Chemoenzymatic Aldol Reactions

Applications of a four-enzyme-catalytic cascade reaction

Lieke van Hemert

Chemoenzymatic Aldol Reactions
Applications of a four-enzyme-catalytic cascade reaction

Jacoba Cornelia van Hemert

Chemoenzymatic Aldol Reactions
Applications of a four-enzyme-catalytic cascade reaction

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus, prof. mr. S. C. J. J. Kortmann,
volgens besluit van het college van decanen

in het openbaar te verdedigen op donderdag 1 november 2012
om 13.00 uur precies

door

Jacoba Cornelia van Hemert

geboren op 19 april 1980
te Middelburg

Promotor:	Prof. dr. Floris P. J. T. Rutjes
Copromotor:	Dr. Floris L. van Delft
Manuscriptcommissie:	Prof. dr. Pedro H. H. Hermkens
	Prof. dr. Ron Wever (Universiteit van Amstedam)
	Dr. Hans W. Scheeren
Paranimfen:	Lennar van Hemert
	Janton van Hemert

The research described in this thesis was financially supported by the Integration of Bio- and Organic Synthesis (IBOS) of the Netherlands Organization for Scientific Research (NWO).

Printing:	Optima Grafische Communicatie
Cover Design:	Optima Grafische Communicatie

“Do or do not, there is no try”

Yoda

Dit proefschrift is opgedragen aan de RAMA konijntjes
voor hun bijdrage aan de wetenschap

Table of Contents

Chapter 1

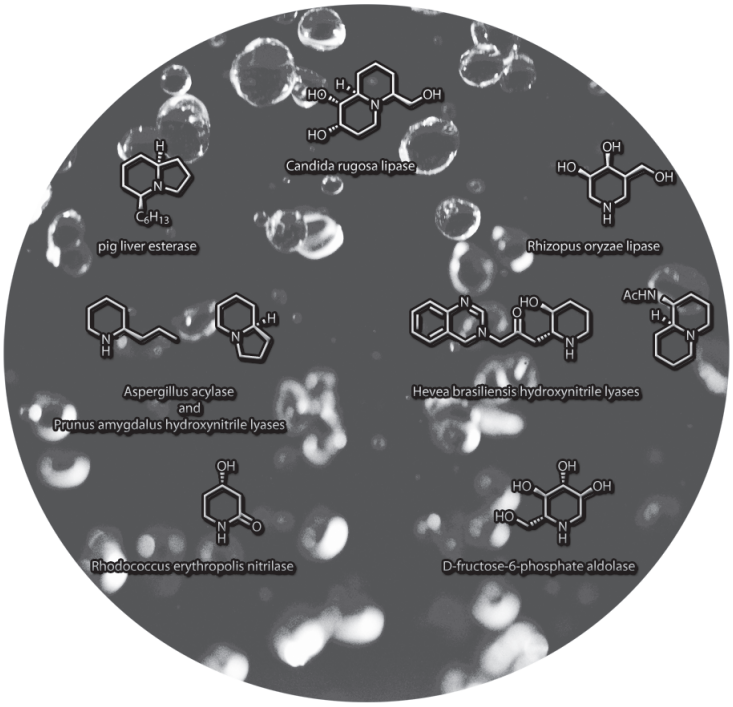
Chemoenzymatic strategies to enantiopure piperidines

Lieke J. C. van Hemert, Stefan van Rootselaar and Floris P. J. T. Rutjes,
Chemoenzymatic strategies to enantiopure piperidines, manuscript submitted.

"As for me, all I know is that I know nothing."
– Socrates

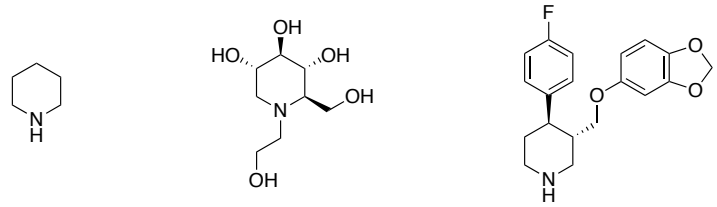
Abstract

Nature has created excellent enzymatic catalysts, which are increasingly applied by organic chemists in chemoenzymatic strategies to generate enantio- and/or diastereomerically pure compounds. In this chapter, an overview is given of the enzymes that have been used in the synthesis of several types of piperidines. This is an interesting compound class, containing many members that either have relevant biological properties on their own, or have been used as a scaffold to create libraries of biologically active ompounds. The enzyme classes that will be discussed include hydrolases, hydroxynitrilases, hydroxylases, nitrilases and in particular aldolases, which have been extensively applied in this thesis.



1.1 — Introduction

Nature has created sophisticated biosynthetic systems to synthesize molecules with excellent control over the stereochemistry.[1] A structural unit that is frequently produced, either on itself or as part of larger polycyclic systems, in a wide variety of organisms, is the piperidine moiety. As a result, piperidine structures are important targets for synthetic and medicinal chemistry in the search for specific therapeutic potential (Figure 1).



1, piperidine **2**, Miglitol **3**, Paroxetine

Figure 1 — Examples of piperidine structures.

Pathological conditions that have been treated with piperidine-derived small molecules include HIV,[2] diabetes type 2 (Miglitol, **2**),[3] lysosomal storage disorders,[4] autoimmune diseases,[5] malaria,[6] depression (Paroxetine, **3**)[7] and recently examples have been reported for the treatment of cancer.[8] Numerous approaches have been developed to synthesize piperidine-containing bioactive compounds, for example via hydrogenation of pyridine derivatives,[9] reduction of lactam derivatives[10] and ring formation via reductive amination.[11] The synthesis of piperidines was also accomplished with strategies involving metal catalysis such as ring closing metathesis with the Hoveyda-Grubbs catalyst,[12] Pd-catalyzed intramolecular hydroamination of unactivated alkenes,[13] and gold(I)-catalyzed hydroamination of unactivated olefins.[14] The biggest challenge in synthesizing these molecules is generally the stereoselective introduction of suitable functional groups. Mimicking nature in a chemoenzymatic approach to these compounds provides an excellent alternative to classical synthesis routes.[15] For example, enzymatic resolution to synthesize enantiopure piperidines is nowadays a method that is frequently applied on industrial scale.[16] Enzymatic resolution is one of the many options to construct enantiopure piperidine scaffolds and nowadays enzymes have become fully accepted as powerful means to do so.[17] This chapter provides a review of recent literature concerning chemoenzymatic approaches to synthesize enantiomerically pure hydroxylated piperidines, based on the different classes of enzymes that have been employed.

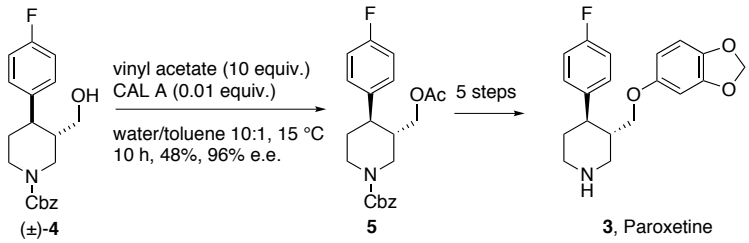
1.2 — Hydrolases

Enzymes from the class of hydrolases have been commonly applied in the synthesis of chiral piperidines via enzymatic kinetic resolution. The hydrolases can be divided into three subclasses, being lipases, esterases and acylases based on substrate specificity.

1.2.1 — Lipases

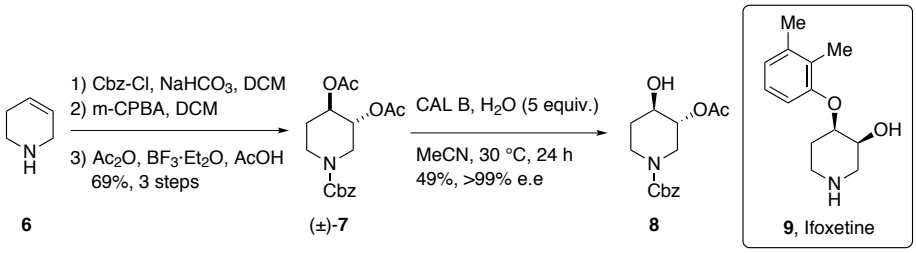
The first subclass is represented by lipases, which often display a broad substrate range and are capable of hydrolyzing a variety of ester groups. *In vivo* they play an important role in the degradation of fatty acids via hydrolysis of esters of glycerol. Particularly their tolerance towards a large variety of reaction conditions, including organic solvents, renders them useful for application in chemoenzymatic strategies.[18] Two well-investigated hydrolases are *Candida antarctica* lipase A and B (CAL A and CAL B, respectively), but *Candida rugosa* lipase (CRL), *Rhizopus oryzae* lipase (ROL) and *Pseudomonas cepacia* lipase (PCL) will be covered in this section as well.

Over the years CAL A has proven its use in many biotechnological applications.[19] One of the substituted piperidines synthesized in enantiopure form through kinetic resolution by CAL A is (–)-paroxetine (**3**, Scheme 1).[20] (–)-Paroxetine is used to treat major depression, obsessive-compulsive disorder, panic disorder, social anxiety, post-traumatic stress disorder and generalized anxiety disorder.7[21] Key step in the synthesis was a selective acylation of one of the *trans*-enantiomers of **4** by CAL A, which resulted in acetate **5** in good yield (48%) and excellent enantioselectivity (96% e.e.).[22] It should be noted that this reaction involved a reversed enzymatic reaction: instead of hydrolysis synthesis took place. This intermediate was then transformed into paroxetine (**3**) in five steps.[23] Unfortunately, this compound, marketed as Paxil, became topic of discussion in a misleading advertisement[24] and in 2008 severe side effects were observed, which tempered the interest, but nevertheless the drug is still on the market.[25]



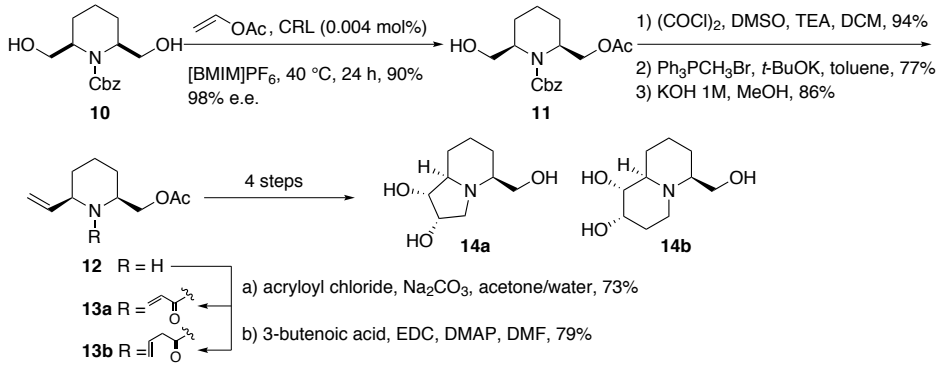
Scheme 1 — CAL A catalyzed synthesis of paroxetine (3).

Another well investigated lipase is Cal B, which has a wide substrate tolerance and catalyzes numerous reactions with large variations in pH and temperature.[19] CAL B can even operate in pure organic solvents, which is ideal for applications in organic synthesis.[26] CAL B was employed by the group of Gotor to gain access to enantiopure 3,4-disubstituted piperidines,[27] which possess interesting inhibitory activities on β-galactosidases,[28] and xylanases.[29] These disubstituted piperidines also act as gastrointestinal stimulants.[30] A particular example is ifoxetine (**9**, Scheme 2), which possesses antidepressant activity.[31] The 3,4-disubstituted piperidine scaffold was synthesized in four steps starting from tetrahydropyridine **6**, which was epoxidized with *m*-CPBA, followed by ring-opening with acetic anhydride and $BF_3{\cdot}Et_2O$ to provide the racemic *trans*-diacetate **7**. An enzymatic resolution was carried out with CAL B to give almost quantitative conversion of only one of the enantiomers into monoacetate **8**. With this strategy the synthesis of *cis*-dihydroxypiperidines was also investigated, but was less successful. [27]



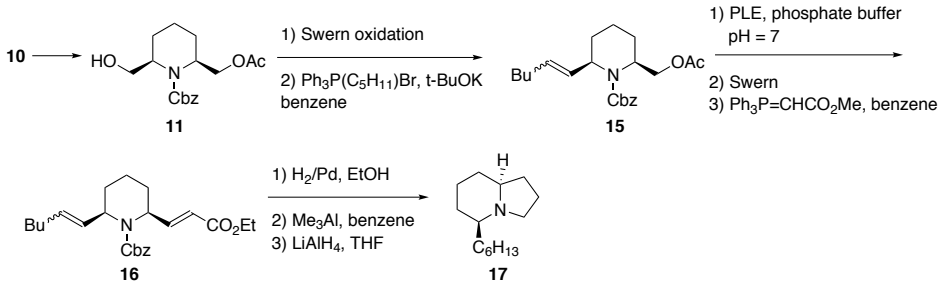
Scheme 2 — Synthesis of *trans*-3,4-dihydroxypiperidine scaffol 8.

Another lipase frequently used in biosynthetic pathways was isolated from *Candida rugosa* (formerly known as *Candida cylindracea*)[32] and has been named accordingly *Candida rugosa* lipase (CRL). Soon after it was discovered, the reliability of this enzyme was questioned due to irreproducible results, but careful analysis revealed that several isoenzymes of CRL were present in different sources causing these variations.[33] Recent investigations indicated that use of this enzyme is viable even on industrial scale.[33c] The application of CRL was amongst others demonstrated in the synthesis of trihydroxylated azabicycles **14a** and **14b**,[34] which are common pharmacophores (Scheme 3).[35] *Trans*-diol **10** was selectively desymmetrized through monoacylation with CRL.[34] The first alkene was introduced via Swern oxidation of the primary alcohol, followed by a Wittig reaction and deprotection of the amine, providing alkene **12** in good yield. The second alkene was introduced on the amine, after which ring-closing metathesis with the Grubbs-II catalyst, reduction of the amide and Upjohn dihydroxylation provided the bicyclic heterocycles **14a** and **14b**.[34]



Scheme 3 — Synthesis of pharmacophores 14a and 14b.

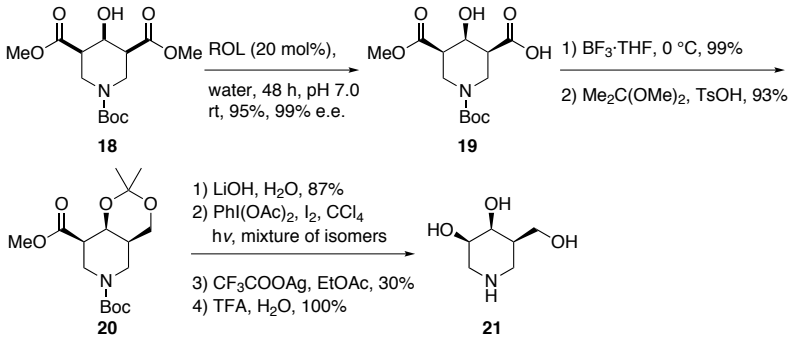
The desymmetrization of **10** was also applied in the synthesis of alkaloid (+)-indolizidine 209D (**17**, Scheme 4) which was isolated from dendrobatid frogs in Central America.[36] Alkaloid **17** and its epimers are highly active blockers of the nicotinic acetylcholine receptors.[37] Enzyme-mediated asymmetric syntheses of all four stereoisomers were developed by different groups.[37,38] The group of Chênevert developed a method to obtain the alkaloid starting from piperidine **11**.[39] After Swern oxidation and Wittig reaction, a pig liver esterase (PLE) catalyzed deprotection of the acetate group and subsequent Swern oxidation and another Wittig reaction, resulted in piperidine **16**. The final alkaloid **17** was obtained via hydrogenation, lactam formation and reduction of the bicyclic lactam.



Scheme 4 — Synthesis of (+)-indolizidine 209D (17).

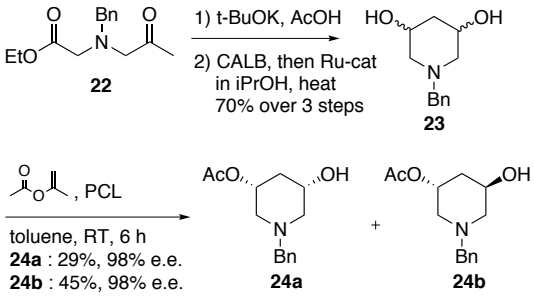
The third lipase, *Rhizopus oryzae* lipase (ROL, formerly known as *Rhizopus delemar*)[40] is known to exclusively react with primary alcohols in generally high enantioselectivity and is therefore able to discriminate between the different esters of glycerides *in vivo*.[41] This enzyme played a crucial role in the synthesis of the β-galactosidase inhibitor[42] isogalactofagomine (**21**), in which it was used to hydrolyze one of the precursors (Scheme 5).[43] Alternative synthetic strategies started from carbohydrates[43] or chiral separation of race-

mates.[44] The group of Bols applied ROL in their synthetic strategy to desymmetrize *cis*-diester **18** providing (*R*)-acid **19** in excellent yield (95%) and enantioselectivity (99% e.e.). In the subsequent steps the acid was reduced to the corresponding alcohol and the diol was protected with an acetone clip (**20**). The synthetic route was continued via hydrolysis of the acid, followed by oxidative decarboxylation with concomitant replacement by iodine through a radical intermediate,[45] resulting in a mixture of diastereoisomers that were separated with column purification. Nucleophilic substitution of the *cis*-iodide with silver trifluoroacetate in EtOAc proceeded smoothly with retention of configuration,[46] after which the trifluoroacetate and dioxolane were hydrolyzed with aqueous TFA to obtain piperidine **21**.



Scheme 5 — Synthesis of isogalactofagomine (21) with ROL as desymmetrizing enzyme.

