L-rhamnulose-1-phosphate and L-fuculose-1-phosphate aldolases mediated multi-enzyme cascade systems for nitrocyclitol syntheses.

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Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; DMSO, dimethyl sulfoxide; FucA, L-fuculose-1-phosphate aldolase; RhuA, L-rhamnulose-1-phosphate aldolase; DHAK, dihydroxyacetone kinase; AK, acetate kinase; ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide reduced form; GPDH/TPI, glycerophosphate dehydrogenase/triosephosphate isomerase; Pase, acid phosphatase; FBA, fructose-1,6-*bis*phosphate aldolase; L-R1P, L-rhamnulose-1-phosphate; L-F1P, L-fuculose-1-phosphate; L-Lact, L-lactaldehyde; L-GOP, L-glycerophosphate; DMA, dimethoxyacetaldehyde; IMAC, immobilised metal ion affinity chromatography; NTA, nitrilotriacetic acid.

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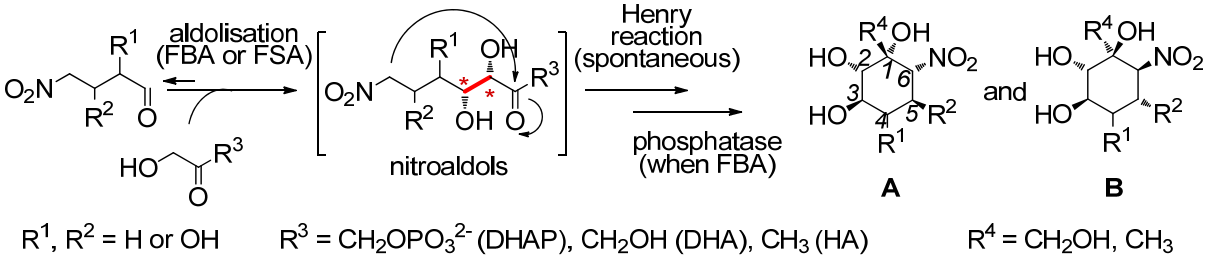
Abstract:

One-pot multistep stereoselective cascade reactions were implemented for the straightforward synthesis of various nitrocyclitols. Two kinases, an aldolase and a phosphatase were involved in this process together with a spontaneous intramolecular Henry reaction to provide a nitrocyclitol moiety. The C-C bond formation catalyzed by the aldolase and the nitroaldol reaction were key steps to build the carbocycle stereoselectively. The acceptor substrates for aldolases were all 4-nitrobutanal structurally based, hydroxylated or not on C2 and/or C3 positions. On the one side, L-fuculose-1-phosphate aldolase (FucA) has catalyzed the formation of the expected (*R*,*R; D-erythro*) aldol except in the case of 4-nitrobutanal from which (*R*,*S; L-threo*) aldol was also present. On the other side, L-rhamnulose-1-phosphate aldolase has always provided the expected (*R*,*S; L-threo*) aldol together with minor amount of (*R*,*R; D-erythro*) aldol thus being less stereoselective than FucA. The intramolecular Henry reaction has occurred spontaneously on the aldol due to the presence of both ketone and terminally positioned nitro group. This cyclisation was stereoselective and correlated to the presence or not of a hydroxyl group in β to the nitro group. From 4-nitrobutanal as substrate and for one defined stereochemistry by the aldolase (*R*,*R* or *R*,*S*) one cyclitol was formed. In the other cases, two diastereomeric series were formed due to the presence of an *R* and *S* configured alcohol function in β to the nitro group. The combination of this set of reactions successfully furnished 11 nitrocyclitols never described before in literature.

1. Introduction

The high stereoselectivity of aldolases in C-C bond formation gives them tremendous synthetic applications as biocatalysts. Among the different families of aldolases, dihydroxyacetone phosphate (DHAP)-dependent aldolases are particularly attractive since two new stereocentres are made during the formation of the new C-C bond between two carbonyl compounds. Thus a set of four possible diastereoisomers of keto vicinal diols can be prepared.[1-5] Among them L-fuculose-1-phosphate and L-rhamnulose-1-phosphate aldolases (FucA and RhuA both from *Escherichia coli*) providing respectively (*R*,*R*) and (*R*,*S*) configurations were recently and efficiently used for the synthesis of iminosugars. These wild-type or variant ($FucA_{F131A}$) aldolases have shown a broad tolerance towards acceptor substrates, even accepting in some cases sterically constrained aldehydes.[6-8] The strict requirement for the donor substrate DHAP, a rather expensive and unstable compound has led to the development of various strategies for its synthesis. Thus, well documented chemical or enzymatic DHAP preparations are accessible[9] and have facilitated the use of DHAP-dependent aldolases in organic synthesis. Enzymatic preparations are of particular interest as they can be combined in cascade reactions with other enzymes to provide highly functionalized molecules.[10] A preferred choice, however not applicable in all cases, is the direct use of dihydroxyacetone (DHA) as donor due to its low cost and easy availability. Wild-type RhuA from *E. coli* can accept the unphosphorylated donor with a low activity that could be increased in the presence of

borate buffer.[11] FucA had no detectable activity with DHA even in the presence of borate.[12, 13] A RhuA variant was recently built in order to improve this promiscuous catalytic property.[14]
Nitrocyclitols belong to the large family of cyclitols (also found as carbocyclic polyols or carbasugars).[15-18] They are essentially found as synthetic precursors of aminocyclitols.[19, 20] However natural nitrosugars moieties do exist and are derived from aminosugars via an oxidation step. They are found in ~50 bioactive natural products.[21, 22] Cyclitols often acting as glycosidase inhibitors are of large interest as they exhibit antitumor, antibacterial, antifungal, antimalarial, and antiviral activities.[17, 23, 24] Consequently, there have been continuous efforts in the last decade to prepare natural cyclitols and aminocyclitols as well as analogues with enhanced or more selective biological profiles in order to study in depth the influence of this moiety on their specific receptors.[15-18, 25-27]
Few years ago, we have reported that nitrocyclitols can be efficiently prepared by a one-pot multi-step chemoenzymatic synthesis, consisting of an aldol reaction catalyzed by an aldolase and an intramolecular spontaneous Henry reaction as key steps (scheme 1). On the one hand, starting from several nitrobutanals fructose-1,6-*bis*phosphate aldolase (FBA)[28-30] and fructose-6-phosphate aldolase (FSA)[20, 31] have led to nitroaldols with 3*S*,4*R* (becoming 2*S*,3*R* in the final cyclitol) defined configurations. On the other hand, the following spontaneous intramolecular Henry reaction has provided stereoselectively two main cyclitol families (scheme 1, **A** and **B**). These two families are correlated to the *R* and *S* configurations ("racemic") when R^2=OH on the aldehyde acceptor subtrate. FSA has advantageously replaced FBA, thus avoiding tedious DHAP preparation. In addition, as this aldolase can accept other donors than DHA, the use of hydroxyacetone (HA) as donor has enlarged the scope of the process with variation on R^3 group. However, the nitroaldol reaction in the presence of the methyl group was less stereoselective.[20]



