehyde was removed and aldol adducts **11**, **14a–d**, **23a** and **23b** were treated with acid phosphatase. Afterwards, aldols were purified by reverse-phase HPLC, paying special attention to prevent the losses of possible diastereoisomers formed during the reaction. Then, aldol adducts were submitted to Cbz deprotection/intramolecular

Table 2. Overall yield and stereochemical outcome obtained in the synthesis of iminocyclitols catalysed by Rhu1PATm.

Aldehyde	Iminocyclitol	Iminocyclitol overall yield [%] ^[a]	L-threo:D-erythro [%] ^[b]
10	12a + 12b	32	53:47
13a	15a	19	> 95:5
13b	16b + 17b	47	67:33
13c	18c + 19c^[c] + 20c	18	32:68
13d	21	12	> 95:5
22a	24a + 24b	9	> 95:5
22b	24c + 24d	12	65:35

^[a] Purification procedures were not optimized.

^[b] Ratio corresponding to the aldol adducts inferred from the iminocyclitol compounds and determined within the limits of NMR detection.

^[c] **18c** and **19c** are epimers at C-2 formed during reductive amination.

lecular reductive amination to afford the corresponding iminocyclitols, following the method previously described.^[21b] This procedure provided overall yields ranging from 9 to 47% (Table 2). The structure and relative stereochemistry of the iminocyclitols were determined by NMR and in all cases were consistent with those previously described in the literature.^[12a,21a,c,23] As in previous examples of aldol additions catalysed by DHAP-dependent aldolases, it can be assumed from mechanistic considerations that the absolute configuration at C-3 (Scheme 4) (i.e., the stereogenic centre generated from the DHAP donor) will be conserved throughout with any DHAP-aldolases and with no exceptions known so far.^[5,21,22] Moreover, the specific absolute configuration introduced by some *N*-Cbz-aminoaldehyde chiral centres (e.g., **13a–d**, **22a** and **22b**, Scheme 4) allowed the unequivocal assessment of the absolute stereochemistry of the iminocyclitols. Thus, from the stereochemical configuration of the iminocyclitols, it was possible to infer the stereochemical outcome of both the enzymatic aldol addition and the reductive amination.

Mixtures of *L*-threo and *D*-erythro diastereoisomers were obtained, which depended upon the structure of the acceptor aldehyde (Table 2). Rhu1PATm provided a good stereoselectivity for the (*S*)-configured *N*-Cbz-aminoaldehydes furnishing always the *L*-threo configured aldol adducts while *L*-threo:*D*-erythro mixtures were formed with the (*R*)-configured ones (Table 2). This behaviour was similar to that observed for Rhu1PAEc using both DHAP or DHA in the presence of borate buffer as donors.^[12a,21a,22,23] The aldol addition of DHAP to (*R*)-*N*-Cbz-valinal (**13c**) gave a mixture of *L*-threo:*D*-erythro (32:68) being the *D*-erythro the major one (Table 2). Interestingly, the same reaction catalysed by Rhu1PAEc was fully *D*-erythro stereoselective.^[12a] For **13c** both enzymes have a preference

for the *D*-erythro stereochemistry, which is typically obtained with Fuc1PAEc catalyst.

In another example, the aldol addition of DHAP to (*R*)-*N*-Cbz-prolinal (**22b**), Rhu1PATm gave a mixture of *L*-threo:*D*-erythro 65:35, whereas using Rhu1PAEc the reaction was reported to be fully stereoselective.^[23] Given the high sequence identity and structural homology between the Rhu1PA from *T. maritima* and *E. coli*, it could be argued that the lack of selectivity in the Rhu1PATm enzyme is determined for similar reasons to those already determined for Rhu1PAEc. Thus, according to Kroemer et al.,^[24] *L*-lactaldehyde binds to the catalytic centre of the Rhu1PAEc through two hydrogen bonds, directing the methyl group to the hydrophobic surroundings. As a result, the substrate is anchored to the active site of the enzyme favouring the nucleophilic attack by just one enantiotopic face. Restrictions imposed by the structure of the different *N*-Cbz-aminoaldehydes, as well as the lack of the hydroxy group, may induce an incorrect orientation of the aldehyde in the catalytic pocket.^[21a] Moreover, some important differences may occur in the active site of both aldolases since Rhu1PATm was not able to use DHA in presence of borate buffer, an ability so far restricted to Rhu1PAEc.^[22]

As previous studies have reported, the stereochemistry of the iminocyclitols at C-2 [C-3 in pyrrolizidines **24–27**] revealed that the reductive amination with H₂ in the presence of Pd/C depended on the structure of the aldol adduct precursor. In most cases, for example, iminocyclitols **12a**, **12b**, **15a**, **16b**, **17b**, **21d**, **26b** and **27b** reductive amination was highly diastereoselective. Here, the hydrogenation took place from the face opposite to the C-4 hydroxy group (C-1 hydroxy group for the pyrrolizidine derivatives), regardless of the relative stereochemistry of the other substituents. Exceptions were observed in the iminocyclitols **18c**, **19c**, **24a** and **25a**.