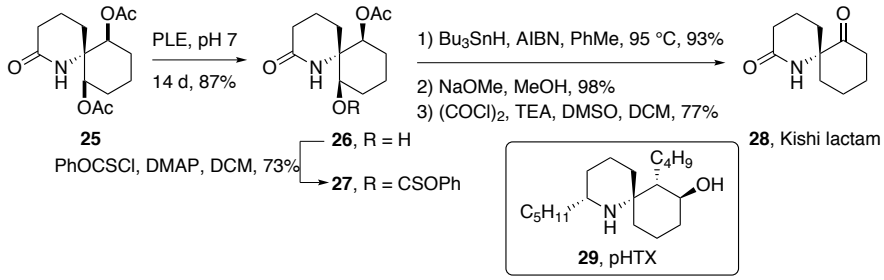
3,5-Dihydroxypiperidines form another interesting class of piperidines too, as they possess similar glucosidase inhibiting properties as the 3,4-disubstituted piperidines.[47] Recent studies also indicate possible treatment of Alzheimer's disease and schizophrenia with this class of piperidines.[48] Another application of these compounds is their use as chiral ligands in the enantioselective addition of diethylzinc onto aldehydes.[49] Syntheses of both the *cis*- and *trans*-dihydroxylated piperidines **24a** and **b**, respectively (Scheme 6), were published earlier without using enzymes.[50,51] A chemoenzymatic route to these enantiopure piperidine moieties was realized with *Pseudomonas cepacia* lipase (PCL),[52] which was isolated from the bacterial strain *Pseudomonas cepacia* (also called *Burkholderia cepacia*). This lipase accepts both primary and secondary alcohols,[41,53] and these properties were used in the synthesis of the 3,5-disubstituted piperidine derivatives **24a** and **b**. The group of Bäckvall started from glycinate **22**, which was ring-closed in a Dieckmann condensation. Next, a one pot deacetylation by CAL B of the intermediate enolacetate and transfer hydrogenation by a ruthenium catalyst resulted in racemic diol **23**.[47] Desymmetrization was carried out with PCL to yield each of the (3*R*)-acetylated diastereoisomers **24a** and **b** in high enantioselectivities.



Scheme 6 — Diastereoselective acetylation to afford 21a and 21b.

1.2.2 — Esterases

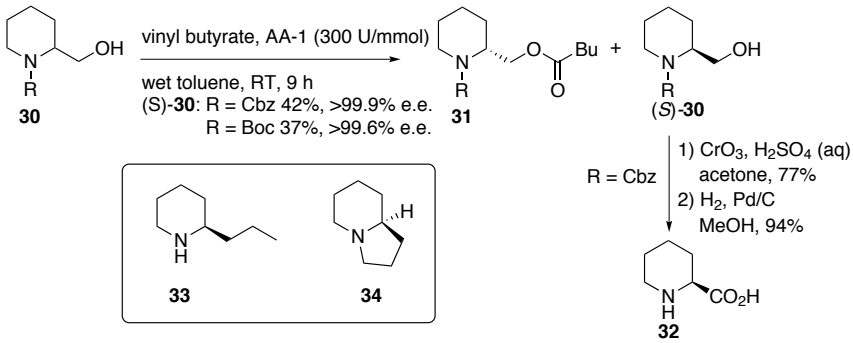
Esterases, another subclass of hydrolases, are known to react with short chain acyl esters. A frequently used esterase is pig liver esterase (PLE), which has been applied in synthetic routes for several decades.[54] This enzyme has also shown inconsistencies in reactivity due to the presence of isoenzymes.[55] These problems were overcome by the development of a recombinant source and nowadays this enzyme shows remarkable stability and reproducibility.[56] It accepts a wide variety of substrates due to its *in vivo* function to hydrolyze carboxylic esters present in the pig diet.[56] One of the targets synthesized in enantiopure form using PLE is the alkaloid (–)-Kishi lactam (**28**, Scheme 7), a versatile intermediate for the synthesis of perhydrohistrionicotoxin alkaloids (pHTX, **29**). These alkaloids were isolated from the "arrow poison frog" *Dendrobates histrionicus* in South-America and have been used extensively as neurological probes for the acetylcholine receptor channel.[57] The synthesis of diacetal protected spiro compound **25**, precursor for the synthesis of Kishi lactam **28**, was previously reported by the same group.[58] Key step in the synthesis of lactam **28** was the selective deprotection of **25** with PLE, which proceeded in high yields. Treatment of **26** with phenyl thionochloroformate and DMAP in DCM resulted in lactam **27**. Kishi lactam (**28**) was obtained via treatment of **27** with tri-*n*-butyltin hydride and AIBN, followed by deacylation and subsequent oxidation.



Scheme 7 — Synthesis of Kishi lactam (28).

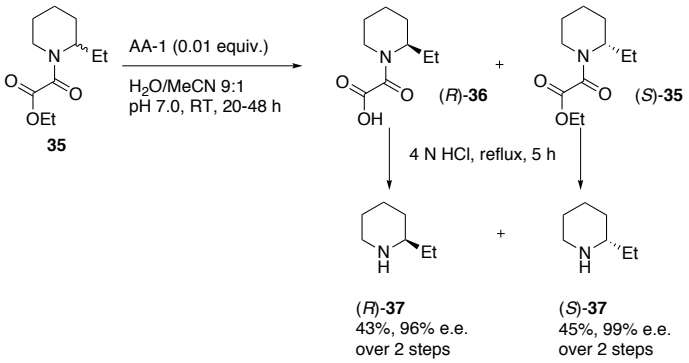
1.2.3 — Acylases

The last subclass of hydrolases, the acylases, catalyzes the formation and cleavage of *N*-acylamino acids and ester bonds, which is illustrated by the use of Aspergillus acylase 1 (AA-1).[59] AA-1 was isolated from *Aspergillus niger* and the enzyme was applied in the chemical synthesis of numerous compounds, such as the non-proteinogenic amino acid (*S*)-pipecolic acid (**32**, Scheme 8) which is present in various natural peptides[60] and anti-inflammatory pharmaceuticals.[61] Acid **32** is an intermediate formed especially in the mammalian central nervous system via a specific degradation pathway,[62] which implies a role in important neurological functions.[63]



Scheme 8 — Synthesis of (*S*)-pipecolic acid (32).

The novel route to (*S*)-pipecolic acid by Herradón *et al*. commenced with a racemic mixture of carbamate-protected piperidine **30**, which was subjected to AA-1 to produce the desired (*S*)-enantiomer in excellent purity and good yield.[64] The corresponding carboxylic acid **32** was obtained via Jones oxidation and carbamate deprotection. From pipecolic acid **32** also (*S*)-δ-coniceine (**34**) and (*R*)-coniine (**33**) were synthesized.[65] Another example of the use of AA-1 was seen in the synthesis of 2-ethylpiperidines **37** (Scheme 9).[66] Both products were obtained in high yield and purity over two steps.

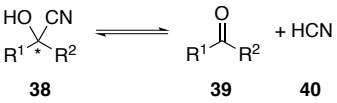


Scheme 9 — Preparation of enantiomerically pure 2-ethylpiperidines 37.

The desymmetrization methods described in this section are elegant examples of the synthesis of enantiopure piperidines, leading to 100% yield of one of the enantiomers. The enzymatic kinetic resolutions of racemic mixtures, on the other hand, result in a maximum yield of 50% one of the desired enantiomer. In order to reach 100% yield in the latter case, a racemization method for the starting material needs to be developed, which then results in a dynamic kinetic resolution (DKR).[15a,67]

1.3 — Hydroxynitril lyases

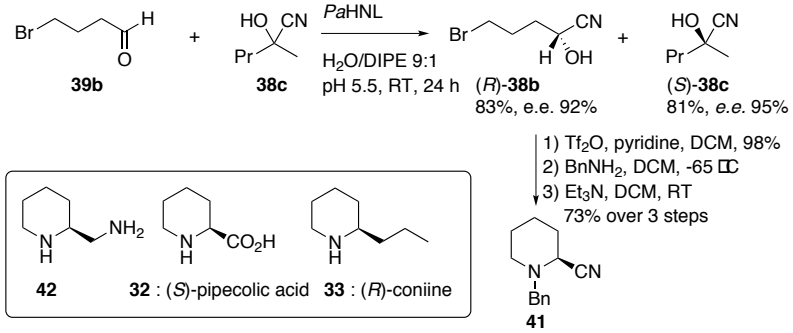
Hydroxynitril lyases (HNLs), also referred to as oxynitrilases, catalyze *in vivo* the hydrolysis of a wide range of cyanohydrins (**38**) into the corresponding aldehydes or ketones (**39**) and toxic hydrogen cyanide (**40**, Scheme 10). Nature created this defense mechanism to protect the organism when it is under attack.[68] For synthetic purposes the equilibrium can be reversed to give access to enantiopure cyanohydrins via addition of HCN onto prochiral carbonyl compounds **39**. The first application of HNLs was published in 1908 and its use has increased rapidly in particular over the last two decades.[69]



Scheme 10 — *In vivo* degradation of cyanohydrins 38.

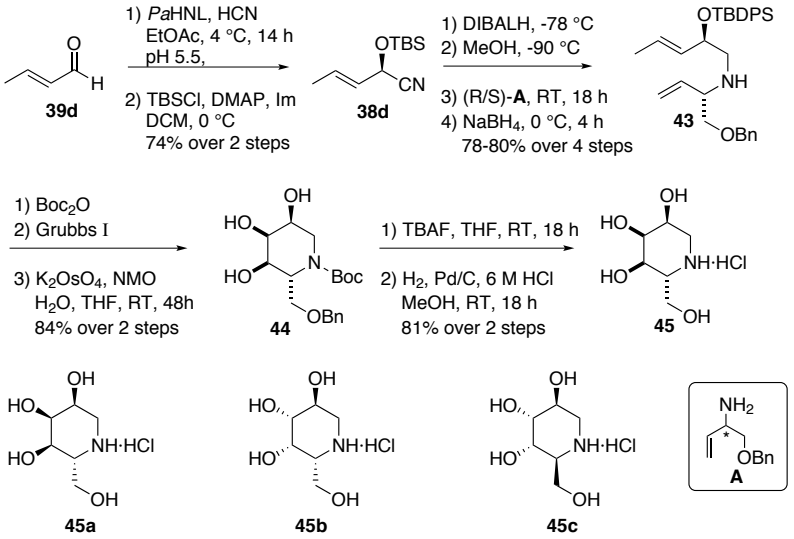
The HNLs have been divided into four classes based on sequence, structure, catalytic mechanism,[70] and enantioselectivity. The (*S*)-selective enzymes are derived from *Hevea brasiliensis* (*Hb*HNL, isolated from leaves of the tropical rubber tree), *Manihot esculenta* (*Me*HNL, isolated from the cassava) and *Sorghum bicolor* (*Sb*HNL, isolated from millet).[71]

The complementary (*R*)-selective enzymes include *Linum usitatissimum* (*Lu*HNL, originating from flax), *Phlebodium aureum* (*Pha*HNL, found in fern plants), *Prunus amygdalus* (*Pa*HNL, isolated from almonds) and other *Prunus* species.[69,72] *Hb*HNL and *Pa*HNL display a broad substrate range, which due to the opposite selectivities provide excess to both enantiomeric configurations in generally excellent enantiomeric excess (>99%). The group of Gotor used *Pa*HNL to synthesize various naturally occurring bioactive piperidines.[11c,73] These natural products were constructed via a transcyanation reaction starting from ω-bromoaldehyde **39b**, cyanohydrin **38c** and *Pa*HNL to give the desired (*R*)-cyanohydrin **38b**, which was used as precursor for the synthesis of (*S*)-2-aminomethylpiperidine (**42**), (*S*)-pipecolic acid (**32**) and (*R*)-coniine (**33**, Scheme 11). Diamine **42** was subsequently applied as a ligand in asymmetric catalysis.[74] The construction of piperidines **32** and **33** with HNL is an alternative to the acylase from the *Aspergillus* species mentioned previously (Section 1.2.3).



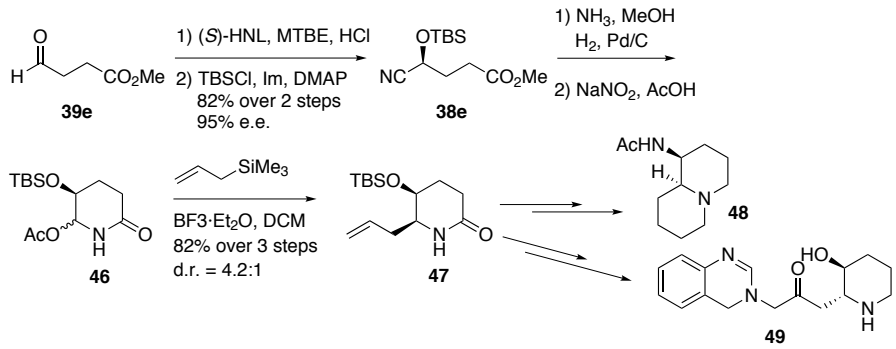
Scheme 11 — Synthesis of naturally occurring piperidines with *Pa*HNL.

*Pa*HNL was also applied in the synthesis of the biologically active iminosugars L-*altro*-1-deoxynojirimycin (**45a**), D-*allo*-1-deoxynojirimycin (**45b**) and D-*galacto*-1-deoxynojirimycin (**45c**, Scheme 12).[75] The first two iminosugars possess moderate inhibitory effects on the α-glucosidase enzyme of different organisms, while piperidine **45c** inhibits multiple enzymes.[76] The synthesis of these iminosugars started from crotonaldehyde (**39d**), which was treated with a crude extract from almonds containing *Pa*HNL to give TBS-protected cyanohydrin **38d** after silyl protection.[75] Subsequent DIBAL-H reduction and transimination, followed by sodium borohydride reduction afforded amine **43** in high yield. Boc-protection of the amine and RCM with the Grubbs-I catalyst, followed by Upjohn dihydroxylation[77] resulted in compounds **44a–c**, after which deprotection produced iminosugars **45a–c**.



Scheme 12 — Synthesis of iminosugars 45a–c via a chemoenzymatic strategy involving HNL.

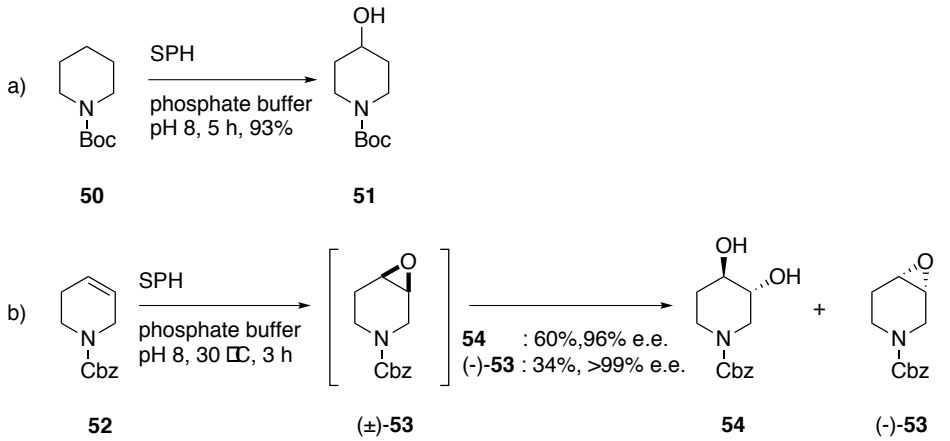
In order to reach the opposite stereochemistry, *Hb*HNL was used extensively by Rutjes *et al.* in the synthesis of the natural products (+)-epiquinamide[78] (**48**, Scheme 13) and (+)-febrifugine[79] (**49**). Alkaloid **48** was isolated from the Ecuadorian poison frog *Epipedobates tricolor* in 2003.[80] Shortly after its isolation conflicting reports were published about its ability to mimic the function of nicotine.[81] Further research on the activity of epiquinamide demonstrated that contamination with traces of the alkaloid epibatidine caused the actual activity.[82] Alkaloid **49** was obtained in 1946 for the first time from *Dichroa febrifuga*[83] and later from the more abundant *Hydrangea umbellate*[84] and showed potent antimalarial activity.[85] In 1999, epimers of alkaloid **49** were obtained, which led to the unambiguous assignment of all chiral centers.[86] With this achievement the development of other synthetic pathways was stimulated, unfortunately, side effects have hampered clinical trials so far.8[b,86,87] Key step in the synthesis of both natural products is the treatment of methyl-4-oxobutyrate (**39e**) under acidic conditions with *Hb*HNL, followed by TBS-protection of the hydroxyl group to produce **38e** in excellent yield and e.e.[88] Subsequent reduction and diazotation resulted in the formation of *N*,*O*-acetal **46**. Using *N*-acyliminium ion chemistry, the allyl chain was introduced leading predominantly to *cis*-isomer **47**, which served as a precursor for both alkaloids **48** and **49**.



Scheme 13 — Synthesis of alkaloids 48 and 49 with (*S*)-HNL.

1.4 — Hydroxylases

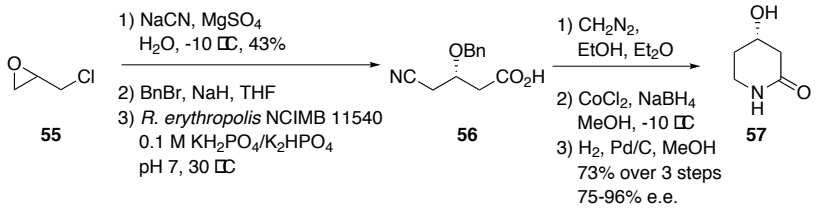
Hydroxylases, also known as oxygenases, constitute a class of enzymes that catalyze the chemo-, regio- and stereoselective oxygenation of alkanes and many other compounds.[89] Among them is *Sphingomonas* sp. HXN-200 (SPH), which was isolated with a trickling bed bioreactor. This enzyme is present in a parent cell line, whose activity was appointed to the P450 oxygenase CYP153.[89] The employment of SPH allowed the transformation of various non-activated molecules into their corresponding hydroxylated descendants,[89] among which a few piperidine derivatives.[90] The first application of SPH was the regioselective transformation of *N*-protected piperidine **50** into alcohol **51** (Scheme 14, a). Unfortunately, this approach did not result in a chiral compound. In route b a diastereoselective dihydroxylation was carried out with the same cell lysate, starting from *N*-protected 1,2,5,6-tetrahydropyridine **52** affording almost exclusively the (3*R*,4*R*)-enantiomer **54** via the racemic epoxide intermediate **53**.[90b] Scaffold **54** was also prepared with hydrolases as demonstrated in Section 1.2.