Scheme 1: One-pot cascade reactions for the synthesis of nitrocyclitols

Encouraged by these interesting results we envisaged such stereoselective cascades using RhuA and FucA as key biocatalysts for C-C bond formation. The overall process would lead to different stereocenters at C2 and C3 cyclitol positions due to aldolase stereoselectivity together with two other stereocentres (C1 and C6) established by the nitroaldolisation. Instead of using chemically synthetized DHAP,[28] a second bi-enzymatic system developed by García-Junceda's group has been coupled for *in situ* generation of DHAP from DHA as initial donor.[32] This system is based on the use of the recombinant ATP-dependent dihydroxyacetone kinase (DHAK from *Citrobacter freundii* CECT 4626).[33] The regeneration of ATP is catalyzed by acetate kinase (AK). The new one-pot multi-enzyme system is depicted scheme 2.

2. Materials and methods

2.1. Organisms and enzyme production.

Acetyl phosphate, acetate kinase from *E. coli* (E.C.2.7.2.1) and acid phosphatase from wheat germ (E.C. 3.1.3.2) were purchased from Sigma-Aldrich Company.
RhuA, FucA from *E. coli* and DHAK from *C. freundii* were all produced and purified by the same protocol as described below.
E.coli BL21 (DE3) strains overexpressing the corresponding enzyme were grown on 5 mL of Luria-Bertani medium (yeast: 5 g/L, tryptone: 10 g/L and NaCl: 10 g/L), bacteriologic agar (15 g/L) and an antibiotic (0.1 mg/mL of ampicillin for DHAK and 0.06 mg/mL of kanamycin for RhuA or FucA). Those pre-cultures were used to inoculate 1L of sterile Luria-Bertani medium containing the antibiotic and allowed to stir for 3h in 37°C till a 0.5 OD value (at 600 nm) was reached. Then 2 mL (for DHAK) or 1 mL (for the aldolases) of a 0.5M solution of IPTG was added and the mixture shaked overnight at 30°C. The culture was then centrifuged (8,000 rpm) for 15 min at 4°C and the supernatant was discarded. The harvested cells were washed with buffer Tris-HCl 50 mM pH 8 buffer and centrifuged again. Washed cells were suspended in buffer (8 mL /gram of cells), disrupted by ultrasonication at 4°C and centrifuged at 12,000 rpm during 30 min at 4°C. The cell-free extract obtained was loaded onto a Ni^{2+}-NTA IMAC and the stationary phase was then washed with phosphate buffer (20 mM, pH 7.5) containing 500 mM of NaCl. The protein was gradiently eluted using the previous buffer containing 50-500 mM of imidazole. Positive fractions checked by the Bradford test were pooled together and ultrafiltrated under pressure through a 30 kDa cut-off membrane using phosphate buffer. Dialysis could also be

performed on small volumes. The solution was finally freeze-dried. The obtained white powder can be stored at 4°C for months without loss of enzyme activity.

DHAK activity assay.

The DHAK activity was spectrophotometrically measured by enzymatic quantification of the DHAP formed in the phosphorylation reaction. This assay was run at room temperature following NADH absorbance at 340 nm (ε_{NADH}= 6220 $cm^{-1}M^{-1}$) for 15 minutes in a cuvette of 1 mL containing TRIS-HCl (800 µL, 50 mM, pH 8.0), NADH (10 µL, 20 mM, 0.2 µmol), ATP (75 µL, 50 mM, 3.75 µmol), $MgSO_4$ (100 µL, 50 mM, 5 µmol), GPDH (2 µL, 630 U/ml, 1.3 U), TPI (2 µL, 6300 U/ml, 12.6 U), DHAK (1-2 µL of purification aliquot, 2.5-5.0 µg of protein), and DHA (2,5 µL, 1 M, 2.5 µmol). One unit was defined as the amount of kinase that catalyzes the phosphorylation of 1µmol of DHA in one min.

RhuA and FucA activity assays.

One unit was defined as the amount of the enzymes able to retroaldolize 1µmol/min of L-fuculose-1-phosphate or L-rhamnulose-1-phosphate respectively producing dihydroxyacetone phosphate (DHAP).[34] Accumulated DHAP from retroaldol reaction was detected and quantified using the GPDH/TPI spectrophotometric assay previously described. The enzymatic assays were run at room temperature following the decrease of absorbance at 340 nm for 20 minutes in a cuvette of 1 mL containing Tris-HCl buffer (800 µL, 50 mM, pH 8.0), NADH (10 µL, 20 mM, 0.2 µmol), GPDH (2 µL, 630 U/ml, 1.3 U), TPI (2 µL, 6300 U/ml, 12.6 U), RhuA (1.0-2.5 µL of purification aliquot, 0.05-0.13 mg of protein) or FucA (1.0-2.5 µL of purification aliquot, 0.05-0.17 mg of protein), $ZnCl_2$ (1 µL, 10 mM, 0.01 µmol), and the corresponding substrate (2.5 µL, 50 mM, 1.25 µmol).

Alternatively a rapid activity assay is described in this work. Aldolization between DHAP and dimethoxacetaldehyde (DMA) is spectrophotometrically monitored following the DHAP consumption by GPDH reaction. In this case, one unit was defined as the ability of the enzymes to aldolize 1µmol/min of dimethoxacetaldehyde and DHAP producing the corresponding aldol.