Conclusions

The work presented herein describes the synthetic scope of the Rhu1PATm. Since this aldolase maintains a significant percentage of activity at temperatures much lower than its optimum (95 °C), it is compatible with multienzyme systems for C–C bond formation involving mesophilic enzymes. This enzyme was successfully tested in two different chemo-enzymatic cascades for the synthesis of nitrocyclitols and iminocyclitols. In both cases, Rhu1PATm was able to catalyse the aldol addition using the corresponding aldehydes to provide the desired products with similar or higher yields than Rhu1PAEc. Additionally, the stereoselectivity shown by the aldolase from *T. maritima* was, in general, similar to the one of the Rhu1-

PAEC, even though there were significant differences on specific substrates. Our data indicated that the formation of the L-threo isomer was faster than that of the D-erythro isomer. No effect of the temperature was observed in this process.

Aldol adduct precursors of iminocyclitols **14a**, **14d** and **23a** were obtained with full L-threo (3R,4S) stereoselectivity. The rest aldol adducts from nitroaldehydes and N-Cbz-aminoaldehydes were obtained as mixtures of L-threo (3R,4S) and D-erythro (3R,4R) diastereoisomers. The major diastereomer formed was the one with the natural stereopreference of the enzyme (L-threo), with the exception of aldehyde **13c**.

Experimental Section

Materials

All solvents and reagents were purchased from Sigma-Aldrich or Acros. Triosephosphate isomerase (TIM), α -glycerophosphate dehydrogenase (α GDH), phosphatase acid from potato and acetate kinase (AK) was purchased from Sigma-Aldrich. Rhu1PA from *T. maritima* and DHAK from *C. freundii* were expressed and purified as previously described.^[10,18] DHAP was enzymatically prepared from DHA as previously described by Oroz-Guinea et al.^[11c] with slight modifications. Acetyl phosphate (7.15 mmol), MgSO₄ (1.25 mmol) and DHA (5 mmol) were added to phosphate buffer (30 mL; 60 mM; pH 7.5) and water to a final reaction volume of 90 mL. DHAK (431 U) and AK (225 U) were suspended in ultra-pure water (pH \approx 7.0) and added to the reaction. To start the reaction ATP (163 μ mol) was added. The phosphorylation reaction was completed after 30 min. For the storage of the mixture the pH was adjusted to 5.0 to avoid the degradation of DHAP and was subsequently frozen. Nickel/iminodiacetic acid (Ni²⁺/IDA) agarose was supplied by Agarose Bead Technologies (Spain). Dialysis was performed in SPECTRA/Por® membranes (Spectrum) with 12,000–14,000 Da cut-off.

Rhu1PATm Activity Assay

The retro-aldol activity of Rhu1PATm was spectrophotometrically measured using rhamnulose-1-phosphate (Rhu-1P) as substrate. Rhu-1P was synthesised using the multienzyme system described by Sánchez-Moreno et al.^[11a] at 1 mmol of DHA scale. The reaction was carried out in 30 mL of HEPES buffer (20 mM; pH 7.5) containing DHA (1 mmol), L-lactaldehyde (1.5 mmol), acetyl phosphate (2 mmol), ATP (68 pmol), MgSO₄ (250 pmol), ZnCl₂ (0.3 pmol), DHAK (15 U), AK (30 U) and Rhu1PA from *E. coli*. Activity assays were run at 45 °C following the decrease in absorbance at $\lambda = 340$ nm ($\epsilon^{\text{NADH}} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) for 10 min in a reaction mixture (1 mL) containing Tris-HCl buffer (40 mM; pH 8.0), NADH (0.2 mmol), α GDH/TIM (2.4 U of α GDH and 24.8 U of TIM), Rhu-1P (10 μ mol), and Rhu1PATm. 1 unit aldolase activity was defined as the amount of enzyme that converts 1 μ mol of Rhu-1P in DHAP and L-lactaldehyde per minute in the above described conditions.