Scheme 14 — Hydroxylation of *N*-protected piperidine derivatives 51 and 54.

1.5 — Nitrilases

Nitrilases and nitrile hydratases are enzymes that transform aromatic and aliphatic nitriles into the corresponding acids and amides, respectively, in a chemo- and enantioselective fashion.[91,92] In the synthesis of piperidines, the reactivity of a nitrilase present in *Rhodococcus erythropolis* NCIMB 11540[93] was demonstrated by Rutjes *et al.* in an enantioselective synthesis of 4-hydroxypiperidone (**57**, Scheme 15).[67] Nucleophilic attack of cyanide onto racemic epichlorohydrin (**55**) resulted in a prochiral dicyanide, which was *O*-protected with a benzyl group. Enantioselective monohydrolysis with *Rhodococcus erythropolis* NCIMB 11540 resulted in mono acid **56**. Esterification and reduction finally led to enantiopure lactam **57**, which can be transformed into a plethora of other druglike building blocks *via* well-established chemistry.[67]



Scheme 15 — Chemoenzymatic synthesis of hydroxypiperidone 57.

1.6 — Aldolases

Aldolases are widely abundant in many organisms and involved in the degradation of carbohydrates.[94] For synthetic purposes, especially the synthesis of sugar-mimicking polyhydoxylated piperidines, aldolases are a useful tool. These so-called 'iminosugars' for are defined as cyclic sugar derivatives in which the ring oxygen atom has been replaced by nitrogen.[95] Iminosugars are potent glucosidase and glycosyltransferase inhibitors and therefore may have potent pharmaceutical applications. Furthermore, they are useful probes in mechanistic studies of the glycosidase and glycosyltransferase pathway.[96] Aldolases facilitate the stereospecific cleavage of a carbon-carbon bond via a retro aldol reaction. In Nature, β-hydroxy carbonyl adducts undergo this reaction in which amino acids and carbohydrates are degraded to primary and secondary metabolites.[97a] These enzymes can be classified in different ways for example by analysis of the activation mechanism: class I aldolases form an enamine with the substrate, while class II aldolases activate the substrate with a Lewis acid.[97] This mechanism will be discussed in more detail in Chapter 2. Another way to distinguish between the different aldolases is by the substrate specificity. The enzymes that are most commonly used are dihydroxyacetone phosphate (DHAP)-dependent aldolases, dihydroxyacetone (DHA)-dependent aldolases, (phenyl)pyruvate-dependent aldolases, glycine-dependent threonine aldolases (ThrA) and acetaldehyde aldolases.[97] For synthetic purposes the reactivity of these enzymes can be reversed by tuning the conditions resulting in the aldol adduct for synthetic purposes. Because these enzymes operate with high stereoselectivity, they are remarkably useful as catalysts in synthetic pathways towards both natural and non-natural carbohydrates such as iminosugars.[96,97b] Nature provided four complementary DHAP-aldolases named after the degradation (red bond) of their natural substrate: D-fructose 1,6-biphosphate aldolase (**58**, FruA), D-tagatose 1,6-biphosphate aldolase (**59**, TagA), L-rhamnulose 1-phosphate aldolase (**60**, RhuA) and L-fuculose 1-phosphate aldolase (**61**, FucA, Figure 2).

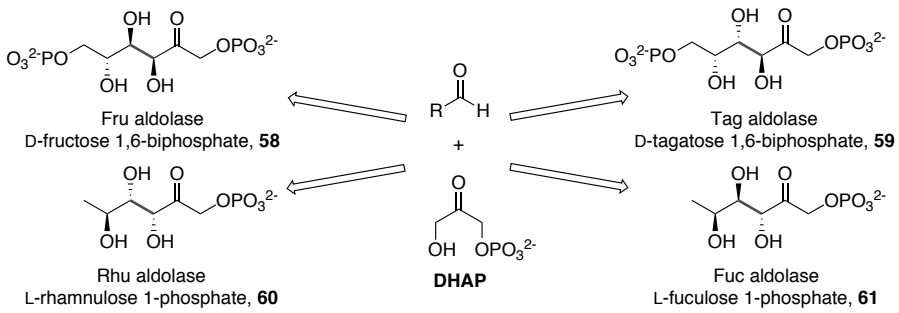
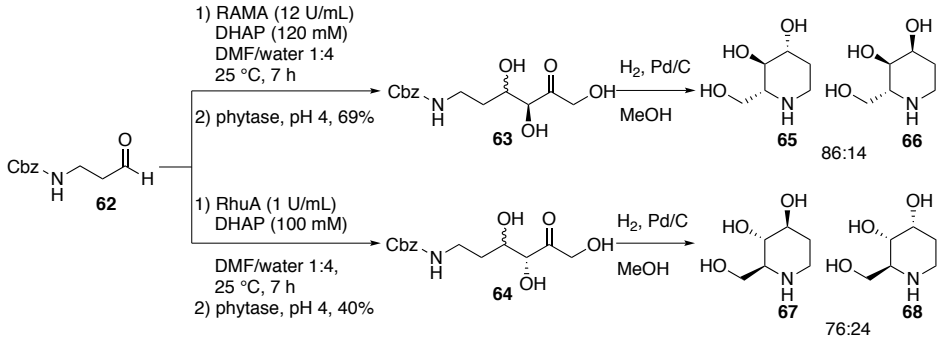


Figure 2 — The four stereocomplementary DHAP-dependent aldolases and their natural substrates.

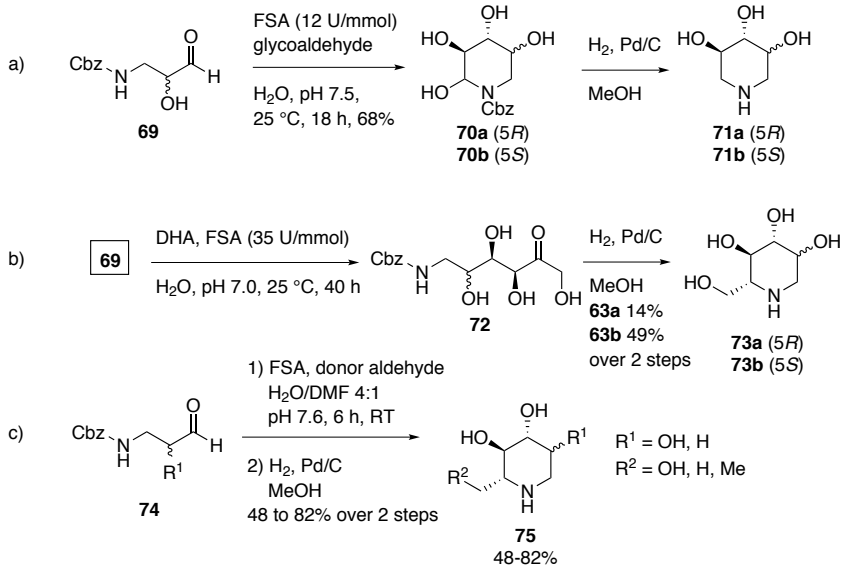
One extensively investigated iminosugar is D-fagomine (**65**) and its epimers **66–68** (Scheme 16), which was accessible *via* catalysis by different DHAP-dependant aldolases. Naturally occurring iminosugar D-fagomine (**65**) was isolated for the first time from seeds of *Fagopyrum esculentum* and its pharmacochemical potential is significant. D-Fagomine inhibits several enzymes that are important in the mammalian glucose level-regulation. The *N*-alkyl derivatives of **65** have also been synthesized and showed interesting biological activity.[98,99] Chemoenzymatic syntheses of iminosugars **65–68** started with an aldol condensation of *N*-Cbz-aminopropanal **62** and DHAP in the presence of FruA rabbit muscle aldolase (RAMA), which provided the stereochemistry of the natural compound **65** and epimer **66** in a 84:16 ratio after dephosphorylation and hydrogenation.[100] The other epimers were synthesized with RhuA and resulted in compounds **67** and **68**.[101]



Scheme 16 — Synthesis of D-fagomine and its epimers via an aldolase catalyzed reaction.

Drawback of this approach was the use of DHAP, which is an unstable and reactive compound.[102] In 2001, D-fructose-6-phosphate aldolase (FSA) was isolated from *E. coli*,[103] and this enzyme was able to accept the non-phosphorylated donor DHA. This enzyme provided an excellent alternative to circumvent the use of DHAP in the synthesis of D-fagomine (**65**). The alternative route also reduced the synthetic strategy by one step, since dephosphorylation was no longer required.[102] Besides DHA, other donor aldehydes and ketones were accepted to construct a number of other biologically active molecules, for example *nor*-fucodeoxynojirimycin (**71a**) and its epimer **71b** (Scheme 17, route a). The former inhibits the α-L-fucosidase enzyme at low μM levels[104] and epimer **71b** was isolated from the plant *Eupatorium fortunei*.[105] The synthesis of both compounds consisted of an FSA-catalyzed aldol reaction between aldehyde **69** and donor glycoaldehyde to produce the desired aldol products **70**, which upon hydrogenation gave iminosugars **71**.[106] With FSA syntheses of 1-deoxynojirimycin (DNJ, **73b**) and its epimer 1-deoxymannojirimycin (DMJ, **73a**) were also readily realized (route b). These iminosugars widely abundant in Nature have range of activities,[98b,107] including inhibiting a range of glycosidases.[108,109] For

instance iminosugar **73b** is an effective inhibitor of human α-glucosidase and its *N*-EtOH derivative is commercially marketed as Miglitol (**2**), a drug for Gaucher's disease, which will be discussed in more detail in Section 4.1.[109,110] DMJ (**73a**) also exhibits inhibitory effects against an α-L-fucosidase in the low μM range.[109] Clapés *et al.* developed route b towards iminosugar **73** via an aldol reaction of racemic aldehyde **69** and DHA in the presence of FSA. Subsequent hydrogenation and separation of both isomers resulted in the desired iminosugars in moderate to good yield.[11d] Derivatives of **73** were synthesized with FSA by Wong *et al.* using analogues of both the acceptor aldehydes **74** and donor DHA (route c).[111] Some of these analogues showed μM range inhibition of various enzymes.[111]



Scheme 17 — Synthesis of iminosugars catalyzed by FSA.

1.7 — Outline and purpose of this thesis

Being part of the policy of the Netherlands Organization for Scientific Research (NWO), the Integration of Biosynthesis and Organic Synthesis (IBOS) program was set up with the aim to create sustainable synthetic strategies that combined both organic chemistry and modern biochemistry. Our project focused on using enzyme catalyzed aldol reactions to develop novel, sustainable and industrially viable synthetic pathways to versatile enantiomerically pure heterocyclic building blocks. This project was a collaboration between the University of Amsterdam (UvA), Wageningen University (WUR), Radboud University (RU) and the industrial partners DSM (Geleen, The Netherlands), MSD (Oss, The Netherlands) and Syncom (Groningen, The Netherlands). Key element in these aldol reactions was the

chemoenzymatic generation of DHAP, which in combination with stereocomplementary aldolases would provide access to all four diastereoisomers of the aldol product. Our aim was to establish the scope and limitations of these strategies and use the carbohydrate structures for the synthesis of functionalized heterocyclic building blocks.

To gain more insight in the mechanistic properties of RAMA, computational studies were performed, which are described in detail in **Chapter 2**. In **Chapter 3** an alternative route to DHAP is presented, proceeding via *in situ* generation from glycerol in a cascade reaction with a total of four enzymes in one pot. Our goal was to synthesize industrially relevant heterocycles and **Chapter 4** includes optimization of the synthesis of D-fagomine and DNJ, as well as derivatization of DNJ into several fluorinated analogues. Application of the one-pot four enzyme cascade approach towards a synthesis of the naturally occurring iminosugar batzellaside is discussed in detail in **Chapter 5**. Besides the synthesis of piperidines, **Chapter 6** describes a chemoenzymatic synthesis of azepanes, seven-membered ring iminosugars. Finally, **Chapter 7** provides a perspective on the current issues of integrating biocatalysis with organic synthesis.

1.8 — Acknowledgements

Stefan van Rootselaar is kindly acknowledged for his contributions to this chapter.

1.9 — References

1. Newman, D. J. and Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461–477.
2. Ratner, L.; Heyden, N. V. and Dedera, D. *Virology* **1999**, *181*, 180–192 and references cited therein.
3. a) Felpin, F.-X.; Bouberkeur, K. and Lebrenton, J. *J. Org. Chem.* **2004**, *69*, 1497–1503 and references cited therein; b) Anzeveno, P. B.; Creemer, L. J.; Daniel, J. K.; King, C.–H. R. and Liu, P. S. *J. Org. Chem.* **1989**, *54*, 2539–2542; c) Balfour, J. A. and McTavish, D. *Drugs* **1993**, *46*, 1025–1054.
4. Butters, T. D.; Dwek, R. A. and Platt, F. M. *Chem. Rev.* **2000**, *100*, 4683–4696.
5. The application of (poly)hydroxylated piperidines in various therapeutic areas is reviewed in: Pearson, M. S. M.; Mathé-Allainmat, M.; Fargeas, V. and Lebreton, J. *Eur. J. Org. Chem.* **2005**, 2159–2191.
6. Takaya, Y.; Tasaka, H.; Chiba, T.; Uwai, K.; Tanitsu, M. A.; Kim, H. S.; Wataya, Y.; Miura, M.; Takeshita, M. and Y. J. Oshima, *J. Med. Chem*. **1999**, *42*, 3163–3166.
7. Dechant, K. L. and Clissold, S. P. *Drugs* **1991**, *41*, 225–253.
8. Gross, P. E.; Baker, M. A.; Carver, J. P. and Dennis, J. W. *Clin. Cancer Res.* **1995**, *1*, 935–944.
9. Wang, X.; Kauppi, A. M.; Olsson, R. and Almqvist, F. *Eur. J. Org. Chem.* **2003**, 4586–4592.
10. Micouin, L.; Varea, T.; Riche, C.; Chiaroni, A.; Quirion, J.-P.and Husson, H.-P. *Tetrahedron Lett*. **1994**, *35*, 2529–2532.

11. a) Monterde, M. I.; Brieva, R. and Gotor, V. *Tetrahedron: Asymmetry* **2001**, *12*, 525–528; b) Concia, A. L.; Lozano, C.; Castillo, J. A.; Parella, T.; Joglar, J. and Clapés, P. *Chem. Eur. J.* **2009**, *15*, 3808–3816. c) Castillo, J. A.; Calveras, J.; Casas, J.; Mitjans, M.; Vinardell, M. P.; Parella, T.; Inoue, T.; Sprenger, G. A.; Joglar, J. and Clapés, P. *Org. Lett.* **2006**, *8*, 6067–6070.
12. Fustero, S.; Jiménez, D.; Sánchez-Roselló, M. and del Pozo, C. *J. Am. Chem. Soc.* **2007**, *129*, 6700–6701.
13. Michael, F. E. and Cochran, B. M. *J. Am. Chem. Soc.* **2006**, *128*, 4246-4247.
14. a) Han, X. and Widenhoefer, R. A. *Angew. Chem. Int. Ed.* **2006**, *45*, 1747-1749; b) Bender, C. F. and Widenhoefer, R. A. *Org. Lett.* **2006**, *8*, 5303-5305.
15. a) Schoemaker, H. E.; Mink, D. and Wubbolts, M. G. *Science* **2003**, *299*, 1694–1697; b) Faber, K. *Biotransformations in Organic Chemistry*; Springer-Verlag: Berlin, 1997; b) Wong, C.-H. and Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon Press: Oxford, **1994**.
16. Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M. and Witholt, B. *Nature* **2001**, *409*, 258–268.
17. Wohlgemuth, R. *Curr. Opin. Microbiol.* **2010**, *13*, 283–292 and references cited therein.
18. Joseph, B.; Ramteke, P. W. and Thomas, G. *Biotechnol. Adv.* **2008**, *26*, 457–470 and references cited therein.
19. Dominguez de Maria, P.; Carboni-Oerlemans, C.; Tuin, B.; Bargeman, G.; van der Meer, A. and van Gemert, R. *J. Mol. Cat. B: Enzym*. **2005**, *37*, 36–46
20. De Risi, C.; Fanton, G.; Pollini, G. P.; Trapella, C.; Valente, F. and Zanirato, V. *Tetrahedron: Asymmetry* **2008**, *19*, 131–155.
21. a) Katzman, M. A. *CNS Drugs,* **2009***, 23*, 103–120; b) http://us.gsk.com/products/assets/us_paxil.pdf; retrieved 2011-07-11.
22. In this thesis, stereochemical assignments in the structural drawings were made with emboldened or dashed wedges for enantio- or diastereomerically pure compounds with known absolute configuration. Straight emboldened or dashed lines were used for indicating relative stereochemistry. Waving lines indicated a racemic mixture. wedged: lined: waved:
23. a) de Gonzalo, G.; Brieva, R.; Sánchez, V. M.; Bayod, M. and Gotor, V. *J. Org. Chem.* **2001**, *66*, 8947–8953; b) Gotor, V. *Org. Process Res. Dev.* **2002**, *6*, 420–426.
24. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/EnforcementActivitiesbyFDA/WarningLettersandNoticeofViolationLetterstoPharmaceuticalCompanies/ucm055293.pdf; retrieved 28-3-2012.
25. a) Barbui, C.; Furukawa, T. A. and Cipriani, A. *Can. Med. Assoc. J.* **2008**, *178*, 296–305.
26. a) Anderson, E. M.; Larsson, K. M. and Kirk, O. *Biocatal. Biotransfor.* **1998**, *16*, 181–204 and references cited therein; b) Gotor-Fernández, V.; Busto, E. and Gotor, V. *Adv. Synth. Catal.* **2006**, *348*, 797–812 and references cited therein.
27. Solares, L. F.; Lavandera, I.; Gotor-Fernández, V.; Brieva, R. and Gotor, V. *Tetrahedron* **2006**, *62*, 3284–3291.
28. Ichikawa, Y.; Igasashi, Y.; Ichikawa, M. and Suhara, Y. *J. Am. Chem. Soc.* **1998**, *120*, 3007–3018.
29. Williams, S. J.; Hoos, R. and Withers, S. G. *J. Am. Chem. Soc.* **2000**, *122*, 2223–2235.
30. Cossy, J.; Molina, J. L. and Desmurs, J.-R. *Tetrahedron Lett.* **2001**, *42*, 5713–5715.
31. Waldmeier, P. C.; Maitre, L.; Baumann, P. A.; Hauser, K.; Bischoff, S.; Bittiger, H. and Paioni, R. *Eur. J. Pharmacol.* **1986**, *130*, 1–10.
32. Lambusta, D.; Nicolosi, G.; Patti, A. and Sanfilippo, C. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 271–277.