2.2. Substrates syntheses and domino reactions.

Modified procedure for preparation of 4-nitrobutanal **1**

To a solution of freshly distilled acrolein (4 mL, 60 mmol, 1 eq.) in 290 mL of water were added nitromethane (32.2 mL, 600 mmol, 10 eq.) and bovin serum albumin (1 g). The reaction mixture was allowed to gentle stir at room temperature for 7 hours. Ethanol 95% was added and the mixture was filtered through a pad of celite. After concentration under vacuum the solution was bulb-to-bulb distilled under reduced pressure to afford compound **1** in 37% yield displaying same spectral characteristics than already mentioned.[29]

General procedure for preparation of nitrocyclitols with FucA and RhuA (one-pot – two-steps procedure):

a) Preparation of DHAP:[35]

To distilled water (2.7 mL), were successively added solutions of DHA (150 µL, 1M, 0.3 mmol, 1 eq.), $MgSO_4$ (150 µl, 0.5M, 0.075 mmol), acetylphosphate (1.2 mL, 0.5M, 0.6 mmol, 2 eq.) and phosphate buffer (1.5 mL, 60 mM, 1.5 mL, pH 7). The pH was adjusted to 7.5 and a solution of DHAK (160 µL, 36 U/mL, 6 U), AK (100 µL, 170 U/mL, 18 U) and ATP (40 µL, 0.5M, 9.8 µmol) were added. The total phosphorylation of DHA was spectrophotometrically measured by GPDH enzymatic quantification of DHAP. This spectrophotometric assay was run at room temperature during 10 min in a final volume of 1 mL, containing Tris-HCl 50 mM pH 8 (975 µL), NADH (20 µL), aliquot of the reaction (2.5-5.0 µL), and a mixture of GPDH/TPI (2 µL).

b) Synthesis of nitrocyclitols:

To the freshly prepared DHAP solution, aldehyde (450 µmol, 1.5 eq.) was added to the solution along with a solution of $ZnCl_2$ (10 µL, 10 mM) and DMSO (900 µL, for water insoluble aldehyde, otherwise DMSO was replaced by water). The reaction was then started by aldolase (FucA or RhuA, 200 µL, 43 U/mL = 9 U) addition and the reaction was shaken until total disappearance of DHAP (DHAP spectrophotometric assay). Then, the pH was lowered to 3.9 and the solution was centrifuged (20,000 rpm, 40 min, 5°C) to recover the supernatant. To this last solution, acid phosphatase (18 mg, 20 U) was added and the mixture was shaken for 24 h. The solution was centrifuged (20,000 rpm, 40 min, 5°C) and the supernatant was concentrated under reduced pressure at 30°C. The residue was purified by flash chromatography (CH_2Cl_2/MeOH 9:1) to afford the desired nitrocyclitols; RhuA: **2** (192 mg,77 %), **3** (11 mg, 4 %), **5** (314 mg, 67 %), **6** (61 mg, 13 %), **9** (266 mg, 62 %), **10** (73 mg, 17 %), **13** (98 mg, 41 %), **14** (98 mg, 41 %), FucA: **2** (164 mg, 66 %), **3** (42 mg 17 %), **6** (271 mg, 58 %), **7** (112 mg, 24 %), **10** (167 mg, 39 %), **11** (189 mg, 44 %), **14** (95 mg, 44 %), **15** (81 mg, 34 %).

(1*R*,2*R*,3*S*,6*S*)-1-Hydroxymethyl-6-nitrocyclohexane-1,2,3-triol **2**: R_f = 0.60 (DCM/MeOH 9:1). IR: ν = 3367, 2495, 1553 cm^{-1}. $[\alpha]_D^{25} = -20$ (c = 1, CH_3OH). (**2** is the enantiomer of the already described nitrocyclitol obtained with FBA[29])

(1*S*,2*R*,3*R*,6*R*)-1-Hydroxymethyl-6-nitrocyclohexane-1,2,3-triol **3**: R_f = 0.65 (DCM/MeOH 9:1). IR: ν = 3367, 2495, 1553 cm^{-1}. $[\alpha]_D^{25}$ = + 18 (c = 1, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.75 (dd, *J* = 12.4, 4.0 Hz, 1H, 6ax-H), 4.00 (m, 1H, 3ax-H), 3.88 (d, *J* = 2.6 Hz, 1H, 2eq-H), 3.83 (d, *J* = 11.5 Hz, 1H, 7-H), 3.55 (d, *J* = 11.5 Hz, 1H, 7-H), 2.41 (m, 1H, 5eq-H), 1.93 (m, 2H, 5ax-H, 4eq-H), 1.76 (m, 1H, 4ax-H) ppm.^{13}C NMR (100 MHz, MeOD): δ = 87.8 (6-C), 76.6 (1-C), 74.8 (2-C), 71.3 (3-C), 65.1 (7-C), 25.8 (4-C), 25.7 (5-C) ppm. IR: ν = 3367, 2495, 1553 cm^{-1}, 1047 (CO) cm^{-1}. HRMS (ES+): *m/z* calcd. for $C_7H_{13}NO_6$+Na^+: 230.0641 [M+Na^+], found 230.0632.

(1*R*,2*R*,3*S*,5*R*,6*S*)-1-Hydroxymethyl-6-nitrocyclohexane-1,2,3,5-tetraol **5**: IR: ν = 3481, 1545, 1378, 1064 cm^{-1}. $[\alpha]_D^{25}$ = – 26 (c = 1, CH_3OH). (**5** is the enantiomer of the already described nitrocyclitol obtained with FBA[30])

(1*S*,2*R*,3*R*,5*S*,6*R*)-1-Hydroxymethyl-6-nitrocyclohexane-1,2,3,5-tetraol **6**: IR: ν = 3357, 1554, 1375, 1053 cm^{-1}. $[\alpha]_D^{25}$ = + 22 (c = 1, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.55 (d, *J* = 10.2 Hz, 1H, 6ax-H), 4.43 (ddd, *J* = 10.2,10.8,4.2 Hz, 1H, 5ax-H), 4.06 (m, 1H, 3ax-H), 3.82 (d, *J* = 2.4 Hz, 1H, 2eq-H), 3.79 (d, *J* = 11.3 Hz, 1H, 7-H), 3.50 (d, *J* =11.3 Hz, 1H, 7-H), 2.02 (m, 1H, 4eq-H), 1.82 (ddd, *J* = 11.9,11.9,10.8 Hz, 1H, 4ax-H) ppm.^{13}C NMR (100 MHz, MeOD): δ = 93.2 (6-C), 76.4 (1-C), 73.5 (2-C), 67.4 (5-C), 66.9 (3-C), 65.7 (7-C), 36.4 (4-C) ppm. HRMS (ES+): *m/z* calcd. for $C_7H_{13}NO_7$+Na^+: 246.0590 [M+Na^+], found 246.0588.