Stereochemistry Study of the Rhu1PATm-Catalysed Reaction

Stereochemistry of the Rhu1PATm was evaluated by measuring the percentage of each diastereoisomer formed depending on the reaction time and temperature. Benzyloxyacetaldehyde **2** (100 μ mol) was dissolved into 600 μ L of DMSO (10% final volume) and, then, added to 200 μ mol of DHAP. Afterwards, the reaction pH was adjusted to 6.9. Finally, aldolase (0.4 U) was added to start the reaction. Four different temperatures were chosen to carry out the study: 4 °C, room temperature, 45 °C and 80 °C. Aliquots of each reaction were taken at different times and formation of the diastereoisomer was monitored by HPLC, using an RP-HPLC XBridge® C18, 5 μ m, 4.6 \times 250 mm column (Waters). The elution system used was: solvent (A); 0.1% trifluoroacetic acid (TFA) in H₂O (v/v) and solvent (B); 0.095% (v/v) TFA in CH₃CN/H₂O 4:1; gradient elution from 10% to 70% B over 30 min. Flow rate was 1 mL min⁻¹, detection was at 215 nm. The amount of aldol adduct produced was quantified from the peak areas using an external standard.

Synthesis of Nitrocyclitols 5a, 5b, 7a and 7b and Nitrocyclitol Precursors 9a and 9b

Nitroaldehyde **4** was prepared by mixing acrolein with an excess of nitromethane with KF as a mild base at -35 °C, as described by El Blidi et al.^[16a] Nitroaldehyde (\pm)-**6** was obtained starting from aldehyde **8**, as reported by El Blidi et al.^[16b] Finally, aldehyde **8** was prepared by ozonolysis of the corresponding alkene,^[25] previously obtained using the procedure of Hoaglin et al.^[26]

0.5 mmol of the corresponding aldehyde – **4** (58.5 mg), (\pm)-**6** (66.5 mg) or **8** (73.1 mg) – and 3 U of Rhu1PATm were added to 1 mmol of DHAP, previously adjusted to pH 6.9. Distilled water was added to a final volume of 30 mL. DMSO (10% in the final volume) was added to the reaction media to dissolve aldehyde **4**. The reaction was kept at room temperature for 24 h with stirring. The pH was then adjusted to 4.7 with HCl (1 M) and Rhu1PATm was eliminated by centrifugation. Subsequently, acid phosphatase from potato (200 U) was added and the reaction was carried out during 24 h. After this time, the phosphatase was removed and the resulting solution was concentrated under vacuum. Reactions were monitored spectrophotometrically measuring the disappearance of DHAP^[11b] and by TLC (Merck 60 F254 silica gel TLC plates). The residue was purified by column chromatography with Merck 60/230–400 and 60/40–63 mesh silica gel, using a solvent gradient from 9:1 to 8:2 of CH₂Cl₂/MeOH to afford the nitrocyclitols **5a**, **b**, and **7a**, **b** and 95:5 of CH₂Cl₂/MeOH for the ketone diacetal **9a**, **b**. Overall yields of the desired products were: **5a**+**5b** (72 mg, 70%), **7a**+**7b** (79.2 mg, 71%) and **9a**+**9b** (77.1 mg, 67%). ¹H and ¹³C NMR spectra of these products were recorded on a Bruker Avance 400 spectrometer in CDCl₃, D₂O, and CD₃OD. In all cases, resonance spectra were consistent with those previously described for these compounds.^[17,19]

1-(Hydroxymethyl)-6-nitrocyclohexane-1,2,3-triol: 2 isomers. (2R,3S):(2R,3R) ratio **5a**:**5b**, 95:5. Isomer **5a** (1R,2R,3S,6S): ¹H NMR (400 MHz, MeOD, 298 K): δ = 4.79 (dd, J = 13.0 Hz, J = 4.0 Hz, 1H, H-6), 3.82 (d, J = 11 Hz, 1H, H-7_b), 3.73 (ddd, J = 9 Hz, J = 11.5 Hz, J = 4.6 Hz, 1H, H-3),

3.37 (d, $J=9.3$ Hz, 1H, H-2), 3.33 (d, $J=11.0$ Hz, 1H, H-7_A), 2.43 (m, 1H, H-5), 2.01 (m, 1H, H-5), 1.97 (m, 1H, H-4_B), 1.32 (m, 1H, H-4_A). **Isomer 5b** (1*R*,2*R*,3*R*,6*S*): ¹H NMR (400 MHz, MeOD, 298 K): $\delta=4.75$ (dd, $J=12.4$ Hz, $J=4.0$ Hz, 1H, H-6), 4.00 (m, 1H, H-3), 3.88 (d, $J=2.6$ Hz, 1H, H-2), 3.83 (d, $J=11.5$ Hz, 1H, H-7_B), 3.55 (d, $J=11.5$ Hz, 1H, H-7_A), 2.41 (m, 1H, H-5), 1.93 (m, 2H, H-5, H-4_B), 1.76 (m, 1H, H-4_A).