33. a) Mala, J. G. S. and Takeuchi, S. *Anal. Chem. Insights* **2008,** *3*, 9–19 and references cited therein; b) Dave, R. and Madamwar, D. *Biocatal. Biotransfor.* **2010**, *28*, 157–166 and references cited therein; c) de María, P. D.; Sánchez-Montero, J. M.; Sinisterra, J. V. and Alcántara, A. R. *Biotechnol. Adv.* **2006** *24,* 180–196 and references cited therein.

34. Lesma, G.; Colombo, A.; Landoni, N.; Sacchetti, A. and Silvani, A. *Tetrahedron: Asymmetry* **2007**, *18*, 1948–1954 and references cited therein.

35. a) Michael, J. P. *Nat. Prod. Rep.* **2007**, *24*, 191–222 and previous reports; b) Michael, J. P. In *The Alkaloids: Chemistry and Pharmacology* Cordell, G., Ed.; Academic Press: New York, **2001**; *55*, 91.

36. Daly, J. W. and Spande, T. F. *Alkaloids: Chemical and Biological Perspectives* Wiley & Sons: New York, **1986**; Vol. 4, Ch. 1.

37. a) Takahata, H.; Kubota, M.; Ihara, K.; Okamoto, N.; Momose, T.; Azer, N.; Eldefrawi, A. T. and Eldefrawi, M. E. *Tetrahedron-Asymmetry* **1998**, *9*, 3289–3301; b) Aronstam, R. S.; Daly, J. W.; Spande, T. F.; Narayanan, T. K. and Albuquerque, E. X. *Neurochem. Res.* **1986**, *11*, 1227–1240.

38. a) Bubnov, Y. N.; Klimkina, E. V. and Ignatenko, A. V. *Russ. Chem. B+* **1998**, *47*, 941–949; b) Jefford, C. W. and Wang, J. B. *Tetrahedron Lett*. **1993**, *34*, 3119–3122; c) Ahman, J. and Somfai, P. *Tetrahedron* **1995**, *51*, 9747–9756; d) Ahman, J. and Somfai, P. *Tetrahedron Lett*. **1995**, *36*, 303–306; e) Polniaszek, R. P. and Belmont, S. E. *J. Org. Chem*. **1990**, *55*, 4688–4693; f) Pearson, W. H.; Walavalkar, R.; Schkeryantz, J. M.; Fang, W. and Blickensdorf, J. D. *J. Am. Chem. Soc*. **1993**, *115*, 10183–10194; g) Comins, D. L. and Zhang, Y. *J. Am. Chem. Soc*. **1996**, *118*, 12248–12249; h) Jefford, C. W.; Sienkiewicz, K. and Thornton, S. R. *Helv. Chim. Acta*. **1995**, *78*, 1511–1524; i) Lee, E.; Li, K. S. and Lim, J. *Tetrahedron Lett*. **1996**, *37*, 1445–1446.

39. Chênevert, R.; Ziarani, G. M.; Morin, M. P. and Dasser, M. *Tetrahedron: Asymmetry* **1999**, *10*, 3117–3122.

40. Schipper, M. A. A. *Stud. Mycol.* **1984**, *25*, 1–19.

41. Bornscheuer, U. T. *Enzymes in Lipid Modification* Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, **2000**; Vol. 1, Ch. 4.

42. Ichikawa, Y.; Igarashi, Y.; Ichikawa M. and Suhura Y. *J. Am. Chem. Soc.* **1998**, *120*, 3007–3018.

43. Liang, X.; Lohse, A. and Bols, M. *J. Org. Chem.* **2000**, *65*, 7432–7437.

44. Hansen, S. U. and Bols, M. *J. Chem. Soc., Perkin Trans. 1* **2000**, 911–915.

45. a) Freire, R.; Marrero, J. J.; Rodriguez, M. S. and Suárez, E. *Tetrahedron Lett.* **1986**, *27*, 383–386; b) de Armas, P.; Francisco, C. G. and Suárez, E. *J. Am. Chem. Soc.* **1993**, *115,* 8865–8866 and references cited therein.

46. a) Baker, R. K.; Rupprecht, K. M.; Armistead, D. M.; Boger, J.; Frankshun, R. A.; Hodges, P. J.; Hoogsteen, K.; Pisano, J. M. and Witzel, B. E. *Tetrahedron Lett*. **1998**, *39*, 229–232; b) Bloodsworth, A. J.;Bowyer, K. J. and Mitchell, J. C. *J. Org. Chem*. **1987**, *52*, 1124–1128.

47. Olofsson, B.; Bogár, K.; Fransson, A.-B. L. and Bäckvall, J.-E. *J. Org. Chem.* **2006**, *71*, 8256–8260.

48. a) John, V.; Moon, J. B.; Pulley, S. R.; Rich, D. H.; Brown, D. L.; Jagodzinska, B. and Jacobs, J. S. WO Patent 2003043987, 2002-US37037, 2003; b) Hoffmann, T.; Koblet, A.; Peters, J.-U.; Schnider, P.; Sleight, A. and Stadler, H. U.S. Patent 2005/ 0090533, 2005; c) Herbert, N. U.S. Patent 6828427 B1, 2004; d) Shepherd, R. G.; White, A. C. GB Patent 2060617 A, 1981.

49. Roudeau, R.; Pardo, D. G. and Cossy, J. *Tetrahedron* **2006**, *62*, 2388–2394.

50. *Cis*-variants: a) Bentley, N.; Dowdeswell, C. S. and Singh, G. *Heterocycles* **1995**, *41*, 2499–2506 and reference 48a.

51. *Trans*-variants: a) Suhara, Y.; Kittaka, A.; Ono, K.; Kurihara, M.; Fujishima, T.; Yoshida, A. and Takayama, H. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 3533–3536; b) Cossy, J.; Dumas, C.; Michel, P.

and Pardo, D. G. *Tetrahedron Lett*. **1995**, *36*, 549–552; c) Cossy, J.; Dumas, C. and Pardo, D. G. *Eur. J. Org. Chem*. **1999**, 1693–1699.

52. Dalal, S.; Singh, P. K.; Raghava, S.; Rawat, S. and Gupta M. N. *Biotechnol. Appl. Biochem.* **2008**, *51*, 23–31.

53. Schulz, T.; Pleiss, J. and Schmid, R. D. *Protein Sci.* **2000**, *6*, 1053–1062.

54. Chahinian, H.; Nini, L.; Boitard, E.; Dubès, J.-P.; Comeau, L.-C. and Sarda, L. *Lipids* **2002**, *37*, 653–662 and references cited therein.

55. a) Bloch, R.; Guibe-Jampel, E. and Girard, C. *Tetrahedron Lett.* **1985**, *26*, 4087–4090; b) Schneider, M.; Engel, N.; Honicke, P.; Heinemann, G. and Gorisch, H. *Angew. Chem. Int. Ed.* **1984**, *23*, 67–68; c) Zemlicka, J.; Craine, L. E.; Heeg, M. J. and Oliver, J. P. *J. Org. Chem.* **1988**, *53*, 937–942; d) Hutchinson, E. J.; Roberts, S. M. and Thorpe, A. J. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2245–2246; e) Hill, J. M.; Hutchinson, E. J.; Le Grand, D. M.; Roberts, S. M.; Thorpe, A. J. and Turner, N. J. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1483–1487; f) Hultin, P. G.; Mueseler, F.-J. and Jones, J. B. *J. Org. Chem.* **1991**, *56*, 5375–5380; g) Jones, J. B.; Hinks, R. S. and Hultin, P. G. *Can. J. Chem.* **1985**, *63*, 452–456; h) Sabbioni, G.; Shea, M. L. and Jones, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 236–238.

56. Domínguez de María, P.; García-Burgos, C. A.; Bargeman, G. and van Gemert, R. W. *Synthesis* **2007**, 1439–1452 and references cited therein.

57. Luzzio, F. A. and Fitch, R. W. *J. Org. Chem.* **1999**, *64*, 5485–5493 and references therein.

58. Luzzio, F. A. and Fitch, R. W. *Ultrason. Sonochem*. **1997**, *4*, 99–107.

59. Ors, M.; Morcuende, A.; Jiménez-Vacas, M. I.; Valverde, S. and Herradón, B. *Synlett* **1996**, 449–451 and references cited therein.

60. a) Schmidt, U.; Mundinger, K.; Mangold, R. and Lieberknecht, A. *J. Chem. Soc., Chem. Comm*. **1990**, 1216–1219; b) Gupta, S.; Krasnoff, S. B.; Roberts, D. W. and Renwick, J. A. A. *J. Am. Chem. Soc.* **1991**, *113*, 707–709; c) Lee, K. K.; Gloer, J. B.; Scott, J. A. and Malloch, D. *J. Org. Chem.* **1995**, *60*, 5384–5385; d) Boger, D. L.; Chen, J.-H. and Sainnonz, K. W.; *J. Am. Chem. Soc.* **1996**, *118*, 1629–1644.

61. Belshaw, P. J.; Meyer, S. D.; Johnson, D. D.; Romo, D.; Ikeda, Y.; Andrus, M.; Alberg, D. G.; Schultz, L. W.; Clardy, J. and Schreiber, S. L. *Synlett* **1994**, 381–392.

62. Wickwire, B. M.; Harris, C. M.; Harris, T. M. and Broquist, H. P. *J. Biol. Chem*. **1990**, *265*, 14742–14747, and references cited therein.

63. Zabriskie, T. M.; Kelly, W. L. and Liang, X. *J. Am. Chem. Soc.* **1997**, *119*, 6446–6447, and references cited therein.

64. Sánchez-Sancho, F. and Herradón, B. *Tetrahedron: Asymmetry* **1998**, *9*, 1951–1965.

65. Panchgalle, S. P.; Bidwai, H. B.; Chavan, S. P. and Kalkote, U. R. *Tetrahedron: Asymmetry* **2010**, *21*, 2399–2401.

66. Hu, S.; Tat, D.; Martinez, C. A.; Yazbeck, D. R. and Tao, J. *Org. Lett.* **2005**, *7*, 4329–4331.

67. Vink, M. K. S.; Schortinghuis, C. A.; Luten, J.; van Maarseveen, J. H.; Schoemaker, H. E.; Hiemstra, H. and Rutjes, F. P. J. T. *J. Org. Chem.* **2002**, *67*, 7869–7871.

68. Blum, M. S. and Porter Woodring, J. *Science* **1962**, *138*, 512–513.

69. a) Purkarthofer, T.; Skranc, W.; Schuster, C. and Griengl, H. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 309–320, and references cited therein; b) Holt, J. and Hanefeld, U. *Curr. Org. Synth.* **2009**, *6*, 15–37.

70. a) Dreveny, I.; Gruber, K.; Glieder, A.; Thompson, A. and Kratky, C. *Structure* **2001**, *9*, 803–815; b) Dreveny, I.; Kratky, C. and Gruber, K. *Protein Sci.* **2002**, *11*, 292–300; c) Trummler, K. and Wajant, H. *J. Biol. Chem.* **1997**, *272*, 4770–4774.

71. a) Gruber, K. and Kratky, C. *J. Polym. Sci. A1* **2004**, *42*, 479–486; b) Lauble, H.; Miehlich, B.; Förster, S.; Wajant, H. and Effenberger, F. *Biochemistry* **2002**, *41*, 12043–12050.

72. a) Xu, L.-L.; Singh, B. K. and Conn, E. E. *Arch. Biochem. Biophys*. **1988**, *263*, 256–263; b) Wajant, H.; Förster, S.; Selmar, D.; Effenberger, F. and Pfizenmaier, K. *Plant Physiol.* **1995**, *109*, 1231–1238.

73. a) Menédez, E.; Brieva, R.; Rebolledo, F. and Gotor, V. *J. Chem. Soc., Chem. Commun.* **1995**, 989–990; b) Nazabadioko, S.; Pérez, R. J.; Brieva, R. and Gotor, V. *Tetrahedron: Asymmetry* **1998**, *9*, 1597–1604.

74. Froelich, O.; Desos, P.; Bonin, M.; Quirion, J.-C. and Husson, H.-P. *J. Org. Chem.* **1996**, *61*, 6700–6705 and references cited therein.

75. van den Nieuwendijk, A. M. C. H.; Ruben, M.; Engelsma, S. E.; Risseeuw, M. D. P.; van den Berg, R. J. B. H. N.; Boot, R. G.; Aerts, J. M.; Brussee, J.; van der Marel, G. A. and Overkleeft, H. S. *Org. Lett.* **2010**, *12*, 3957–3959 and references cited therein.

76. a) Asano, N.; Yamauchi, T.; Kagamifuchi, K.; Shimizu, N.; Takahashi, S.; Takatsuka, H.; Ikeda, K.; Kizu, H.; Chuakul, W.; Kettawan, A. and Okamoto, T. *J. Nat. Prod.* **2005**, *68*, 1238–1242; b) Legler, G. and Pohl, S. *Carbohydr. Res.* **1986**, *155*, 119–129; c) Kato, A.; Kato, N.; Kano, E.; Adachi, I.; Ikeda, K.; Yu, L.; Okamoto, T.; Banba, Y.; Ouchi, H.; Takahata, H. and Asano, N. *J. Med. Chem.* **2005**, *48*, 2036–2044.

77. Van Rheenen, V.; Kelly, R. C. and Cha, D. Y. *Tetrahedron Lett*. **1976**, *23*, 1973–1976

78. a) Wijdeven, M. A.; Botman, P. N. M.; Wijtmans, R.; Schoemaker, H. E.; Rutjes, F. P. J. T. and Blaauw, R. H. *Org. Lett.* **2005**, *7*, 4005–4007; b) Wijdeven, M. A.; Wijtmans, R.; van den Berg, R. J. F.; Noorduin, W.; Schoemaker, H. E.; Sonke, T.; van Delft, F. L.; Blaauw, R. H.; Fitch, R. W.; Spande, T. F.; Daly, J. W. and Rutjes, F. P. J. T. *Org. Lett.* **2008**, *10*, 4001–4003.

79. Wijdeven, M. A.; van den Berg, R. J. F.; Wijtmans, R.; Botman, P. N. M.; Blaauw, R. H.; Schoemaker, H. E.; van Delft, F. L. and Rutjes, F. P. J. T. *Org. Biomol. Chem.* **2009**, *7*, 2976–2980.

80. Fitch, R. W.; Garraffo, H. M.; Spande, T. F.; Yeh, H. J. C. and Daly, J. W. *J. Nat. Prod.* **2003**, *66*, 1345–1350.

81. Kanakubo, A.; Gray, D.; Innocent, N.; Wonnacott, S. and Gallagher, T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4648–4651.

82. Fitch, R. W.; Sturgeon, G. D.; Patel, S. R.; Spande, T. F.; Garraffo, H. M.; Daly, J. W. and Blaauw, R. H. *J. Nat. Prod.* **2009**, *72*, 243–247.

83. a) Jang, C. S.; Fu, F. Y.; Wang, C. Y.; Huang, K. C.; Lu, G. and Chou, T. C. *Science* **1946**, *103*, 59; b) Koepfli, J. B.; Mead, J. F. and Brockman, J. A. *J. Am. Chem. Soc.* **1947**, *69*, 1837; c) Chou, T. Q.; Fu, F. Y. and Kao, Y. S. *J. Am. Chem. Soc.* **1948**, *70*, 1765–1767; d) Kuehl, J.; Frederick, A.; Spence C. F. and Folkers, K. *J. Am. Chem. Soc.* **1948**, *70*, 2091–2093; e) Koepfli, J. B.; Mead, J. F. and Brockman, J. A. *J. Am. Chem. Soc.* **1949**, *71*, 1048–1054.

84. Ablondi, F.; Gordon, S.; Morton II, J. and Williams, J. H. *J. Org. Chem.* **1952**, *17*, 14–18.

85. For examples of syntheses of febrifugine and analogues, see: a) Takeuchi, Y.; Abe, H. and Harayama, T. *Synthesis* **1999**, 1814–1818; b) Taniguchi, Y. and Ogasawara, K. *Org. Lett.* **2000**, *2*, 3193–3195; c) Ooi, H.; Urushibara, A.; Esumi, T.; Iwauchi, Y. and Hatakeyama, S. *Org. Lett.* **2001**, *3*, 953–955; d) Okitsu, O.; Suzuki, R. and Kobayashi, S. *J. Org. Chem.* **2001**, *66*, 809–823; e) Takeuchi, Y.; Azuma, K.; Oshige, M.; Abe, H.; Nishioka, H.; Sasakiand K. and Harayama, T. *Tetrahedron* **2003**, *59*, 1639–1646; f) Huang, P.-Q.; Wei, B.-B. and Ruan, Y.-P. *Synlett* **2003**, 1663–1667; g) Kikuchi, H.; Yamamoto, K.; Horoiwa, S.; Hirai, S.; Kasahara, R.; Hariguchi, N.; Matsumoto, M. and Oshima, Y. *J. Med. Chem.* **2006**, *49*, 4698–4706; h) Zhu, S.; Meng, L.; Zhang, Q. and Wei, L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1854–1858; i) Sieng, B.; Ventura, O.