(1*R*,2*R*,3*R*,5*R*,6*S*)-1-Hydroxymethyl-6-nitrocyclohexane-1,2,3,5-tetraol **7**: IR: ν = 3357, 1554, 1375, 1053 cm^{-1}. $[\alpha]_D^{25}$ = – 29 (c = 1, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.75 (ddd, *J* = 10.6,10.8,4.6 Hz, 1H, 5ax-H), 4.70 (d, *J* = 10.6 Hz, 1H, 6ax-H), 4.15 (m, 1H, 3eq-H), 3.76 (d, *J* =12.4 Hz, 1H, 7-H), 3.65 (d, *J* = 2.2 Hz, 1H, 2ax-H), 3.17 (d, *J* = 12.4 Hz, 1H, 7-H), 2.26 (m, 1H, 4eq-H), 1.52 (ddd, *J* = 12.1,12.1,10.8 Hz, 1H, 4ax-H) ppm.^{13}C NMR (100 MHz, MeOD): δ = 93.4 (6-C), 79.1 (1-C), 72.2 (3-C), 69.0 (2-C), 64.6 (5-C), 61.8 (7-C), 38.1 (4-C) ppm. HRMS (ES+): *m/z* calcd. for $C_7H_{13}NO_7$+Na^+: 246.0590 [M+Na^+], found 246.0586.

(1*R*,2*R*,3*S*,4*R*,5*S*,6*S*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **9**: IR: ν = 3418, 1556, 1378, 1109 cm^{-1} $[\alpha]_D^{25}$ = –10 (c = 1.0, CH_3OH). (**9** is the enantiomer of the already described nitrocyclitol obtained with FBA or FSA).[28]

On the mixture of 1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **10** and **11** obtained with FucA as catalyst: HRMS (ES+): *m/z* calcd. for $C_7H_{13}NO_8$+Na^+: 262.0539 [M+Na^+], found 262.0551.

(1*S*,2*R*,3*R*,4*R*,5*R*,6*R*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **10**: IR: ν = 3418, 1556, 1378, 1109 cm^{-1}. $[\alpha]_D^{25}$ = +21 (c = 1.0, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.71 (d, *J* = 10.8 Hz, 1H, 6ax-H), 4.47 (dd, *J* = 10.8, 3.1 Hz, 1H, 5ax-H), 4.15 (m, 1H, 4eq-H), 4.06 (dd, *J* = 3.1, 3.3 Hz, 1H, 3ax-H), 3.99 (d, *J* = 3.3 Hz, 1H, 2eq-H), 3.76 (d, *J* = 11.0 Hz, 1H, 7-H), 3.20 (d, *J* = 11.0 Hz, 1H, 7-H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 91.1 (6-C), 79.3 (1-C), 76.4 (4-C), 75.4 (2-C), 69.4 (5-C), 67.1 (3-C), 65.2 (7-C) ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*,6*S*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **11**: IR: ν = 3418, 1556, 1378, 1109 cm^{-1} $[\alpha]_D^{25}$ = – 20 (c = 1.0, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.47 (d, *J* = 9.7 Hz, 1H, 6ax-H), 4.55 (dd, *J* = 9.7, 10.4 Hz, 1H, 5ax-H), 4.15 (m, 1H, 3eq-H), 3.85 (d, *J* = 11.5 Hz, 1H, H-7), 3.72 (d, *J* = 2.2 Hz, 1H, H-2ax-H), 3.52 (d, *J* = 11.5 Hz, 1H, H-7), 3.44 (dd, *J* = 10.4, 2.7 Hz, 1H, 4ax-H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 91.1 (6-C), 76.4 (1-C), 76.0 (4-C), 75.4 (2-C), 69.4 (5-C), 67.1 (3-C), 61.5 (7-C) ppm.

(1*R*,2*R*,3*S*,4*S*,5*S*,6*S*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **13**: $[\alpha]_D^{25}$ = – 13 (c = 1.0, CH_3OH). (**13** is the enantiomer of the already described nitrocyclitol obtained with FBA or FSA).[28]

On the mixture of 1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **14** and **15** obtained with FucA as catalyst: HRMS (ES+): *m/z* calcd. for $C_7H_{13}NO_8$+Na^+: 262.0539 [M+Na^+], found 262.0546.

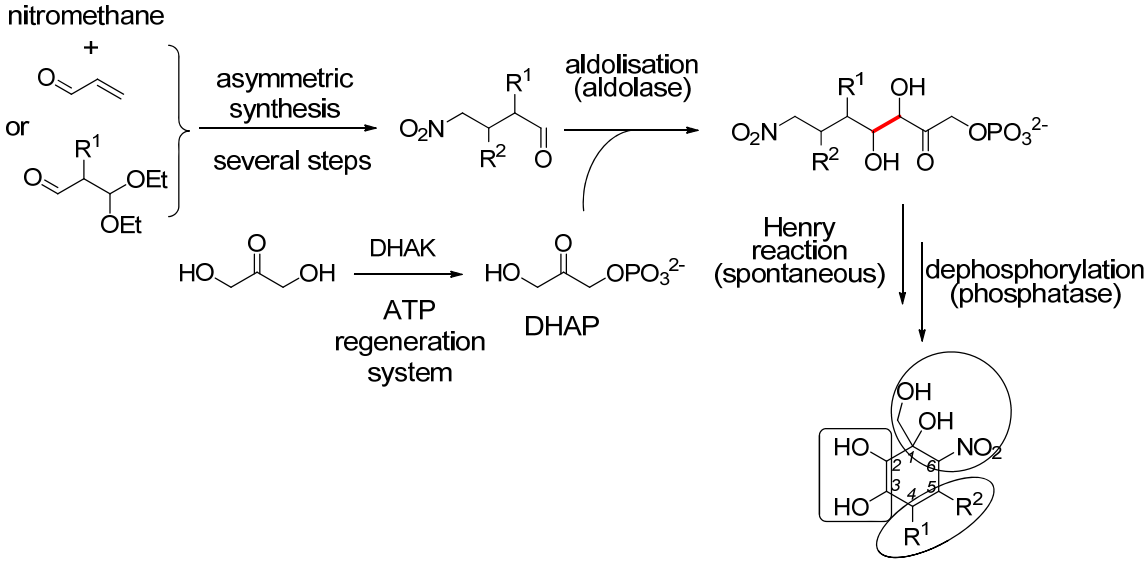
(1*S*,2*R*,3*R*,4*S*,5*R*,6*R*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **14**: $[\alpha]_D^{25}$ = + 11 (c = 1.0, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.69 (d, *J* = 10.6 Hz, 1H, 6ax-H), 4.47 (dd, *J* = 10.6, 10.6 Hz, 1H, 5ax-H), 4.03 (dd, *J* = 10.1, 3.4 Hz, 1H, 3ax-H), 3.92 (d, *J* = 3.4 Hz, 1H, 2eq-H), 3.80 (dd, *J* = 10.6, 10.1 Hz, 1H, 4ax-H), 3.74 (d, *J* = 11.0 Hz, 1H, H-7), 3.25 (d, *J* = 11.0 Hz, 1H, H-7) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 91.0 (6-C), 78.1 (1-C), 75.4 (4-C), 73.6 (3-C), 72.9 (2-C), 69.9 (5-C), 65.0 (7-C) ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*,6*S*)-1-hydroxymethyl-6-nitrocyclohexane-1,2,3,4,5-pentol **15**: $[\alpha]_D^{25}$ = – 18 (c = 1.05, CH_3OH). ^1H NMR (400 MHz, MeOD): δ = 4.82 (d, *J* = 10.0 Hz, 1H, 6ax-H), 4.55 (dd, *J* = 10.0, 3.1 Hz, 1H, 5ax-H), 4.15