1-(Hydroxymethyl)-6-nitrocyclohexane-1,2,3,5-tetraol: 2 isomers. (2*R*,3*S*):(2*R*,3*R*) ratio **7a:7b**, 75:25. **Isomer 7a** (1*R*,2*R*,3*S*,5*R*,6*S*): ¹H NMR (400 MHz, MeOD, 298 K): $\delta=4.67$ (d, $J=10.6$ Hz, 1H, H-6), 4.50 (ddd, $J=11.0$ Hz, $J=11.0$ Hz, $J=4.9$ Hz, 1H, H-5), 3.86 (m, 1H, H-3), 3.77 (d, $J=11.2$ Hz, 1H, H-7_B), 3.41 (d, $J=9.5$ Hz, 1H, H-2), 3.24 (d, $J=10.8$ Hz, 1H, H-7_A), 2.26 (m, 1H, H-4_B), 1.41 (pq, $J=12.1$ Hz, $J=12.1$ Hz, $J=12.1$ Hz, 1H, H-4_A). **Isomer 7b** (1*R*,2*R*,3*R*,5*R*,6*S*): ¹H NMR (400 MHz, MeOD, 298 K): $\delta=4.55$ (d, $J=10.6$ Hz, 1H, H-6), 4.43 (ddd, $J=11.0$ Hz, $J=11.0$ Hz, $J=4.9$ Hz, 1H, H-5), 4.06 (m, 1H, H-3), 3.82 (d, $J=2.4$ Hz, 1H, H-2), 3.79 (d, $J=11.3$ Hz, 1H, H-7_B), 3.50 (d, $J=11.3$ Hz, 1H, H-7_A), 2.02 (m, 1H, H-4_B), 1.82 (ddd, $J=11.9$ Hz, $J=11.9$ Hz, $J=10.8$ Hz, 1H, H-4_A).

6,6-Diethoxy-1,3,4-trihydroxyhexan-2-one: 2 isomers. (3*R*,4*S*):(3*R*,4*R*) ratio **9a:9b**, 70:30. **Isomer 9a** (3*R*,4*S*): ¹H NMR (400 MHz, CD₃OD, 298 K): $\delta=4.66$ (dd, 1H, $J=4.5$ Hz, $J=6.8$ Hz, H-6), 4.50 (d, 1H, $J=19.4$ Hz, H-1_A), 4.40 (d, 1H, $J=19.4$ Hz, H-1_B), 3.88 (m, 1H, H-3), 3.59 (m, 4H, OCH₂-7, OCH₂-9), 3.27 (m, 1H, H-4), 1.82 (m, 2H, CH₂-H-5), 1.15 (m, 6H, CH₃-8, CH₃-10); ¹³C NMR (100 MHz, CD₃OD, 298 K): $\delta=213.47$ (C-2), 102.38 (C-6), 79.67 (C-3), 70.29, 67.95, 63.29, 62.72 (C-1, C-4, C-7, C-9), 38.57 (C-5), 15.66 (C-8, C-10). **Isomer 9b** (3*R*,4*R*): ¹H NMR (400 MHz, CD₃OD, 298 K): $\delta=4.69$ (dd, 1H, $J=4.1$ Hz, $J=7.5$ Hz, H-6), 4.48 (d, 1H, $J=19.4$ Hz, H-1_A), 4.37 (d, 1H, $J=19.4$ Hz, H-1_B), 4.05 (m, 6H, H-3, H-4, OCH₂-7, OCH₂-9), 1.77 (m, 2H, CH₂-H-5), 1.15 (m, 6H, CH₃-8, CH₃-10); ¹³C NMR (100 MHz, CD₃OD, 298 K): $\delta=212.94$ (C-2), 102.25 (C-6), 79.77 (C-3), 70.89, 68.12, 63.02, 62.80 (C-1, C-4, C-7, C-9), 37.79 (C-5), 15.66 (C-8, C-10).