L.; Bellosta, V. and Cossy, J. *Synlett* **2008**, 1216–1218; j) Wee, A. G. H. and Fan, G.-J. *Org. Lett.* **2008**, *10*, 3869–3872; k) Sudhakar, N.; Srinivasulu, G.; Rao, G. S. and Rao, B. V. *Tetrahedron: Asymmetry* **2008**, *19*, 2153–2158; l) Sukemoto, S.; Oshige, M.; Sato, M.; Mimura, K.-I.; Nishioka, H.; Abe, H.; Harayama, T. and Takeuchi, Y. *Synthesis* **2008**, 3081–3087.

86. Kobayashi, S.; Ueno, M.; Suzuki, R.; Ishitani, H.; Kim, H. S. and Wataya, Y. *J. Org. Chem.* **1999**, *64*, 6833–6841 and references cited therein.

87. Kikuchi, H.; Tasaka, H.; Hirai, S.; Takaya, Y.; Iwabuchi, Y.; Ooi, H.; Hatakeyama, S.; Kim, H. S.; Wataya, Y. and Oshima, Y. *J. Med. Chem.* **2002**, *45*, 2563–2570.

88. Vink, M. K. S.; Schortinghuis, C. A.; Mackova-Zabelinskaja, A.; Fechter, M.; Pöchlauer, P.; Castelijns, A. M. C. F.; van Maarseveen, J. H.; Hiemstra, H.; Griengl, H.; Schoemaker, H. E. and Rutjes, F. P. J. T. *Adv. Synth. Catal.* **2003**, *345*, 483–487.

89. van Beilen, J. B. and Funhoff, E. G. *Curr. Opin. Biotech.* **2005**, *16*, 308–314 and references cited therein.

90. a) Chang, D.; Feiten, H.-J.; Engesser, K.-H.; van Beilen, J. B.; Witholt, B. and Li, Z. *Org. Lett.* **2002**, *4*, 1859–1862; b) Chang, D.; Heringa, M. F.; Witholt, B. and Li, Z. *J. Org. Chem.* **2003**, *68*, 8599–8606; c) Li, Z.; Feiten, H.-J.; van Beilen, J. B.; Duetz, W. and Witholt, B. *Tetrahedron-Asymmetry* **1999**, *10*, 1323–1333 and references cited therein.

91. Vink, M. K. S.; Wijtmans, R.; Reisinger, C.; van den Berg, R. J. F.; Schortinghuis, C. A.; Schwab, H.; Schoemaker, H. E. and Rutjes, F. P. J. T. *Biotechnol. J.* **2006**, *1*, 569–573 and references cited therein.

92. Wang, M.-X. *Top. Organomet. Chem*. **2011**, *36*, 105–122.

93. Boesten, W. H. J. and Cals, M. J. US Patent 4705752, 1987; *Chem. Abstr.* **1987**, *105*, 170617.

94. Bugg, T. D. H. *Introduction to Enzyme and Coenzyme Chemistry*; Blackwell Publishing Ltd.: Oxford, **2004**, 2nd Edition, Chapter 7 and references cited therein.

95. McNaught, A. D. *Pure Appl. Chem*. **1996**, *68*, 1919–2008.

96. Clapés, P.; Sprenger, G. A. and Joglar, J. *Modern Biocatalysis: Stereoselective and Environmentally Friendly Reactions*; Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, **2009**, Chapter 19.

97. a) Gijsen, H. J. M.; Qiao, L.; Fitz, W. and Wong, C.-H. *Chem. Rev.* **1996**, *96*, 443–473 and references cited therein; b) Clapés, P.; Fessner, W.-D.; Sprenger, G. A. and Samland, A. K. *Curr. Opin. Chem. Biol.* **2010**, *14*, 154–167 and references cited therein.

98. Fan, J.-Q.; Ishii, S.; Asano, N. and Suzuki, Y. *Nature Med*. **1999**, *5*, 112–115.

99. Castillo, J. A.; Calveras, J.; Casas, J.; Mitjans, M.; Vinardell, M. P.; Parella, T.; Inoue, T.; Sprenger, G. A.; Joglar, J. and Clapés, P. *Org. Lett.* **2006**, *8*, 6067–6070 and references cited therein.

100. Bednarski, M. D.; Simon, E. S.; Bischofberger, N.; Fessner, W.-D.; Kim, M.-J.; Lees, W.; Saito, T.; Waldmann, H. and Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627–635.

101. a) Espelt, L.; Bujons, J.; Parella, T.; Calveras, J.; Joglar, J.; Delgado, A. and Clapés, P. *Chem. Eur. J.* **2005**, *11*, 1392–1401; b) Espelt, L.; Parella, T.; Bujons, J.; Solans, C.; Joglar, J.; Delgado, A. and Clapés, P. *Chem. Eur. J.* **2003**, *9*, 4887–4899.

102. Schumperli, M.; Pellaux, R. and Panke, S. *Appl. Microbiol. Biotechnol*. **2007**, *75*, 33–45.

103. Schürmann, M. and Sprenger, G. A. *J. Biol. Chem.* **2001**, *276*, 11055–11061.

104. Legler, G.; Stütz A. E. and Immich, H. *Carbohydr. Res.* **1995**, *272*, 17–30

105. Sekioka, T.; Shibano, M. and Kusano, G. *Natural Medicines (N. Y.)* **1995**, 332.

106. Garrabou, X.; Castillo, J. A.; Guérard-Hélaine, C.; Parella, T.; Joglar, J.; Lemaire, M. and Clapés, P. *Angew. Chem. Int. Ed.* **2009**, *48*, 5521–5525.

107. a) Asano, N.; Nash, R. J.; Molyneux, R. J. and Fleet, G. W. J. *Tetrahedron-Asymmetry* **2000**, *11*, 1645–1680; b) Compain, P. and Martin, O. R. *Bioorg. Med. Chem.* **2001**, *9*, 3077–3092; c) Asano, N. *Glycobiology* **2003**, *13*, 93–104.

108. Wennekes, T.; Meijer, A. J.; Groen, A. K.; Boot, R. G.; Groener, J. E.; van Eijk, M.; Ottenhoff, R.; Bijl, N.; Ghauharali, K.; Song, H.; O'Shea, T. J.; Liu, H.; Yew, N.; Copeland, D.; van den Berg, R. J. F.; van der Marel, G. A.; Overkleeft, H. S. and Aerts J. M. *J. Med. Chem.* **2010**, *53*, 689–698.

109. a) Kato, A.; Kato, N.; Kano, E.; Adachi, I.; Ikeda, K.; Yu, L.; Okamoto, T.; Banba, Y.; Ouchi, H.; Takahata, H. and Asano, N. *J. Med. Chem.* **2005**, *48*, 2036–2044; b) Asano, N.; Yasuda, K.; Kizu, H.; Kato, A.; Fan, J.-Q.; Nash, R. J.; Fleet, G. W. J. and Molyneux, R. J. *Eur. J. Biochem.* **2001**, *268*, 35–41.

110. a) Butters, T. D.; Dwek, R. A. and Platt, F. M. *Curr. Top. Med. Chem.* **2003**, *3*, 561–574; b) Platt, F. M.; Neises, G. R.; Karlsson, G. B.; Dwek, R. A. and Butters, T. D. *J. Biol. Chem.* **1994**, *269*, 27108–27114.

111. Sugiyama, M.; Hong, Z.; Liang, P.-H.; Dean, S. M.; Whalen, L. J.; Greenberg, W. A. and Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 14811–14817.

Chapter 2

Rabbit muscle aldolase: computational studies and experimental verification

Lieke J. C. van Hemert, Alexander J. J. Groenen, Luuk J. A. Wilders, Lara Babich, Ron Wever, Sander Nabuurs, and Floris P. J. T. Rutjes, Expanding the scope of one-pot four-enzyme cascade aldol reactions, manuscript in preparation.

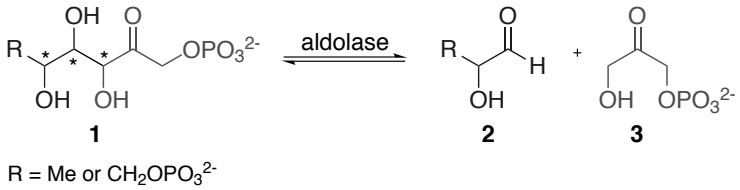
"Life is really simple, but we insist on making it complicated."
-Confucius

Abstract

Due to its excellent stereoselectivity and the acceptance of a broad range of aldehydes, rabbit muscle aldolase is a useful biocatalyst in the synthesis of enantiopure multifunctional molecules via C-C bond formation. This study was focused on computationally determining the scope of aldehydes that are accepted and the structural factors that influence the reaction rate of RAMA catalyzed aldol reactions. The findings were verified in an experimental assay. From these results we concluded that the phosphate group in the natural substrate D-glyceraldehyde-3-phosphate contributes most to the relative reaction rate (V_{rel}). A nitro or carboxylic acid group on the phosphate position had the same accelerating effect. Whereas the computational studies on the α-hydroxy group in the natural substrate revealed that this caused steric interactions with the enzyme and the experimental verification demonstrated a lowered V_{rel}. On the other hand, a strong electron-withdrawing atom on the α-position of the aldehyde greatly accelerated the reaction.

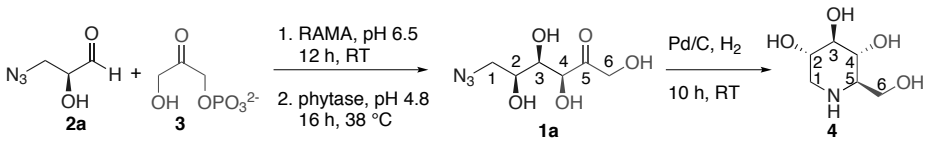
2.1 — Introduction

Dihydroxyacetone phosphate (DHAP)-dependent aldolases are useful enzymes for stereoselective carbon-carbon bond formation.[1] This proceeds through a reversible aldol reaction, which more generally is a powerful reaction for the synthesis of enantiopure multifunctional molecules.1,[2] In Nature, these aldolases catalyze the retro-aldol reaction of carbohydrates (**1**) into aldehydes **2** and DHAP (**3**, Scheme 1) as key element of the glycolysis.[3]



Scheme 1 — General reaction catalyzed by aldolases *in vivo*.

In the synthetic sense, aldolases catalyze the aldol reaction between the nucleophilic donor DHAP (**3**) and an electrophilic aldehyde acceptor (**2**) and are often applied in the synthesis of carbohydrate derivatives such as iminosugars.1 In 1988, Wong *et al.* reported the synthesis of 1-deoxynojirimycin (DNJ, **4**), a potent glycosidase inhibitor, via an enzymatic aldol reaction between azidoaldehyde **2a** and DHAP (**3**, Scheme 2).[4] The stereochemistry of C3 and C4 of tetrahydroxyketone **1a** was introduced in the aldol reaction and directed by the aldolase, while the C2 chirality was predetermined by the starting material. After the aldol reaction and dephosphorylation, the stereochemistry of C5 in the ring (**4**) was introduced via intramolecular reductive amination.



Scheme 2 — Synthesis of DNJ 4 via an aldolase-catalyzed aldol reaction of aldehyde 2a and DHAP 3.

As mentioned in Section 1.6, DHAP-dependent aldolases display excellent stereocontrol over the newly created stereocenters C3 and C4.[5] Nature provides four complementary DHAP-dependent aldolases, which provide access to all four diastereomers (**1b**–**e**, Figure 1).

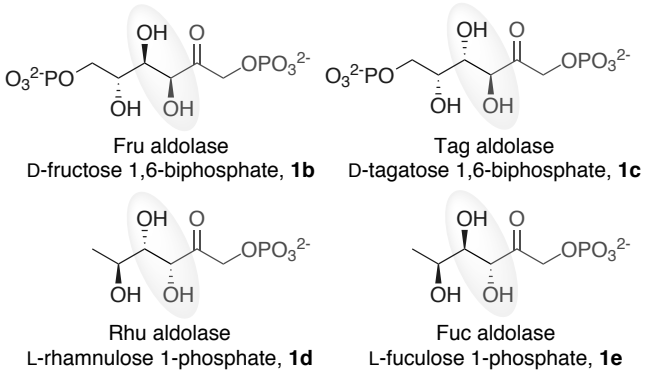
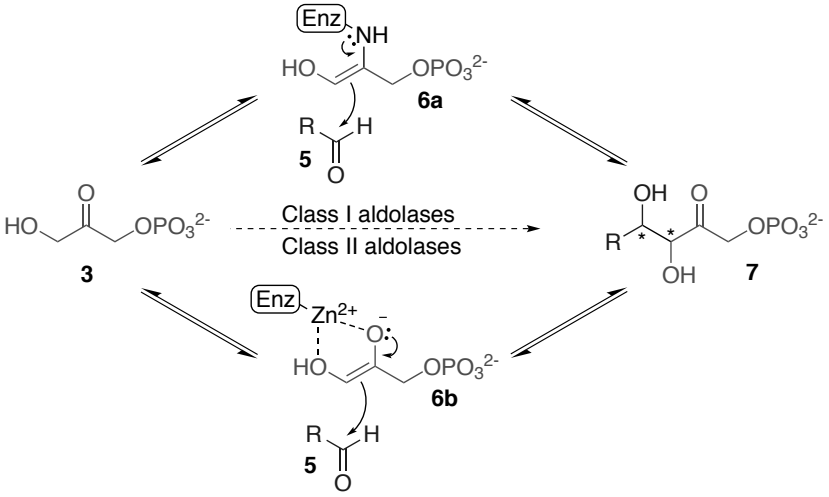


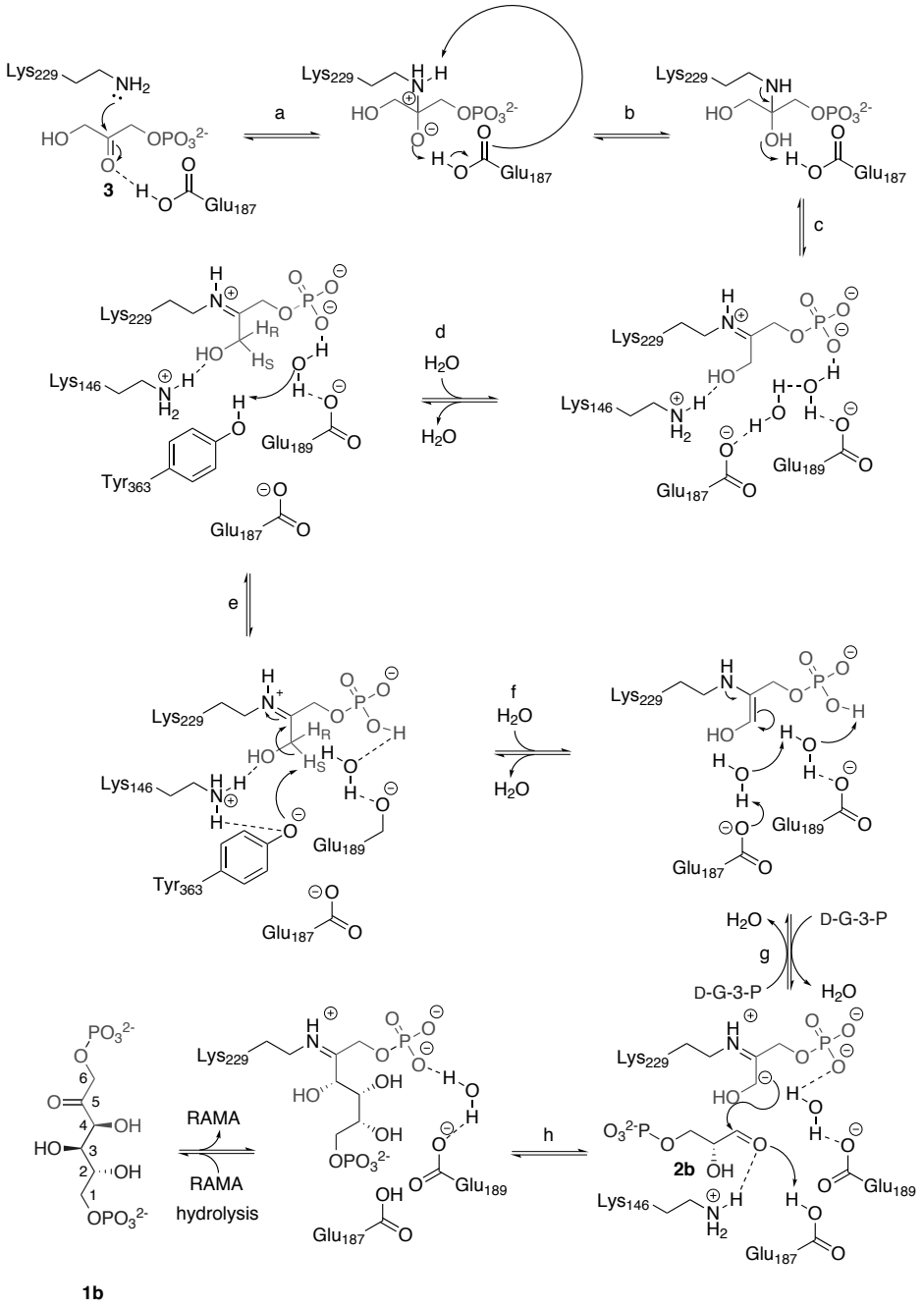
Figure 1 — The four stereocomplementary DHAP-dependent aldolases and their natural substrates.

These DHAP-dependent aldolases are divided into two classes, based on their catalytic mode of action and mechanistic properties. The class I aldolases catalyze the aldol reaction via enamine formation of DHAP with a highly conserved lysine residue in the active site (**6a**, Scheme 3). D-Fructose 1,6-biphosphate aldolase (FruA), such as rabbit muscle aldolase (RAMA), belongs to the class I aldolases.[6]



Scheme 3 — Mechanistic properties of the catalytic center by class I (6a) and II (6b) aldolases.