(m, 1H, 4eq-H), 4.05 (m, 1H, 3eq-H), 3.85 (d, J = 11.5 Hz, 1H, H-7), 3.72 (d, J = 2.3 Hz, 1H, 2ax-H), 3.25 (d, J = 11.5 Hz, 1H, H-7) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 91.5 (6-C), 78.0 (1-C), 76.3 (4-C), 75.4 (2-C), 68.2 (5-C), 65.9 (3-C), 60.3 (7-C) ppm.

3. Results and discussion

The aim of this project was to develop a green methodology enabling the preparation of a variety of nitrocyclitols, controlling the stereochemistry of the asymmetric centers created. Thus we investigated a chemoenzymatic pathway where a spontaneous Henry reaction was flanked by two enzymatic reactions catalyzed first by an aldolase and then by a phosphatase for the final dephosphorylation step (scheme 2). Stereocontrol of C2 and C3 of the nitrocyclitols thus obtained was ensured by an aldolase whereas the one for C1 and C6 was achieved by the Henry reaction. The stereochemistry of C4 and C5 was already defined during the synthesis of nitroaldehydes. Due to the stereoselectivity of each aldolase we would expect the major formation of (2*R*,3*R*) "*D-threo*" from FucA or (2*R*,3*S*) "*L-erythro*" from RhuA configurations for the nitrocyclitol. For achieving this process we first considered enzyme production and then donor and acceptor aldolases substrates syntheses.



Scheme 2: general one-pot multi-steps strategy for the stereocontrol of the asymmetric centers during the preparation of nitrocyclitols. Stereocontrol code, square: aldolases, circle: intramolecular nitroaldolisation, ellipse: asymmetric synthesis.

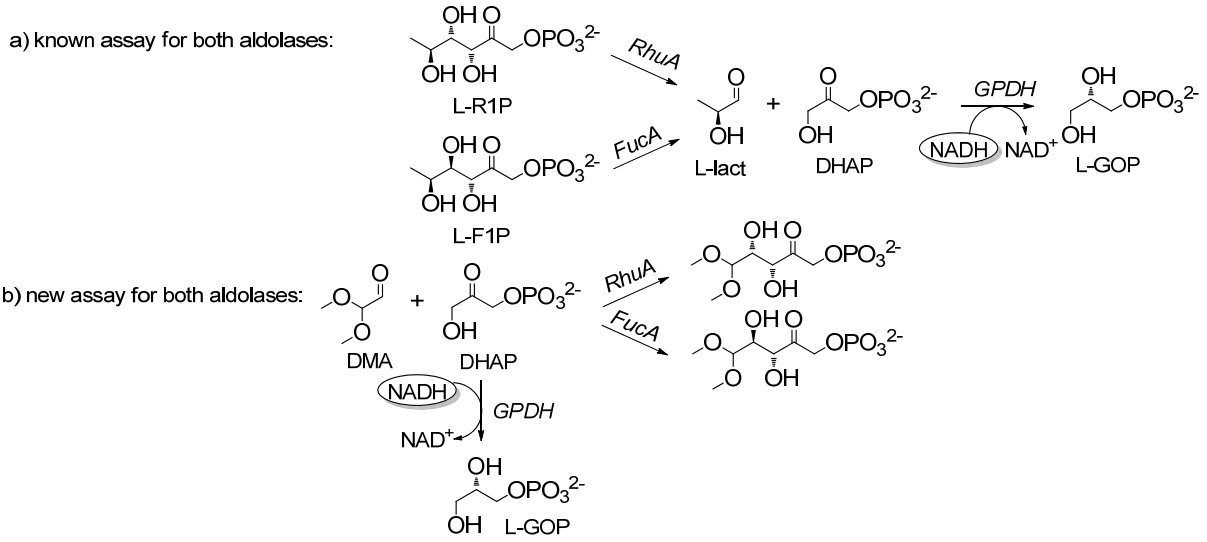
3.1. Syntheses of the substrates for aldolase-mediated reactions.

The DHAP donor substrate was prepared in a straightforward bi-enzymatic phosphorylation system of DHA as already described.[35] The phosphate donor (ATP) was regenerated using AK with acetylphosphate. Another efficient ATP regeneration system based on pyruvate kinase/phosphoenol pyruvate mixture might also be used without any decrease in performance of DHAP preparation as demonstrated by our group for the preparation of phosphorylated sugars.[36]
For preparing the six-members ring nitrocyclitols, linear γ-nitrobutanal acceptor substrates were synthesized as already described,[20, 28, 30] except 4-nitrobutanal for which we have simplified the protocol. Thus the drastic procedure using KF as a base at -35°C in methanol[29] was replaced by the use of bovine serum albumin (BSA) as base in water at room temperature[37] giving similar yields. A total of four nitrobutanals were systematically used with RhuA and FucA (scheme 4 and table 1): 4-nitrobutanal **1** (R^1=R^2=H), (±)-3-hydroxy-4-nitrobutanal **4** (R^1=H, R^2=OH), (2*R*)-2,3-dihydroxy-4-nitrobutanal **8** (R^1=OH, R^2=OH) and (2*S*)-2,3-dihydroxy-4-nitrobutanal **12** (R^1=OH, R^2=OH).