Analytical Scale Synthesis of Iminocyclitols

Analytical scale reactions (1 mL total volume) were conducted in 2-mL test tubes with screw cap incubated at 45 °C. 15 μ mol of aldehyde – **10**, **13a** and **13b** (3.1 mg each); **13c** (3.5 mg); **13d** (3.74 mg); **22a** and **22b** (3.49 mg each) – were dissolved in DMF (300 μ L) and then mixed with freshly prepared DHAP neutralised at pH 6.9. Reaction was started by adding the aldolase (between 0.75 and 1.6 U measured at 45 °C). Reactions were monitored by HPLC, as described above. To monitor those reactions with aldehydes **13c** and **13d**, **22a** and **22b** the gradient elution was changed to 30% to 100% B over 30 min.

Preparative Scale Synthesis of Iminocyclitols

The general procedure for the production of the aldols **11**, **14** and **23** was as described above, with only slight variations of the stoichiometry: 1.2 mmol of the corresponding aldehyde – **10**, **13a** and **13b** (249 mg each); **13c** (282 mg), **13d** (299 mg); **22a** and **22b** (278 mg each) – was dissolved in 24 mL of DMF (35% of final volume), followed by the addition of distilled water (until a final volume of 70 mL),

2 mmol of DHAP (previously synthesized and adjusted to pH 6.9) and 10 U of Rhu1PAT_m. The reaction progress was followed spectrophotometrically, measuring the aldol formed by retro-aldol reaction catalysed by Rhu1PA from *E. coli*. When a maximum of aldol conversion was achieved, 2 volumes of MeOH were added to stop the reaction and precipitate the enzyme (**10**: 105 min; **13a**: 90 min; **13b**: 75 min; **13c**: 150 min; **13d**: 150 min; **22a**: 60 min; **22b**: 90 min). Then, the mixture was filtered through a 0.45 mm cellulose membrane filter and MeOH was removed under vacuum. The filtrate was washed with AcOEt (3 \times 100 mL) to remove the aldehyde that did not react.

Afterwards, the aqueous phase was acidified (pH 4.7) and phosphatase acid from potato was added (5.3 U per mmol of phosphorylated product). Dephosphorylation reaction was followed by HPLC using the same methodology as described above. When the product was completely dephosphorylated the reaction was stopped by adding two volumes of MeOH. After removal of the enzyme by filtration, the filtrate was adjusted to pH 3.0 and loaded onto a semi-preparative HPLC column X-Terra® (19/250 mm). Products were eluted using CH₃CN gradient. The flow rate was 10 mL min⁻¹ and the products were detected at 215 nm. Fractions containing the pure product were pooled and lyophilized. Overall yields of the desired products were: **12a+12b** (112.8 mg, 32%), **15a** (68.9 mg, 19%), **16b+17b** (168.2 mg, 47%), **18c+19c+20c** (70.0 mg, 18%), **21d** (48.1 mg, 12%), **24a+25a** (36.5 mg, 9%), **26b+27b** (48.0 mg, 12%).

The product obtained was dissolved in H₂O/MeOH (1:1 v/v) and treated with H₂ (50 psi) in the presence of Pd/C (2 equiv. w/w) at room temperature during 24 h. After that, the catalyst was removed and the filtrate was adjusted to pH 6.4 with formic acid and lyophilized obtaining a solid material.

Separation of the isomers of iminocyclitols **16b+17b**, **18c+19c+20c** and **26b+27b** was performed in a FPLC system using CM-Sepharose CL-6B (Amersham Pharmacia) packed in a glass column (45 \times 25 cm). The column was washed with four volumes (1 vol \sim 220 mL) of a 100 mM solution of NH₃ and then equilibrated with four additional volumes of distilled H₂O (pH \sim 7.0). Iminocyclitols were dissolved in 10 mL of H₂O and the pH of the solution adjusted to 6.0 with formic acid to be loaded in the column. The column was washed with three volumes of distilled H₂O (pH \sim 7.0) and the products were eluted with a 10 mM solution of NH₃ at a flow of 1 mL min⁻¹.

Fractions containing the compounds were analysed by NMR. ¹H and ¹³C NMR spectra of iminocyclitols were recorded on a Varian System 400 spectrometer. The compounds described were fully characterised using typical gradient-enhanced 2D experiments: COSY, HSQC, recorded under routine conditions (see the Supporting Information for spectra).