L-Rhamnulose 1-phosphate- (RhuA), D-tagatose 1,6-biphosphate- (TagA) and L-fuculose 1-phosphate aldolase (FucA) are class II aldolases and have a divalent metal cation as cofactor in the active site, such as Zn^{2+} or Co^{2+}, which is involved in formation of the corresponding enolate (**6b**, Scheme 3).[7] The research in this chapter is focused on RAMA, a relatively inexpensive aldolase (€ 0.10/U),[8] which accepts a broad range of aldehydes[9]



Scheme 4 — Postulated mechanism of RAMA with induced fit by Tyr_{363}.

with excellent stereoselectivities.[10] RAMA consists of 363 amino acids and has a molecular weight of approximately 60 kDa. A RAMA-catalyzed aldol reaction between DHAP (**3**) and aldehyde **5** results mainly in D-threo (3*R*,4*S*) stereochemistry. The mechanism was studied comprehensively and in 2007 a high resolution crystal structure with the natural substrate was analyzed (PDB entry 2QUU)[11] from which a mechanism could be postulated (Scheme 4). One of the interesting results was the role of the Tyr_{363} at the C-terminus, which acted as a proton acceptor in the active site and caused a structural change in the active site of the enzyme (induced fit, step e).[12] Initially, DHAP (**3**, steps a–c) becomes covalently bound to the enzyme via imine formation between Lys_{229} and the carbonyl group of DHAP, assisted by Glu_{187}, Glu_{189}, Lys_{146} and two water molecules. This step is followed by formation of the enamine through deprotonation of C3 by the C-terminal Tyr_{363} phenolate (steps d–f). D-Glyceraldehyde-3-phosphate (D-G-3-P, **2b**) replaced Tyr_{363} and the enamine attacks the carbonyl of the aldehyde to form the two new chiral centers (steps g–h). Upon hydrolysis, D-fructose-1,6-bisphosphate (FBP, **1b**) is released from the active site.

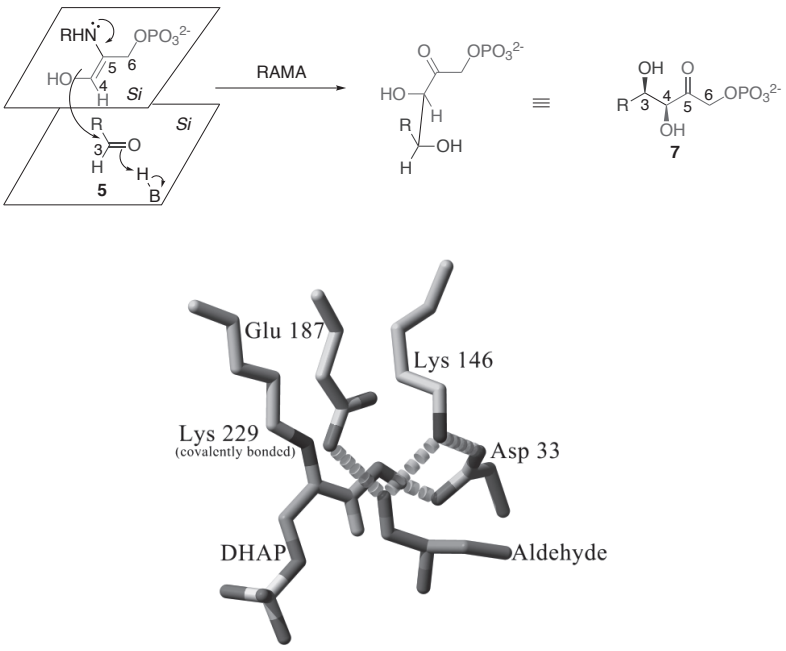


Figure 2 — Crucial step demonstrating the formation of chiral centers C3 and C4 based on PDB entry 1ZAI, with DHAP (green) and 2-hydroxybutanal (orange, R = C(OH)Et). The yellow dotted lines indicate hydrogen bonds.

Figure 2 demonstrates the crucial step in which the formation of two new stereocenters occurs (step h).[13] The *Si*-face of C4 from the activated imine is exposed to the solvent, therefore the aldehyde can only approach the enamine from this direction. The aldehyde is only able to fit in the active site with the *Si*-face towards the enamine (Figure 2), while the carbonyl group from the aldehyde forms two hydrogen-bonds with Glu_{187} and Lys_{146} (step h and Figure 2).[12] The aldehyde is attacked by the activated enamine from the *Si*-face (C3), which results in the formation of the (3*R*,4*S*)-product. Attack on the *Re*-face by a 180° turn of the aldehyde is sterically unfavored.

RAMA is restrictive concerning the nucleophilic donor DHAP, since all functional groups play an important role in the mechanism and binding to RAMA.[12] A few analogs of DHAP have been reported, but were poorly accepted (Figure 3). The first example had an extra methylene group on the C6 position, while also substitution of the C6 oxygen by sulfur or a methylene group yielded poor substrates for RAMA.[9] Replacement of the C4 hydroxy group by a halogen resulted in inhibition of the enzyme. Recently however, fluorohydroxyacetone phosphate (FHAP) was used as a donor substrate for RAMA and aldol product formation was observed, but low yields (7%) were obtained due to poor acceptance.[14] FHAP is missing a hydrogen bond acceptor, which supposedly is crucial for catalysis so that this donor showed a dramatic lowering in reaction rate (V_{rel}).

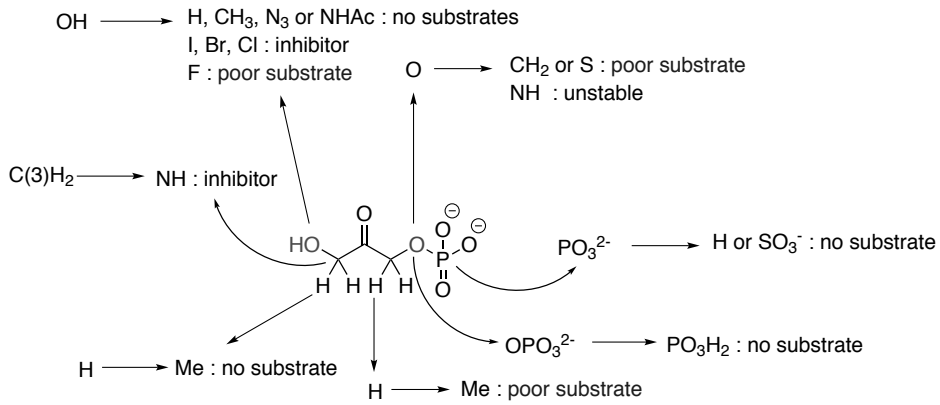


Figure 3 — Various analogues of DHAP tested with RAMA.

In contrast to DHAP derivatives, RAMA has a broad scope for acceptor aldehydes, and over a hundred aldehydes have been reported as substrates for RAMA.[9,15] Little is known about the reaction rate and the influence of the interactions of D-G-3-P with the enzyme (**2b** and Figure 4, orange). Investigation of these interactions by computational studies and experimental verification would reveal insight in which factors influence the V_{rel} in

the product formation catalyzed by RAMA and could lead to predicting new classes of acceptor aldehydes.

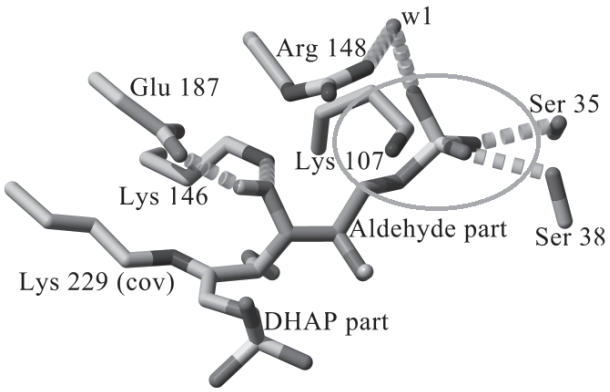


Figure 4 — FBP in the active site of RAMA based on PDB entry 1ZAI.
The phosphate pocket of the aldehyde is marked by a yellow oval.

In 1989 several trends in V_{rel} were reported by Whitesides *et al.*[9] The general trend observed was a decrease in reaction rate with extended chain lengths. However, the natural substrate D-G-3-P (**2b**) appeared an exception to this rule. With a bulky phosphate-group and α-hydroxy group, it showed a V_{rel} comparable with the much smaller propionaldehyde. These predictions were based on experimental results, since no crystal structure was available at that time. The purpose of these computational studies was to explore the structure activity relationship (SAR) of D-G-3-P with RAMA based on the increase or decrease of V_{rel} with various aldehydes using computational studies and experimental verification hereof, which would give insight in the prediction of V_{rel} based on the substitution on the aldehyde (Figure 5).

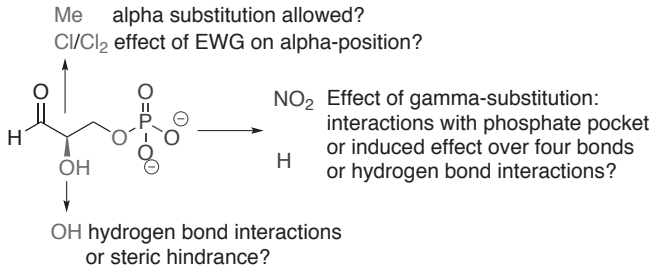


Figure 5 — Structure activity relationship (SAR) of D-G-3-P (**2b**)

2.2 — Results and discussion

2.2.1 — Computational studies

The computational studies were performed with PDB[11] entry 1ZAI in Yasara[16,17] and docking software MOE[18] to predict the substrate specificity of RAMA. The aldehydes reported in the experimental results of the Whitesides group were also calculated.9 Most aldehydes were found to fit in the active site, except for the multiple branched α-carbons, such as pivaldehyde and 2,2,2-trichloroacetaldehyde (both not shown). These aldehydes caused too many steric interactions and thus did not fit into the active site of RAMA according to these calculations. Previous studies revealed that V_{rel} was not only influenced by the stabilizing effects of functional groups, such as phosphate in the natural substrate, but also by the electrophilicity of the carbonyl group.[19] An electron-withdrawing group (EWG) attached to the α-carbon showed a dramatic increase in V_{rel}.[9] Similar results were reported for an EWG on the γ-carbon, which was explained as an inductive activating effect over four bonds. As shown in Figure 4, the phosphate group of the natural substrate has also stabilizing interactions with the enzyme. However, up till now it is not known whether the increase in V_{rel} was caused by electron-withdrawing properties or the interactions with the phosphate pocket and the enzyme. To gain more insight in the factors affecting V_{rel} three sets of aldehydes were studied with a model based on PDB entry 1ZAI (Figure 6, sets **A**–**C**).[11] These aldehydes were modeled after natural substrate **2b** and investigated to predict the effect of the substitution on the V_{rel}. The first set (**A**) gave insight in the effect of hydrogen bonding of the α- and γ-substituent, as well as the effect of the interactions of the phosphate group with the enzyme (**5a**–**d**, **2b**). The substitutions with an expected positive effect on V_{rel} are shown in green and with an expected negative effect in red. The second set (**B**) was designed to predict the influence of EWGs at the β- or γ-position (**5e**–**g**) and to prove the existence of negatively charged interactions with the phosphate pocket of the enzyme. The γ-substitution of aldehydes **5e** ($-NO_2$) and **5g** ($-CO_2^-$) resemble the phosphate group in the natural substrate and these analogues should be able to interact via hydrogen bond interactions with the enzyme, whereas aldehyde **5g**, being one carbon shorter, should not be able to form these hydrogen bonds. The last set (**C**) was designed to reveal the influence of multiple EWGs attached on the α-position (**5i**–**h**).

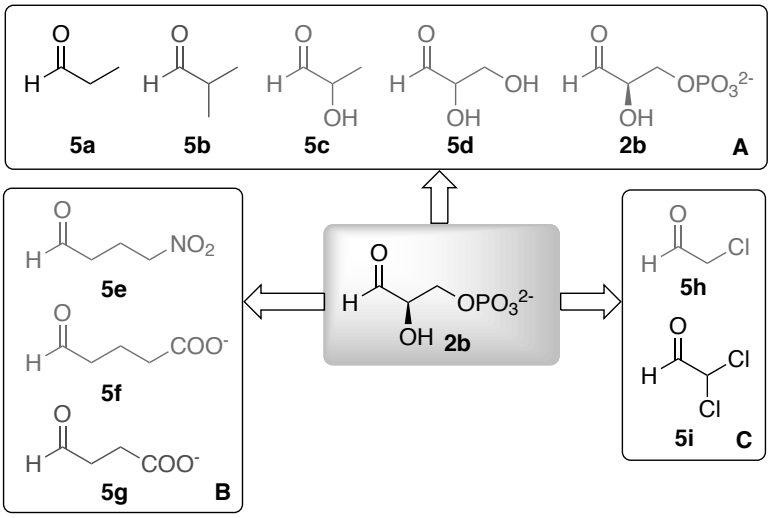
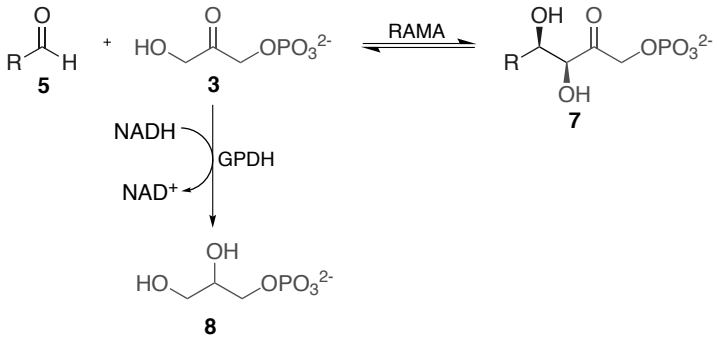


Figure 6 — Sets of aldehydes based on natural substrate 2b.

To our surprise, the α-hydroxyl group of D-G-3-P (**2b**) did not show any hydrogen bond interactions with the enzyme, which led us to conclude that this group did not contribute in stabilizing the substrate in the active site and thus should not accelerate V_{rel}. On the other hand longer chain lengths without α-branching fitted nicely, especially aldehydes bearing a negatively charged group, such as a phosphate group on de γ-position. Energy minimization with Yasara[17b] showed that other reactive groups with hydrogen bond accepting properties or (partial) negative charges, such as nitro or carboxylic acid groups, could occupy the phosphate pocket as well. These groups showed hydrogen bonds with the residues of the phosphate pocket, mimicking the phosphate group of natural substrate **2b** and hence were expected to have a positive influence on V_{rel}. If this effect would cause the relative rate to increase, a comparison between 4-oxobutanoic acid (**5g**) and 5-oxopentanoic acid (**5f**) should demonstrate this with a higher V_{rel} for **5f**. In case of aldehyde **5f**, the negatively charged oxygen was positioned at a comparable location as the negative charge of the phosphate group in natural substrate **2b** (Figure 4). According to the prediction based on this model, aldehyde **5g** bearing a carboxylic acid in the β-position would not be able to access the phosphate pocket of the active site. The distance to both Ser_{35} and Ser_{38} was >4Å and thus this aldehyde could not form hydrogen bonds with the phosphate pocket. Since these calculations cannot be used to produce an accurate V_{rel}, the three sets of aldehydes were experimentally evaluated.

2.2.2 — Experimental verification

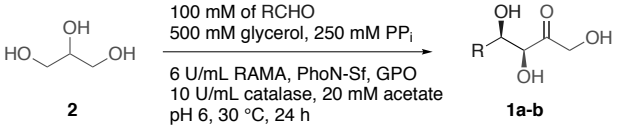
Most aldehydes were purchased as pure compounds, in solution or protected as dimethyl acetal. Lactaldehyde (**5c**) and 4-nitrobutanal (**5e**) were synthesized via a straightforward approach by reducing the corresponding ketone and ester, respectively. The acetal-protected aldehydes were deprotected *in situ* prior to the assay, to prevent degradation or side reactions.



Scheme 5 — RAMA activity test design with NADH as reducing agent.

The DHAP consumption was analyzed by measuring the decrease of DHAP indirectly, using a coupled assay with glycerol-3-phosphate dehydrogenase (GPHD) and NADH in which DHAP was converted into glycerol-3-phosphate (**8**, Scheme 5).[20] The reaction mixture contained 50 mM DHAP (**3**), 100 mM aldehyde **5**, acetate buffer pH 6 and 1 U/mL RAMA and the assay mixture consisted of 1 U/mL GPDH, 0.16 mM NADH and tris/acetate buffer pH 7.5. Every 10 minutes (3-5 times), a diluted 10 µL sample was added to the assay mixture and reacted for 3 minutes, followed by the recording of absorbance at 340 nm. Unfortunately, this assay revealed an initial concentration of 25 mM DHAP instead of the added 50 mM and in addition the obtained results were remarkably irreproducible. Therefore a new assay was designed, based on the actual DHAP consumption, measured via HPLC using UV detection at 254 nm. The relative rate (V_{rel}) was based on the amount of DHAP consumed in time in a 0.5 mL reaction mixture containing 2 U/mL RAMA, 50 mM DHAP (**3**) and 100 mM aldehyde **5** at pH 6 and 20 °C. The course of the reaction was directly analyzed by HPLC, based on the decrease of the peak area of DHAP. All tests were performed in triplo.

Table 1 — Relative reactivities of aldehydes with DHAP (3)



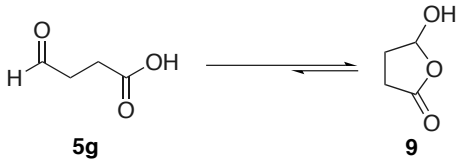
Entry	Set	Aldehyde	R	V_{rel}	V_{rel} reported[b, 9]
1	A	**5a**	-Et	100 ± 7	100
2	A	**5b**	$-CHMe_2$	41 ± 4	18
3	A	**5c**	-CH(OH)Me	30 ± 2	9.5
4	A	**5d**	$-CH(OH)CH_2OH$	25 ± 8	32
5	A	**2b**	$-CH(OH)CH_2OPO_3^{2-}$	n.d.[a]	95
6	B	**5e**	$-C_3H_7NO_2$	234 ± 28	162
7	B	**5f**	$-C_3H_7CO_2^-$	133 ± 21	n.d.
8	B	**5g**	$-C_2H_5CO_2^-$	0	n.d.
9	C	**5h**	$-CH_2Cl$	n.d.	323
10	C	**5i**	$-CHCl_2$	24 ± 14	n.d.