3.2. Enzyme productions and development of a rapid indirect activity assay.

6-Histidine tagged RhuA and FucA were overexpressed in *E. coli* and purified by IMAC using an imidazole gradient for elution. Imidazole was then removed by dialysis or ultrafiltration giving fair yields of each enzyme after freeze-drying (36 U, 171 mg of FucA; 30 U, 252 mg of RhuA per liter of each culture). Up to

now aldolases activity were spectrophotometrically measured at 340 nm using the retro-aldol reaction from their natural substrate (L-fuculose-1-phosphate or L-rhamnulose-1-phosphate, scheme 3a). However the phosphorylated monosaccharides used to measure these activities are not commercially available which means that their syntheses by coupling L-lactaldehyde to DHAP [38, 39] or following other methods described in the literature[40, 41] were necessary. An additional problem was the non-availability on the market of L-lactaldehyde acceptor substrate, thus requiring its preparation following described procedures.[42-44] Due to those major disadvantages, we decided to develop another assay for both aldolases involving a cheap (achiral) and easily available substrate. Several commercially available aldehydes (ethanal, propanal, isobutyraldehyde, benzyloxyacetaldehyde, etc.) were screened and the DHAP conversion level was measured. Dimethoxyacetaldehyde (DMA) was found as good substrate as L-lactaldehyde, leading to more than 95% of DHAP consumed compared to 95% with natural acceptor. In parallel the aldol produced was followed applying a protocole published by Sheldon's group.[45] Similar results were obtained when comparing L-lactaldehyde and DMA as ~94% of aldol was assayed in both cases. Therefore we used DMA to perform the activity assay, more suitable for a rapid evaluation of activities in routine production of enzymes (scheme 3b). An indirect assay was implemented thereby NADH consumption was followed upon time on aliquots of the biocatalysed aldolisation reaction between DMA and DHAP in initial rate conditions. In order to compare with the initial assay[34] and taking into account that indirect assays are less accurate, the same batch of a given aldolase was tested. Thus we have found a FucA specific activity of 0.21±0.01 U/mg using the retroaldolisation assay and 0.24±0.04 U/mg in the aldol way. Proceeding similarly, we have found 0.12±0.01 U/mg for RhuA using the direct assay whereas a value of 0.16±0.04 was obtained with the indirect assay. In conclusion, this method could simplify the quantification of catalytic activity assay and has given an acceptable order of magnitude for routine production of aldolases.



Scheme 3: development of a spectrophotometric activity assay for L-rhamnulose-1-phosphate and L-fuculose-1-phosphate aldolases.

DHAK was produced and purified as already described by Sánchez-Moreno *et al*.[33]

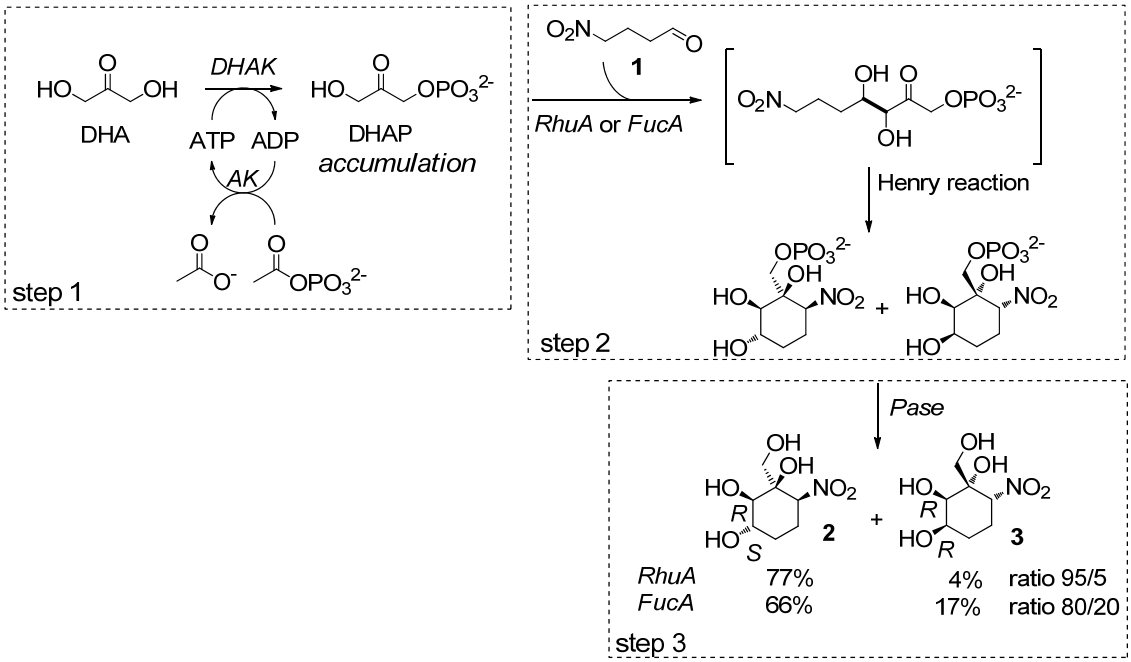
3.3. Biocatalysed cascade aldol reactions towards nitrocyclitols.

3.3.1. Implementation of the reactions.

The first aldehyde involved with DHAP in the cascade process was 4-nitrobutanal **1**. This compound being water insoluble, DMSO was used as co-solvent. DMSO was generally tolerated by most of the DHAP aldolases and up to 50% was accepted for RhuA or 30% for FucA.[39, 46] In our case, a 10% v/v proportion of DMSO in water was sufficient for dissolution of the nitroaldehyde.

Then a first attempt was done to couple DHAK-catalyzed synthesis of DHAP from DHA and RhuA or FucA-catalysed aldolisation with nitrobutanal **1** in a one-pot one-step process. At an analytical level DHA disappearance was spectrophotometrically monitored using DHAK and GPDH/TPI coupled enzymes with NADH. However no DHA consumption was detected, reflecting inhibition of at least one of the kinases involved. Further investigations showed that DMSO was responsible for this inhibition on DHAK (a slighter inhibition due to the nitroaldehyde was also detected). Those initial assays guided us to focus on a one-pot multi-steps process where DHAP was allowed to accumulate and then an aldehyde solution in DMSO was added followed by the aldolase (scheme 4). As a remark, reactions between nitrobutanals and DHA in borate buffer

catalyzed by RhuA have been envisaged following described procedures,[11] unfortunately these attempts stayed unsuccessful.