2-(Hydroxymethyl)piperidine-3,4-diol: 2 isomers. (2*S*,3*S*,4*S*):(2*S*,3*S*,4*R*) ratio **12a:12b** 53:47. **Isomer 12a** (3*S*,4*S*): ¹H NMR (400 MHz, D₂O, 298 K): $\delta=3.87$ (dd, $J=3.2$, $J=12.6$ Hz, 1H, H-7_A), 3.73 (dd, $J=5.7$ Hz, $J=12.7$ Hz, 1H, H-7_B), 3.6 (dt, $J=4.9$ Hz, $J=9.5$ Hz, $J=11.6$ Hz, 1H, H-4), 3.13 (t, 1H, H-3), 3.0 (br, 1H, H-6_A), 3.0 (m, 1H, H-2), 2.95 (dt, $J=3.2$ Hz, $J=13.1$ Hz, 1H, H-6_B), 1.91 (br, 1H, H-5_A), 1.38 (dq, $J=4.3$ Hz, 1H, H-5_B); ¹³C NMR (125 MHz,

D₂O, 298 K): δ = 72.38 (C-4), 72.11 (C-3), 60.57 (C-7), 60.45 (C-2), 44.38 (C-6), 31.44 (C-5). **Isomer 12b** (3S,4R): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.0 (m, 1H, H-3), 3.80 (m, 1H, H-4), 3.2 (m, 1H, H-2), 1.85 (m, 1H, H-5_A), 1.75 (m, 1H, H-5_B), 3.76 (dd, 1H, H-7_A), 3.72 (dd, 1H, H-7_B), 3.18 (m, 1H, H-6_A), 2.95 (m, 1H, H-6_B); ¹³C NMR (125 MHz, D₂O, 298 K): δ = 68.26 (C-4), 66.45 (C-3), 60.17 (C-7), 59.65 (C-2), 42.40 (C-6), 25.13 (C-5).

(5S)-2-(Hydroxymethyl)-5-methylpyrrolidine-3,4-diol

(15a): ¹H NMR (400 MHz, D₂O, 298 K): δ = 3.77 (t, J = 7.0 Hz, 1H, H-3), 3.66 (dd, J = 4.2 Hz, J = 12.0 Hz, 1H, H-7_A), 3.60 (t, J = 7.5 Hz, 1H, H-4), 3.58 (dd, J = 6.4 Hz, J = 12.0 Hz, 1H, H-7_B), 3.13 (dt, J = 4.2 Hz, J = 6.4 Hz, J = 7.0 Hz, 1H, H-2), 3.06 (dq, J = 7.0 Hz, 1H, H-5), 1.16 (d, J = 7.0 Hz, 3H, H-6); ¹³C NMR (125 MHz, D₂O, 298 K): δ = 81.15 (C-4), 76.53 (C-3), 61.67 (C-2), 60.65 (C-7), 56.28 (C-5), 15.95 (C-6).

(5R)-2-(Hydroxymethyl)-5-methylpyrrolidine-3,4-diol: 2

isomers. (2S,3S,4S,5R):(2S,3S,4R,5R) ratio **16b:17b** 67:33. **Isomer 16b** (2S,3S,4S,5R): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.01 (dd, J = 1.4 Hz, J = 3.2 Hz, 1H, H-4), 3.95 (dd, J = 1.8 Hz, J = 3.2 Hz, 1H, H-3), 3.84 (dd, J = 4.9 Hz, J = 11.9 Hz, 1H, H-7_A), 3.48 (dq, J = 3.5 Hz, J = 6.5 Hz, 1H, H-5), 3.71 (dd, J = 8.8 Hz, J = 12.3 Hz, 1H, H-7_B), 3.16 (dt, J = 3.3 Hz, J = 5.1 Hz, J = 8.2 Hz, 1H, H-2), 1.24 (d, J = 6.7 Hz, 3H, H-6); ¹³C NMR (125 MHz, D₂O, 298 K): δ = 76.3 (C-4), 76.3 (C-3), 67.5 (C-2), 59.6 (C-7), 56.51 (C-5), 11.8 (C-6). **Isomer 17b** (2S,3S,4R,5R): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.28 (t, J = 3.7 Hz, 1H, H-3), 3.95 (dd, J = 3.7 Hz, J = 9.0 Hz, 1H, H-4), 3.9 (dd, J = 4.7 Hz, J = 11.8 Hz, 1H, H-7_A), 3.8 (dd, J = 8.4 Hz, J = 11.8 Hz, 1H, H-7_B), 3.58 (m, J = 3.7 Hz, J = 4.7 Hz, J = 8.4 Hz, 1H, H-2), 3.31 (m, 1H, H-5), 1.32 (d, J = 6.7 Hz, 3H, H-6); ¹³C NMR (125 MHz, D₂O, 298 K): δ = 76.7 (C-4), 70.4 (C-3), 61.5 (C-2), 58.4 (C-7), 55.5 (C-5), 16.06 (C-6).