[a]Measurement of the reaction rate was not possible in this setup, due to overlap on HPLC. b) The previously reported V_{rel} was obtained with the indirect DHAP assay depicted in Scheme 5, which could explain the differences with the measured V_{rel}.

A logical standard for V_{rel} would be D-G-3-P (**2b**), however, this aldehyde had a similar retention time as DHAP and also showed absorbance at 254 nm. Fortunately, propanal (**5a**) had a reaction rate comparable with D-G-3-P[9] and was set at a V_{rel} of 100 (Table 1, entry 1). Substitution on the α-position with a methyl group showed a decrease in V_{rel} (entry 2), which was in accordance with the computational predictions, as this caused steric interference with the enzyme. This was also demonstrated by a hydroxyl group on the α-position (entry 3). Instead of the expected hydrogen bonding with the enzyme, which should result in a higher V_{rel} as demonstrated by the computational studies, the effect of steric hindrance was more dominant and caused the V_{rel} to drop. Computational studies revealed that the oxygen of the carbonyl group could form hydrogen bonds with Glu_{187} and Lys_{146}. Glu_{187} was also the hydrogen-donor in the aldol condensation. The α-hydroxyl group was not involved in direct hydrogen bonding with the enzyme and a lower V_{rel} was observed (entry 4). RAMA also did not show any stereoselectivity towards the C3-hydroxy group so that the formation of two diastereoisomers was observed, which is in accordance with previously reported results.[21] This outcome led us to conclude that the phosphate group on the γ-position causes the increase in relative rate due to stabilizing interactions in the phosphate pocket of the enzyme (entry 5). The twofold negatively charged phosphate group of D-G-3-P (**2b**) had electrostatic interactions with the positively charged residues Arg_{148} and Lys_{107} and also the two hydrogen bonds with Ser_{35} and Ser_{38} are shown in the crystal structure (Figure 3). Although no V_{rel} for aldehyde **2b** could be derived with this design, based on these results and earlier work, the phosphate group seemed responsible for the largest share in reactivity. We envisioned that replacement of

the phosphate group by a nitro or carboxylic acid group might have the same accelerating effect. 4-Nitrobutanal (**5e**, entry 6) showed a dramatic increase of V_{rel} with respect to **2b**, which is in accordance with the predictions made, but could also be explained via the inductive effect hypothesis mentioned by Whitesides *et al*.[9]

The effect of a carboxylic acid group on the γ-position was less dramatic (entry 7) and surprisingly the smaller 4-oxobutanoic acid did not show any activity (**5g**, entry 8), indicating it was not accepted by RAMA. According to the computational studies a negatively charged substituent on the γ-position should have a positive effect on the V_{rel} due to interactions with the phosphate pocket of the enzyme. Whereas a negatively charged substituent on the β-position should not show the same effect as it could not interact with the phosphate pocket, however we did not expect to observe a V_{rel} of 0. ^{1}H-NMR analysis of the aldehyde in D_2O, revealed that less than 5% of the substrate was in the aldehyde form and that it mainly occurred in the cyclized form (**9**, scheme 6).



5g **9**

Scheme 6 – 4-oxobutanoic acid (**5**) in linear and ring-closed form (**9**).

As demonstrated by aldehydes **5b** and **5c**, α-branching reduced the reactivity due to steric hindrance, which was also demonstrated in the computational studies with pivaldehyde.9 On the other hand Clapés *et al.* showed that large *N*-amino protecting groups, such as Fmoc or Cbz, on the α-position have no reasonable effect on the conversion rates.[10] It was also known that 2-chloroacetaldehyde (**5h**), bearing an EWG at the α-carbon showed the highest V_{rel} (entry 9), but 2,2,2-trichloroacetaldehyde showed no reactivity due to steric hindrance. The effect of two EWGs attached to the α-carbon could have a positive effect on the relative rate. To verify this effect 2,2,-dichloroacetaldehyde (**5i**, entry 10) was tested as well, which demonstrated a lower V_{rel}, thus the steric effect had a stronger influence than the electron-withdrawing effect. Moreover, when the relative rate of aldehyde **5i** was compared with **5b** and **5c**, the decrease was remarkably high. Possibly the carbonyl group of aldehyde **5i** was predominantly present in the hydrate form, which made the aldehyde less susceptible to nucleophilic attack of DHAP and thus could explain the lower reaction rate. ^{1}H-NMR analysis in D_2O, however, indicated that >80% was in the aldehyde form, perhaps the slightly acidic reaction conditions were enough to cause hydration of the aldehyde. A summary of the computational studies and the experimental verification is shown in Figure 7.

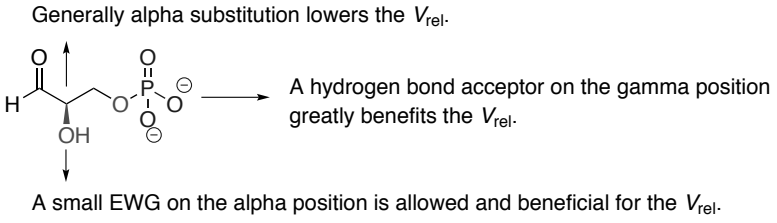


Figure 7 — SAR conclusions based on computational studies and experimental verification of D-G-3-P (**2b**)

2.3 — Conclusions

We have used computational studies in order to be able to predict which parameters influence the relative reaction rate in the aldol reaction catalyzed by RAMA. These predictions were verified in an experimental setup. From the resulting data we concluded that α-branching on the aldehyde resulted in a lower V_{rel}, which is in accordance with previously reported results.9 A small EWG on the α-carbon however resulted in a higher V_{rel} due to enhancing the electrophilicity of the carbonyl group. Unfortunately double substitution with an EWG at the α-carbon lowered the V_{rel}, probably caused by steric hindrance or hydration of the carbonyl group. These results led us to conclude that the high V_{rel} of D-G-3-P (**2b**) was caused by the stabilizing effect of the phosphate group on the γ-position of the aldehyde with the phosphate pocket of the enzyme. This effect was also demonstrated by nitro- and carboxylic acid groups. According to the computational studies, these groups could mimic the hydrogen bond interactions with the enzyme. This was also demonstrated in the experimental verification as both aldehydes showed an increased V_{rel}. When designing a substrate, it is beneficial for the reaction rate to attach a small EWG at the α-position and a negatively charged group or hydrogen bond acceptor at the γ-position.

2.4 — Acknowledgements

Dr. Sander Nabuurs (Centre for Molecular and Biomolecular Informatics, Radboud University Medical Center) and Sander Groenen are gratefully acknowledged for their cooperation and contribution to this chapter.

2.5 — Experimental

2.5.1 — General

Reactions were followed using thin layer chromatography (TLC) on silica gel–coated plates (Merck 60 F254) with the indicated solvent mixture. Detection was performed with UV-light, and/or by charring at ~150 °C after dipping into a solution of $KMnO_4$. Flash column chromatography was carried out using ACROS silica gel (0.035–0.070 mm, and ca 6 nm pore diameter). IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer or a Bruker Tensor 27 FTIR spectrometer. Optical rotations were determined with a Perkin Elmer 241 polarimeter.

NMR spectra were recorded on a Bruker DMX 300 (300 MHz), a Varian 400 (400 MHz) and a Bruker Avance III 500MHz System (500 MHz) spectrometer in $CDCl_3$ or CD_3OD solutions (unless otherwise reported). Chemical shifts are given in ppm with respect to tetramethylsilane (TMS) as internal standard. Coupling constants are reported as *J*–values in Hz.

High resolution mass spectra were recorded on a JEOL AccuTOF (ESI), or a MAT900 (EI, CI, and ESI). Fast Atom Bombardment (FAB) mass spectrometry was carried out using a JEOL JMS SX/SX 102A four-sector mass spectrometer coupled to a JEOL MS-MP9021D/UPD system program.

2.5.2 — Molecular modeling of the (*R*)-2-hydroxypropanal-RAMA complex

The molecular model of (*R*)-2-hydroxypropanal bound to RAMA was constructed on the basis of the crystal structure of a RAMA-fructose 1,6-biphosphate complex (PDB entry: 1ZAI) solved at 1.76 Å resolution.[12] This structure contained the aldol product. The interactions of the carbonyl oxygen of the aldehyde before aldol condensation were not clear. To gain more insight into aldehyde binding in the active site, the carbohydrate product was manually changed into DHAP and (*R*)-2-hydroxypropanal using the Yasara program[17] and finally the resulting complex was minimized in energy using Yasara2 force field.[17c,d]

2.5.3 — Acetal deprotection

Deprotection of a 0.5 M aldehyde solution was performed *in situ* with 0.5 M HCl. After deprotection the solution was neutralized by 0.5 M NaOH to pH 6. To make a fair comparison, NaCl was added to all samples when a deprotection step was not necessary.

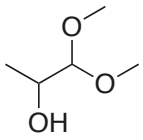
2.5.4 — General procedure for the enzymatic reactions

RAMA-catalyzed reactions were performed at 20 °C in a total volume of 0.5 mL containing acetate buffer (50 mM, pH 6.0), NaCl (100 mM), DHAP (**3**, 50 mM) and aldehyde **5** (100 mM). The reaction was initialized by adding RAMA (2 U/mL) and analyzed by HPLC for 15 min.

2.5.5 — HPLC analyses

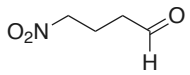
Samples (20 µL) were directly drawn from the reaction mixture and analyzed on a Shimadzu LC 2010 analytical HPLC system equipped with a RP C18 column (Varian, Inertsil ODS-3, 5 µm, 150 × 4.6 mm) and eluted with a mixture of acetonitrile/water (1:99) containing 0.19% trifluoroacetic acid under isocratic conditions at a flow rate of 1.0 mL min^{-1}. The decrease in DHAP concentration was assayed every 2.5 min by following the consumption of DHAP based on the peak area using HPLC. The integrals of the DHAP peak (at 1.75 min) in time were divided by the integral of the DHAP peak on t = 0, to obtain a relative reaction rate (V_{rel}). The UV absorption was analyzed at 254 nm. All experiments were carried out in triplo.

2.5.6 — 2-Hydroxypropanal dimethyl acetal (5c)



To a solution of 1,1-dimethoxypropan-2-one (11.8 mL, 100 mmol) in methanol (50 mL) and THF (50 mL) was added sodium borohydride (3.78 g, 100 mmol) in portions over 30 min at 0 °C. The mixture was allowed to warm to RT and stirred for an additional 30 min. The reaction mixture was then poured into aqueous saturated NH_4Cl (75 mL) and the aqueous layer was extracted with Et_2O (3 × 100 mL). The combined organic layers were concentrated by rotary evaporation. The residue was dissolved in DCM (150 mL), washed with brine (50 mL), dried with Na_2SO_4 and filtered. The solvent was removed by rotary evaporation to give diacetal protected **5c** as a colorless liquid (11.1 g, 88%). R_f: 0.38 (heptane:EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.08 (d, *J* = 6.5 Hz, 1H), 3.76 (qd, *J* = 6.5, 3.5 Hz, 1H), 3.46 (s, 3H), 2.31 (d, *J* = 3.5 Hz, 1H), 1.19 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 108.1, 67.6, 55.3, 55.2, 17.3; υ_{max} (film) cm^{-1} 2933. The analytical data were in accordance with previously reported results.[22]

2.5.7 — 4-Nitrobutanal (5e)



To a solution of methyl 4-nitrobutanoate (0.40 g, 2.7 mmol) in anhydrous DCM (30 mL, -78 °C) was added DIBAL-H (3.0 mL, 3.0 mmol, 1M solution in hexanes) and stirred for 1.5 hour. The temperature was increased to -40 °C and 10% aqueous potassium sodium tartrate (20 mL) was added and stirred for 30 min and warmed to RT. The aqueous layer was extracted with Et_2O (3 × 50 mL) and the combined organic layers were washed with water (100 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (heptane:EtOAc 9:1→2:1) to afford **5e** (204 mg, 64%) as a yellow liquid. R_f: 0.42 (EtOAc:heptane 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.80 (s, 1H), 4.46 (t, *J* = 6.6 Hz, 2H), 2.67 (t, *J* = 6.6 Hz, 2H), 2.31 (q, *J* = 6.6 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.6, 74.3, 40.0, 19.7; υ_{max} (film) cm^{-1} 2850, 1718, 1550, 1372. The analytical data were in accordance with previously reported results.[23]

2.6 — Reference

1. Clapés, P.; Fessner, W-D.; Sprenger G. A. and Samland, A. *Curr. Opin. Chem. Biol.* **2010**, *14*, 154–167.
2. a) Gijsen, H. J. M. and Wong, C.-H. *J. Am. Chem. Soc.* **1995**, *117*, 7585–7591; b) Koeller, K. M. and Wong, C.-H. *Chem. Rev*. **2000**, *100*, 4465–4494.
3. Suau, T.; Álvaro, G.; Dolors Benaiges, M. and López-Santín, J. *Biotechnol. Bioeng.* **2006**, *93*, 48–55.
4. Pederson, R. L.; Kim, M. J and Wong, C.-H. *Tetrahedon Lett.* **1988**, *29*, 4645–4648.
5. Gijssen, H. J. M.; Qiao, L.; Fitz, W. and Wong, C.-H. *Chem. Rev.* **1996**, *96*, 443–469.
6. Wagner, J.; Lai, C. Y. and Horecker, B. L. *Arch. Biochem. Biophys.* **1976**, *1*, 398–403.
7. Rutter, W. J. *Fed. Proc*. **1964**, *23*, 1248–1257.
8. Unit definition: one unit will convert 1.0 μmole of fructose 1,6-biphosphate to dihydroxyacetone phosphate and glyceraldehydes-3-phosphate per min at pH 7.4 at 25 °C.
9. Bednarski, M. D.; Simons, E. S.; Bischofberger, N.; Fessner, W.-D.; Kim, M.-J.; Lees, W.; Saito, T.; Waldmann, H. and Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627–635.
10. Calveras, J.; Bujons, J.; Parella, T.; Crehuet, R.; Espelt, L.; Joglar, J. and Clapés, P. *Tetrahedron* **2006**, *62*, 2648–2656.
11. Protein Data Bank (http://www.pdb.org/).
12. a) St-Jean, M.; Lafrance-Vanasse, J.; Liotard, B. and Sygusch, J. *J. Biol. Chem.* **2005**, *29*, 27262–27270; b) St-Jean, M. and Sygusch, J. *J. Biol. Chem.* **2007**, *42*, 31028–31037; c) St-Jean, M.; Blonski, C. and Sygusch, J. *Biochemistry* **2009**, *48*, 4528–4537.
13. Williams, G. J.; Domann, S.; Nelson, A. and Berry, A. *Proc. Natl. Acad. Sci. USA* **2003**, *6*, 3143–3148.
14. Fischer, M.; Kählig, H. and Schmid, W. *Chem. Commun.* **2011**, *47*, 6647–6649 and references herein.

15. a) Clapés, P. and Garrabou, X. *Adv. Synth. Cat.* **2011**, *353*, 2263–2283; b) van Hemert, L. J. C.; Babich, L.; Bury, A.; Hartog, A. F.; Falcicchio, P.; van der Oost, J.; van Herk, T.; Wever, R. and Rutjes, F. P. J. T. *Green Chem.* **2011**, *13*, 2895–2900; c) Fessner, W.-D.; Anthonsen, T. Eds. *Modern biocatalysis: stereoselective and environmentally friendly reactions*; Wiley-VCH: Weinheim, 2009, Chapter 19.

16. YASARA version 8.1.25.

17. a) Krieger, E.; Koraimann, G. and Vriend, G. *Proteins* **2002**, *47*, 393–402; b) Krieger, E.; Koraimann, G. and Vriend, G. *Proteins Struct. Funct. Bioinf.* **2002**, *47*, 393–402; Forcefields: c) Krieger, E.; Darden, T.; Nabuurs, S. B.; Finkelstein, A. and Vriend, G. *Proteins Struct. Funct. Bioinf*. **2004**, *57*, 578–683; d) Krieger, E.; Joo, K.; Lee, J.; Raman, S.; Thompson, J.; Tyka, M.; Baker, D. and Karplus, K. *Proteins Struct. Funct. Bioinf.* **2009**, *77*, 114–122.

18. MOE: Molecular Operation Environment, version MOE 2010.10, Chemical Computing Group, Montreal, http://www.chemcomp.com/.

19. a) Blom, N. and Sygusch, J. *Nat. Struct. Biol*. **1997**, *4*, 36–39; b) Choi, K. H.; Mazurkie, A. S.; Morris, A. J.; Utheza, D.; Tolan, D. R. and Allen, K. N. *Biochemistry* **1999**, *38*, 12655–12664; c) Choi, K. H.; Shi, J.; Hopkins, C. E.; Tolan, D. R.; Allen, K. N. *Biochemistry* **2001**, *40*, 13868–13875; d) Sherawat, M.; Tolan, D. R. and Allen, K. N. Acta Cryst., Sect. B: Struct. Sci. **2008**, 64, 543–550; e) Maurady, A.; Zdanov, A.; De Moissac, D.; Beaudry, D. and Sygusch, J. *J. Biol. Chem.* **2011**, *277*, 9474–9483.

20. Gutknecht, R.; Beutler, R.; Garcia-Alles, L. F.; Baumann, U. and Erni, B. *EMBO J.* **2001**, *20*, 2480–2486.

21. Van Herk, T. *Bacterial class A acid phosphatases as versatile tools in organic synthesis*, **2008**, PhD thesis, http://dare.uva.nl/en/record/267354.