Scheme 4: one-pot three-steps multi-enzymatic process enabling nitrocyclitol **2** and **3** formation.

At a preparative scale the common compound of both successive reactions of the process was assayed to know when each reaction was complete. Thereby DHAP appearance upon DHAK-catalyzed first step was monitored till a maximum was reached. Then after addition of the other biocatalyst and reactant, its concentration decrease was evaluated upon aldolase-catalyzed step until complete disappearance. At this step, without any isolation of the nitrocyclitol phosphate a fourth enzyme was added to the reaction mixture in order to hydrolyze the phosphate group. Acid phosphatase (Pase) from wheat germ (E.C. 3.1.3.2) was chosen for its large activity spectrum and its low cost. The pH of the reaction mixture was lowered around 5 before adding the hydrolase. As the enzymes (DHAK, AK and aldolase) present in the reaction mixture precipitated, the suspension was centrifuged. The supernatant was stirred overnight in the presence of Pase. With each aldolase, the same two nitrocyclitols **2** and **3** were isolated in good overall yields and separated after flash silica gel chromatography. However the ratio determined from chemical yields were quite different, RhuA has provided the expected 2*R*,3*S* configuration as major one whereas for FucA the 2*R*,3*R* configuration was present in minor proportion (only 20%). This lack of stereoselectivity could be due to steric hindrance considerations between the side chain of the aldehyde and the C-terminal part of the protein as already mentioned.[47] Another reason could be the lack of the hydrogen bond naturally occurring between the hydroxyl group in the second position of L-lact and a Tyr113 residue within the active site.[47]

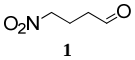
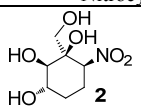
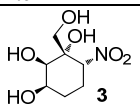
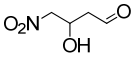
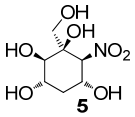
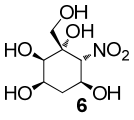
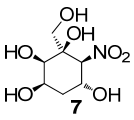
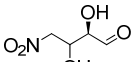
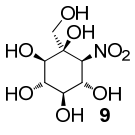
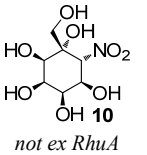
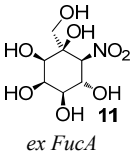
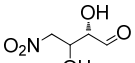
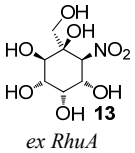
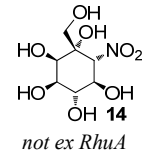
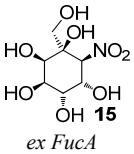
The study was pursued with other nitroaldehydes hydroxylated in position 3 and in position 2 and 3 (**4**, **8** and **12**; Table 1). Even though the acceptor substrate was water soluble like dihydroxynitrobutanal **8** or **12**, the second strategy was adopted to prevent DHAK inhibition noted in the presence of the nitroaldehydes. Following the one-pot multi-steps process, aldehydes **1**, **4**, **8** and **12** were successfully converted to give the corresponding nitrocyclitols in respectively 81, 80, 79 and 82% total yields using RhuA, and 83, 82, 83 and 78% with FucA for the overall four reactions. The results are presented in detail table 1. Each nitrocyclitol was purified by silica gel flash chromatography and characterized by several NMR experiments (in particular NOESY and coupling constants of the chair conformation) referring to the (*R*) configuration of C2 of the nitrocyclitol provided by any aldolase. Even though all aldehydes gave a diastereomeric mixture of nitrocyclitols, they could all be separated from each other by chromatography.

The stereoselectivities shown by the aldolases were different in the presence of the hydroxylated aldehydes **4**, **8** and **12**. In table 1, refereeing to these stereoselectivities, under all cyclitol structures the status “expected” (noted *ex*) and “not expected” (noted *not ex*) was specified. From racemic aldehyde **4** and RhuA as biocatalyst, two diastereomers **5** (2*R*,3*S*) and **6** (2*R*,3*R*) were isolated in 67 and 13 % yield respectively. From **8** and **12**, samely RhuA has furnished two diastereoisomers in each case: **9** and **10** in 62 and 17 % yields respectively and **13** and **14** in both 41 % yields. Thus, in addition to the expected 2*R*,3*S* cyclitols RhuA has always catalyzed the

formation of the "not expected" 2*R*,3*R* cyclitols being less and less stereoselective from **4** to **12**. Even in the presence of a (2S) hydroxyl group (**12**) like found in the natural substrate this enzyme showed no better diastereoselectivity. A rational explanation is that the reaction was under thermodynamic control with total consumption of DHAP. Thus kinetic discrimination could not be observed as reported by Fessner's group.[48] In addition, the diastereoselectivity seems highly substrate-dependent as such low selectivity was also observed by Clapés and coll.[47]

On the contrary, FucA has always catalyzed the formation of the expected 2*R*,3*R* stereocentres from aldehydes **4**, **8** and **12**., Two cyclitols were formed from each aldehyde, **6** and **7** in 58 and 24 % yields, **10** and **11** in 39 and 44 % yields and finally **14** and **15** in 44 and 34 % yields respectively. Thus FucA has been highly stereoselective in presence of hydroxylated aldehydes. It might be explained by the hydroxyl group(s) restoring probably a good positioning of the substrate in the active site due to hydrogen bonds.[47]

Table 1: Nitrocyclitols stereoisomers prepared following the one-pot two-steps cascade reactions

Aldehyde	Nitrocyclitol*			Isolated yield % Aldolase	*Ratio*[#]
 **1**	 **2** *ex RhuA not ex FucA*	 **3** *not ex RhuA ex FucA*		***2/3*** 77/4 RhuA ***2/3*** 66/17 FucA	*95/5* *80/20*
 **4**	 **5** *ex RhuA*	 **6** *not ex RhuA ex FucA*	 **7** *ex FucA*	***5/6*** 67/13 RhuA ***6/7*** 58/24 FucA	*84/16* *71/29*
 (2*R*)-**8**	 **9** *ex RhuA*	 **10** *not ex RhuA ex FucA*	 **11** *ex FucA*	***9/10*** 62/17 RhuA ***10/11*** 39/44 FucA	*79/21* *47/53*
 (2*S*)-**12**	 **13** *ex RhuA*	 **14** *not ex RhuA ex FucA*	 **15** *ex FucA*	***13/14*** 41/41 RhuA ***14/15*** 44/34 FucA	*50/50* *57/43*