2-(Hydroxymethyl)-5-isopropylpyrrolidine-3,4-diol: 3 iso-

mers. (2R,3R,4S,5R):(2R,3R,4S,5S):(2R,3S,4S,5R) ratio **18c:19c:20c**, 42:25:32. **Isomer 18c** (2R,3R,4S,5R): ¹H NMR (400 MHz, D₂O): δ = 4.22 (t, J = 3.9 Hz, 1H), 4.13 (dd, J = 8.5, 4.3 Hz, 1H), 3.88 (dd, J = 11.5, 6.3 Hz, 1H), 3.75 (dd, J = 11.4, 7.1 Hz, 1H), 3.47 (td, J = 6.7, 3.6 Hz, 1H), 3.01 (m, 1H), 1.89 (dq, J = 13.7, 6.8 Hz, 1H), 1.03 (d, J = 6.8 Hz, 3H), 1.01 (d, J = 6.7 Hz, 3H); ¹³C NMR (100 MHz, D₂O): δ = 71.4 (C-3), 74.2 (C-4), 65.8 (C-5), 60.4 (C-2), 59.9 (C-6), 29.9 (C-7), 19.6 (C-8), 18.3 (C-9). **Isomer 19c** (2R,3S,4S,5R): ¹H NMR (400 MHz, D₂O): δ = 4.13 (d, J = 4.7 Hz, 1H), 4.00 (dd, J = 2.8, 0.9 Hz, 1H), 3.82 (dd, J = 11.6, 5.6 Hz, 1H), 3.74 (m, 1H), 3.23 (ddd, J = 7.0, 5.6, 2.8 Hz, 1H), 3.01 (m, 1H), 1.97 (m, 1H), 1.01 (d, J = 3.1 Hz, 3H), 1.00 (d, J = 3.1 Hz, 3H); ¹³C NMR (101 MHz, D₂O): δ = 74.7 (C-3), 74.6 (C-4), 68.2 (C-5), 62.3 (C-2), 58.0 (C-6), 25.7 (C-7), 18.7 (C-8), 18.4 (C-8). **Isomer 20c:** (2R,3S,4S,5R): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.21 (t, J = 4.0 Hz, 1H, H-3), 4.12 (m, 1H, H-4), 3.95 (dd, J = 11.5 Hz, J = 5.0 Hz, 1H, H-6_A), 3.74 (dd, J = 11.2 Hz, J = 7.4 Hz, 1H, H-6_B), 3.46 (dt, J = 3.6 Hz, J = 3.1 Hz, 1H, H-2), 3.00 (t, J = 8.0 Hz, 1H, H-5), 1.88 (dt, J = 6.8, 1H, H-7), 1.00 (m, 6H, H-8, H-9); ¹³C NMR (100 MHz, D₂O): δ = 76.2 (C-4), 73.4 (C-3), 68.7 (C-5), 64.1 (C-2), 60.2 (C-6), 32.0 (C-7), 21.4 (C-8), 20.5 (C-9).

2-(Hydroxymethyl)-5-isobutylpyrrolidine-3,4-diol (21d):

¹H NMR (400 MHz, D₂O, 298 K): δ = 3.93 (t, J = 6.9 Hz, 1H, H-3), 3.83 (m, 1H, H-4), 3.78 (m, J = 12.6, 3.9 Hz, 1H, H-

6_A), 3.83 (m, J = 12.7, 6.0 Hz, 1H, H-6_B), 3.25 (m, 1H, H-2), 3.20 (m, 1H, H-5), 1.71 (m, 1H, H-8), 1.59 (m, 1H, H-7_A), 1.56 (m, 1H, H-7_B), 0.94 (m, 6H, H-9, H-10); ¹³C NMR (100 MHz, D₂O): δ = 80.4 (C-4), 76.3 (C-3), 61.5 (C-2), 60.5 (C-5), 58.5 (C-6), 41.4 (C-7), 24.4 (C-8), 22.4 (C-9), 20.9 (C-10).

(7aS)-3-(Hydroxymethyl)hexahydro-1H-pyrrolizine-1,2-

diol: 2 isomers. (1S,2S,3S,7aS):(1R,2S,3S,7aS) ratio **24a:25a**, 60:40. **Isomer 24a** (1S,2S,3S,7aS): ¹H NMR (400 MHz, D₂O, 298 K): δ = 3.79 (m, J = 12.2 Hz, J = 8.2 Hz, 1H, H-8_A), 3.78 (bs, 1H, H-2), 3.74 (t, J = 8.0 Hz, 1H, H-1), 3.65 (m, J = 11.8, 6.5 Hz, 1H, H-8_B), 3.20 (m, 1H, H-7a), 2.94 (bs, 1H, H-5_A), 2.80 (m, 1H, H-5_B), 2.75 (m, 1H, H-3), 1.96 (m, 1H, H-7_A), 1.88 (m, 1H, H-6_A), 1.79 (m, 1H, H-6_B), 1.76 (m, 1H, H-7_B); ¹³C NMR (100 MHz, D₂O): δ = 79.5 (C-1), 77.5 (C-2), 69.2 (C-3), 66.6 (C-7a), 55.8 (C-8), 54.8 (C-5), 29.3 (C-7), 24.3 (C-6). **Isomer 25a** (1R,2S,3S,7aS): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.33 (dd, J = 3.8 Hz, J = 1.5 Hz, 1H, H-2), 4.26 (bt, J = 1.9 Hz, 1H, H-1), 4.08 (m, 2H, H-8_A, H-8_B), 4.02 (m, 1H, H-3), 3.93 (m, 1H, H-7a), 3.50 (m, 2H, H-5_A, H-5_B), 2.41 (m, 1H, H-7_A), 2.23 (m, 1H, H-6_A), 1.87 (m, 2H, H-7_B, H-6_B); ¹³C NMR (100 MHz, D₂O): δ = 78.5 (C-1), 77.5 (C-2), 74.8 (C-3), 65.8 (C-7a), 55.8 (C-8), 50.1 (C-5), 28.9 (C-7), 25.8 (C-6).

(7aR)-3-(Hydroxymethyl)hexahydro-1H-pyrrolizine-1,2-

diol: 2 isomers. (1S,2S,3S,7aR):(1R,2S,3R,7aR) ratio **26b:27b**, 65:35. **Isomer 26b** (1S,2S,3S,7aR): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.04 (dd, J = 6.3, 1.4 Hz, 1H, H-1), 3.83–3.73 (m, 4H, H-2, H-7a, H-8_A, H-8_B), 3.13 (m, 1H, H-3), 3.04 (m, 1H, H-5_A), 2.83 (dt, J = 6.2 Hz, J = 4.3 Hz, 1H, H-5_B), 1.89 (bs, 1H, H-6_A), 1.79 (m, 2H, H-7_A, H-7_B), 1.65 (m, 1H, H-6_B); ¹³C NMR (100 MHz, D₂O): δ = 75.9 (C-2), 72.9 (C-1), 65.8 (C-7a), 64.6 (C-3), 58.8 (C-8), 54.6 (C-5), 26.9 (C-6), 22.8 (C-7). **Isomer 27b** (1R,2S,3R,7aR): ¹H NMR (400 MHz, D₂O, 298 K): δ = 4.27 (dd, J = 3.6, 1.6 Hz, 1H, H-1), 3.94 (dd, J = 4.4 Hz, J = 1.5 Hz, 1H, H-2), 3.87 (m, 1H, H-7a), 3.70 (m, J = 11.2 Hz, J = 6.5 Hz, 1H, H-8_A), 3.45 (m, 1H, H-8_B), 3.00 (m, 1H, H-5_A), 2.91 (m, 1H, H-3), 2.78 (m, 1H, H-5_B), 1.97 (m, 1H, H-6_A), 1.87 (m, 1H, H-7_A), 1.82 (m, 2H, H-6_B, H-7_B); ¹³C NMR (100 MHz, D₂O): δ = 77.9 (C-2), 74.2 (C-1), 70.3 (C-7a), 69.4 (C-3), 58.8 (C-8), 56.6 (C-5), 29.5 (C-6), 25.4 (C-7).

Acknowledgements

Work at CSIC has been supported by the Spanish Ministerio de Economia y Competitividad (Grant CTQ2010-19073/BQU, CTQ2012-31605), Generalitat de Catalunya (2009 SGR 00281), ERA-IB MICINN, PIM2010EEI-00607 (EIB.10.012. MicroTechEnz-EIB, www.fkit.unizg.hr/miten). COST Action: CM1303 "Systems Biocatalysis" is acknowledged. K. Hernandez and I. Oroz acknowledged the CSIC for a JAE Predoctoral contract program.

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