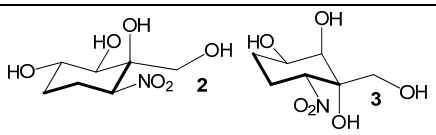
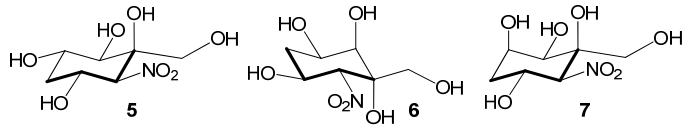
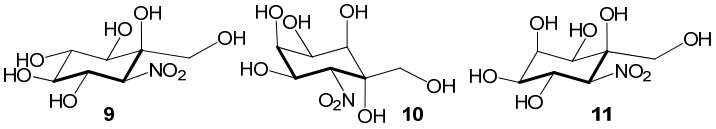
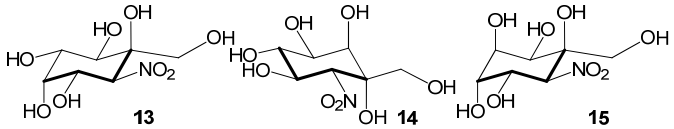
For aldehyde **4**, **8** and **12** preparations see references [28, 30]; * nitrocyclitols **2**, **5**, **9** and **13** are enantiomers of those prepared with FBA or FSA[28]; # calculated from chemical yields; ex: expected; not ex: not expected.

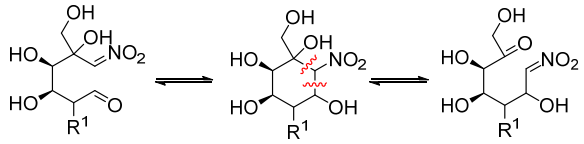
We would like then to focus on the Henry reaction. For a better visualization the nitrocyclitol configurations and conformations deduced from NMR analyses are also proposed in table 2. From aldehyde **1**, considering the expected defined configuration by the aldolase (*R*,*S* for RhuA or *R*,*R* for FucA), one major nitrocyclitol (**2** or **3**) has been obtained. Thus this cyclisation has been highly stereoselective. In the other cases, the substrates **4**, **8** and **12** are bearing a hydroxyl group in β to the nitro group (C3 on the aldehyde, becoming C5 on the cyclitol). Due to the existence of both *R* and *S* configurations on this position C3, two main families of nitrocyclitols have been obtained with the same relative configurations: hydroxymethyl on C1 and $-NO_2$ on C6 *trans* relatives as well as the hydroxyl on C5 and $-NO_2$ on C6. In table 2, these substituants can be clearly seen always in equatorial positions however with the chair conformation inverted. **5** and **6** from RhuA or **6** and **7** from FucA could be cited as example. All these results are consistent with those obtained in our previous work illustrated scheme 1. They were evidently expected with *R*,*S* stereoselective RhuA as all the process could lead to the enantiomers of cyclitols prepared from FSA or FBA. When the multistep process was performed with FucA, the first stereocentres created on the aldols were *R*,*R*. Modification of these configurations when compared with FSA or RhuA case has not influenced the cyclisation as comparable results were obtained. The Henry cyclisation has led to the same two families whatever the first created C-C bond stereochemistry was. In conclusion the spontaneous Henry reaction was also highly stereoselective when additional hydroxyl groups on the aldehydes were present.

Each multistep process has provided two cyclitols on which C5 is *R* or *S*. However, it should be pointed out that the ratio around 70/30 for cyclitols **6** and **7** taken as example is particularly far from an expected 50/50 value considering the racemic aldehyde **4**. This can only be explained by a two-fold retro-Henry reaction illustrated

scheme 5.[20] As we discussed in previous publications,[20, 28] under smooth heating during water removal or at a slight alkaline pH, whatever the C3 configuration of the acceptor aldehyde is, cyclitols **5** to **15** could undergo a retro-Henry reaction. This reversibility has led to a partial enrichment (or total in case of RhuA governed process) of the more stable isomer under thermodynamic control. For example, considering compounds **7** and **15** in table 2, two hydroxyl groups are clearly in 1,3-diaxial interaction thus disadvantaging these stereoisomers when compared with **6** and **14** respectively. This could explain the differences between the 50/50 expected ratio and the experimental results. To note, **10** and **11** in around 50/50 ratio are both showing two hydroxyl substituants in 1,3-diaxial interaction.

Table 2: Nitrocyclitol configurations and conformations

Configurations	Conformations
(1*R*,2*R*,3*S*,6*S*)-**2** and (1*S*,2*R*,3*R*,6*R*)-**3**	
(1*R*,2*R*,3*S*,5*R*,6*S*)-**5**, (1*S*,2*R*,3*R*,5*S*,6*R*)-**6** and (1*R*,2*R*,3*R*,5*R*,6*S*)-**7**	
(1*R*,2*R*,3*S*,4*R*,5*S*,6*S*)-**9**, (1*S*,2*R*,3*R*,4*R*,5*R*,6*R*)-**10** and (1*R*,2*R*,3*R*,4*R*,5*S*,6*S*)-**11**	
(1*R*,2*R*,3*S*,4*S*,5*S*,6*S*)-**13**, (1*S*,2*R*,3*R*,4*S*,5*R*,6*R*)-**14** and (1*R*,2*R*,3*R*,4*S*,5*S*,6*S*)-**15**	



Scheme 5: Retro-Henry reaction possible on both sides of the CH-NO_2 explaining the isomerisation of stereocentres C1, C5 and C6.

4. Conclusions

We finalized herein a powerful and green one-pot four sequential steps process involving two kinases, an aldolase and a phosphatase. It enabled the versatile preparation of several nitrocyclitols in terms of structure and stereochemistry, precursors of aminocyclitols as potential glycosidase inhibitors. This methodology exemplified the synergy by combining chemical and enzymatic steps and has led to a small library of nitrocyclitols by varying only the starting aldehyde and the aldolase. Except with 4-nitrobutanal, FucA was found highly stereoselective, much more than RhuA. Even though the products were obtained as mixtures due to lack of stereoselectivity by the aldolases or to the presence of racemic alcohol, they were all successfully separated by chromatography. The Henry reaction was revealed highly stereoselective, defining two or three stereocenters when a hydroxyl was present on the aldehyde. These one-pot cascade processes reinforce the powerful of biocatalysis by limiting the protection-deprotection steps mainly encountered in the cyclitol synthesis.

Acknowledgments

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