22. Denmark, S. E. and Yang, S.-M. *J. Am. Chem. Soc.* **2002**, *124*, 2102–2103

23. Shechter, H.; Ley, D. E. and Zeldin, L. *J. Am. Chem. Soc.* **1952**, *74*, 3664–3668

Chapter 3

Synthesis of non-natural carbohydrates in a one-pot four-enzyme cascade reaction

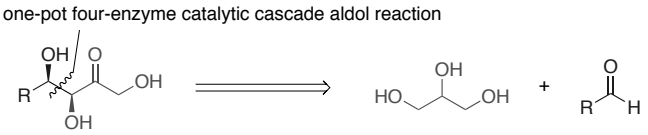
This work was published in: Lieke J. C. van Hemert,[1] Lara Babich,[1] Aleksandra Bury, Aloysius F. Hartog, Pierpaolo Falcicchio, John van der Oost, Teunie van Herk, Ron Wever and Floris P. J. T. Rutjes, Synthesis of non-natural carbohydrates from glycerol and aldehydes in a one-pot four-enzyme cascade reaction, Green Chem. ***2011****, 13, 2895–2900 and Lieke J. C. van Hemert, Alexander J. J. Groenen, Luuk J. A. Wilders, Lara Babich, Ron Wever, Sander Nabuurs, and Floris P. J. T. Rutjes, Expanding the scope of one-pot four-enzyme cascade aldol reactions, manuscript in preparation.*

1 Both authors equally contributed to this work.

"Nature doesn't hurry, Yet everything is accomplished"
– Lao Tzu

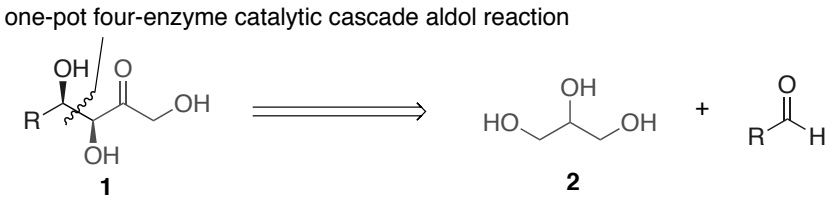
Abstract

A simple procedure has been developed for the synthesis of enantio- and diastereomerically pure carbohydrate fragments from glycerol and a variety of aldehydes in one pot using a four-enzyme cascade reaction. This work demonstrates the potential value of using enzymes in cascade reactions to selectively form complex products that by previous traditional organic chemistry could only be obtained via repeated isolation and purification of intermediates.



3.1 — Introduction

In the search for efficient, sustainable synthetic strategies, chemists increasingly pursue approaches that are inspired by Nature.[1] The ultimate goal is to mimic biosynthetic pathways and construct complex products via ingenious sequences of multiple enzymatic conversions in one pot, preferably proceeding with high chemo- and stereoselectivity.[2] This concept is demonstrated in the synthesis of complex carbohydrate building blocks,[3] which may serve amongst others as intermediates for iminosugars.[4] Besides impressive achievements in the chemical synthesis of carbohydrate fragments using asymmetric catalysis[5] and organocatalysis,[6] use of aldolases could provide direct stereoselective access to carbohydrate structures without going through protecting group manipulations.[7] In order to explore the full potential of existing aldolases in C-C–bond formation, it is crucial to have facile access to phosphorylated substrates.[8] Dihydroxyacetone phosphate (DHAP) is one of the key compounds in aldol condensation using DHAP dependent aldolases and the research groups of Whitesides, Wong and Fessner have used several enzymatic procedures to arrive at the formation of DHAP starting from glycerol,[9] glycerolphosphate[10] or dihydroxyacetone (DHA).[11] These methods have the disadvantage that kinases are needed and that regeneration of ATP is required. The group of Sheldon was among the first to explore the formation of a heptose sugar using a multi-enzyme system in which a phosphorylated intermediate was generated using cheap pyrophosphate (PP_i) as a phosphate donor and in which the DHAP formed was coupled enzymatically to an aldehyde. However, a pH switch was needed to turn off the activity of one of the enzymes and also a very high concentration of glycerol (85%) was required.[12] The group of Wever recently reported the efficient phosphorylation of dihydroxyacetone by the acid phosphatase from *Shigella flexneri* (PhoN-Sf) using PP_i as the phosphate source. In addition, it was shown that this phosphorylation process could be applied in one pot in combination with dihydroxyacetone phosphate (DHAP)-dependent rabbit muscle aldolase (RAMA) to give the desired aldol product.[13] Motivated by this outcome, we now report a one-pot synthesis of chiral carbohydrate fragments **1** by the simultaneous action of four different enzymes starting from inexpensive glycerol (**2**) and an appropriate aldehyde (Scheme 1).

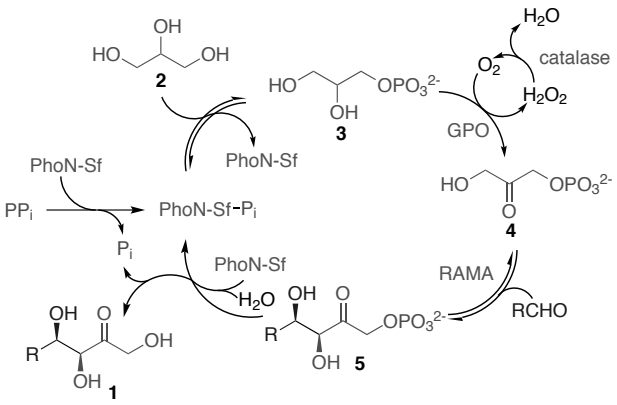


Scheme 1 — One-pot synthesis of carbohydrate fragments 1.

The newly envisaged cascade was based on initial conversion of glycerol into glycerol-1-phosphate,[9,12] followed by L-glycerol-1-phosphate oxidase (GPO)-mediated oxidation to DHAP. Subsequent RAMA-catalyzed C-C–bond formation and dephosphorylation with PhoN-Sf should then give rise to target molecules **1**. Having PhoN-Sf available which operates at pH 6, we envisioned that we should be able to carry out the glycerol–DHAP cascade in a single pot with all enzymes acting simultaneously.[13] Furthermore, we show that it is possible to obtain all the stereocomplementary products by using the corresponding four DHAP-dependent aldolases present in nature.

3.2 — Results and discussion

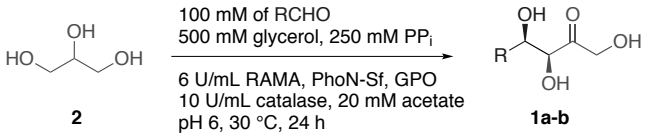
The PhoN-Sf-mediated phosphorylation of glycerol (**2**) with the phosphate donor PP_i produces glycerol-3-phosphate (**3**) in a chemoselective manner, due to the known selectivity of PhoN-Sf for primary hydroxy functions (Scheme 2).[14] The L-enantiomer is then oxidized by GPO in the presence of oxygen to produce DHAP (**4**).[10] The oxidation takes place with concomitant formation of hydrogen peroxide, which is converted into water and oxygen by a third enzyme, catalase. In the next step of the cascade, DHAP reacts with the aldehyde catalyzed by RAMA to provide the phosphorylated aldol product **5**. Under the reaction conditions, the aldol product is ultimately dephosphorylated by PhoN-Sf leading to enantio- en diasteromerically pure triol **1**. The resulting enzyme-bound phosphate may then be either transferred to water (hydrolysis) or to glycerol (transphosphorylation).[13] Hydrolysis is generally preferred over transphosphorylation so that phosphate (P_i) is released. This essentially irreversible step fortuitously shifts the thermodynamic equilibrium of the cascade to aldol product **1** once PP_i becomes exhausted.



Scheme 2 — Four-enzyme one-pot catalytic cascade.

Supposedly, PhoN-Sf-induced phosphorylation of glycerol gives rise to a racemic mixture of D- and L-glycerol-3-phosphate (**3**). Since GPO would oxidize only the L-enantiomer,[10] the D-isomer might accumulate. However, the acid phosphatase possesses strong trans-phosphorylation activity[13] and most likely PhoN-Sf will transfer the phosphate group from D-glycerol-3-phosphate to glycerol resulting in racemic glycerol-3-phosphate so that accumulation of the D-isomer will not occur.

Table 1 – Optimization of the four-enzyme cascade.



Entry	R	Product	PhoN-Sf (U/mL)	GPO (U/mL)	Conv. (%) [a]	Reaction time (h)
1	Et	**1a**	1.5	2	42	24
2	Et	**1a**	1.5	5	50	24
3	Et	**1a**	1.5	10	58	24
4	Et	**1a**	1.5	50	84	24
5	Et	**1a**	3.0	50	87	8
6	Et	**1a**	3.0	50	95 [b]	8
7	Et	**1a**	3.0	50	100 [b,c]	8
8	*i*-Pr	**1b**	3.0	50	48	8

a) Conversions were based on the aldehyde and determined using HPLC on aliquots of the reaction mixture. b) Reaction was performed at 20 °C. c) Reaction was performed in a closed vessel.

The first cascade was conducted at 0.5 mL scale (Table 1, entry 1) with propanal as acceptor aldehyde.[13] A solution of 500 mM glycerol, 250 mM PP_i and 100 mM propanal at pH 6 was incubated with the four enzymes at 30 °C, resulting in 42% conversion after 24 h. By HPLC it was possible to monitor at selected time intervals the ratio of PP_i to P_i, the formation of phosporylated product, dephosphorylated end product, and the amount of aldehyde converted (Figure 1). This allowed us to study in detail the effect of changing the amount of activity of GPO from 2 U/ml to 50 U/ml in the cascade (entries 2-4). Optimal conversion was obtained with 50 U/mL GPO (entry 4).

Encouraged by these results, the effect of changing the activity of the acid phosphatase was also investigated. When the activity of PhoN-Sf in the cascade was doubled, within 8 h a conversion of 87% was reached (entry 5). Changing the activity of GPO or PhoN-Sf had no effect on the diastereoselectivity, a d.r. of 14:1 ((3*S*,4*R*) vs. (3*S*,4*S*)) was observed in all cases. Lowering the temperature to 20 °C resulted in 95% conversion and increased diastereoselectivity (entry 6, d.r. >30:1). Performing the reaction in a closed vessel for 8 h without taking samples led to a conversion of 100% (entry 7). These effects are probably due to evaporation of the rather volatile propanal.

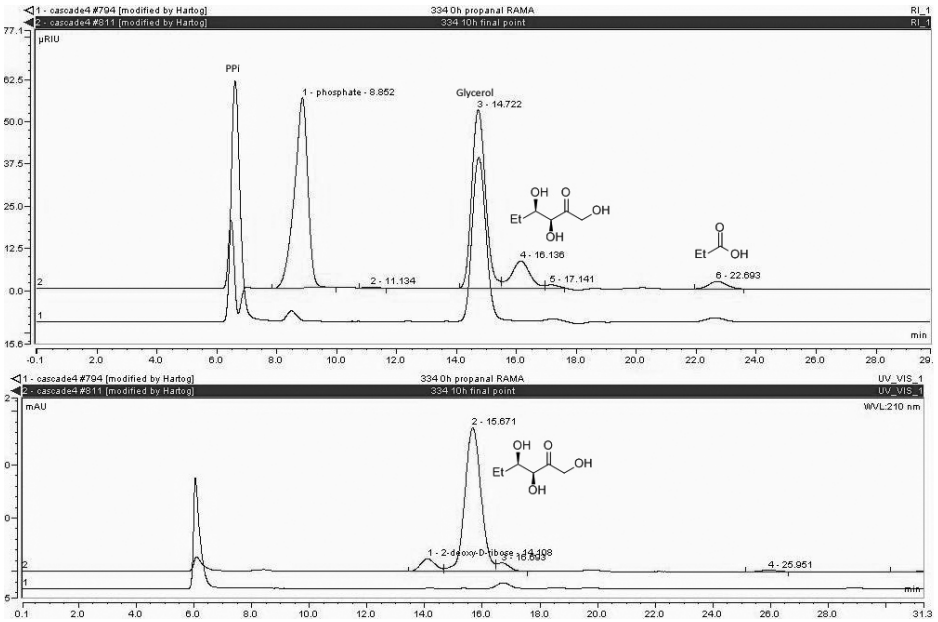


Figure 1 — HPLC chromatograms of propanal cascade at 0 and 10 h, analyzed with RI (above) and UV (below).

A branched aldehyde was also successfully used as a substrate, albeit that the yield was lower compared to propanal (entry 8), which is in line with the aldehyde specificity of RAMA.[15] The cascade reaction using propanal was also performed on 10 mL scale in the presence of 3 U/mL of PhoN-Sf and 50 U/mL of GPO resulting in 87% conversion and an isolated yield of 97 mg (65%).

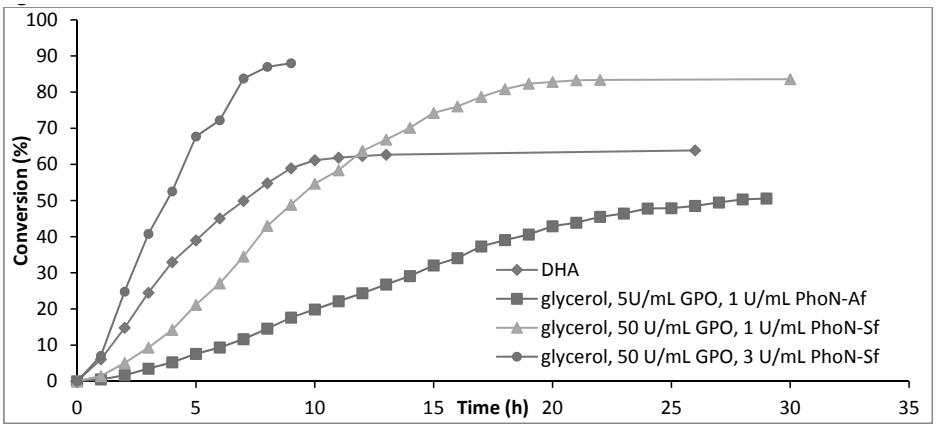


Figure 2 — Time course of the cascade reaction starting from DHA or glycerol. The reactions were carried out with 1.5 U/mL PhoN-Sf, 6 U/mL RAMA, 250 mM PP_i, 100 mM propanal and 0.5 M glycerol or 0.5 M DHA.

Figure 2 shows the time course of the reaction and a comparison between the currently described cascade and the previous cascade starting from DHA instead of glycerol.[13] It is obvious that the glycerol cascade is superior as compared to the DHA one. This may relate to the value of K_m for glycerol which is 0.7 M, while K_m for DHA is 3.6 M.[13] The difference in K_m thus may represent an important factor for the cascade reaction resulting in higher conversions compared to the DHA route. Furthermore, glycerol is known to stabilize most enzymes. The glycerol cascade has the additional advantage that, since DHA is prone to oxidation and side reactions,[8c] significantly fewer by-products are formed.

The effect of the glycerol concentration on the rate of product formation was also investigated (Figure 3). A concentration of 0.5 M glycerol was optimal. At 1 M glycerol both the rate and the yields were lower. The lower activity at low glycerol concentrations probably relates to the K_m value of PhoN-Sf for glycerol of about 0.7 M.

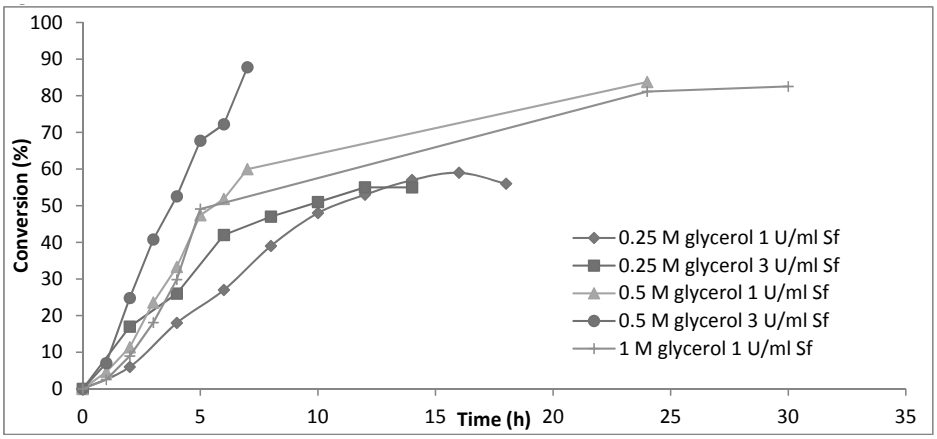
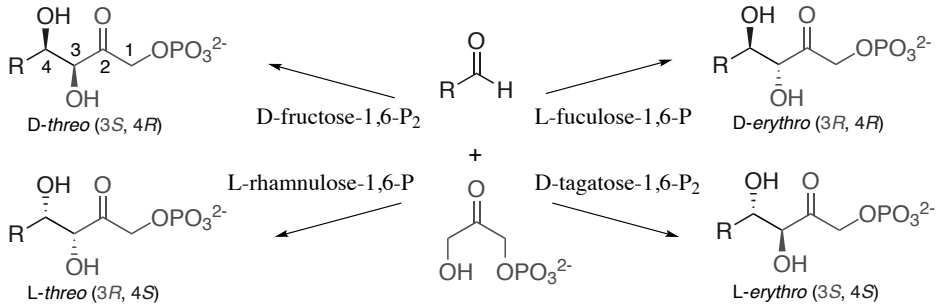


Figure 3 — Effect of glycerol concentration on the yield. 250 mM PPi, 20 mM acetate pH 6, 100 mM propanal, 6 U/ml RAMA, 50 U/ml GPO, 10 U/ml catalase, 1 or 3 U/ml of PhoN-Sf.

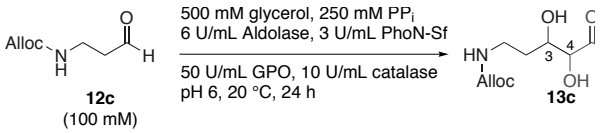
To probe the broader applicability of the glycerol cascade, the reaction was also carried out with three other aldolases in combination with propanal (Table 2). Nature provided four stereocomplementary DHAP-dependent aldolases (Scheme 3), each with their own substrate specificity. These aldolases accept a broad scope of aldehyde substrates making the one-pot four-enzyme cascade a valuable and versatile method towards a plethora of stereochemically different aldol products.[15,16]



Scheme 3 — The four different DHAP dependent aldolases

Rhamnulose-1-phosphate aldolase (RhuA, entry 2) and fuculose-1-phosphate aldolase (FucA, entry 4) gave similar conversions as compared to RAMA (entry 1), although the d.r. in both cases was significantly lower. In all cases the enantioselectivity with respect to the C3 center was complete, while C4 gave a 2:1 mixture in favor of the anticipated diastereoisomer.[17]

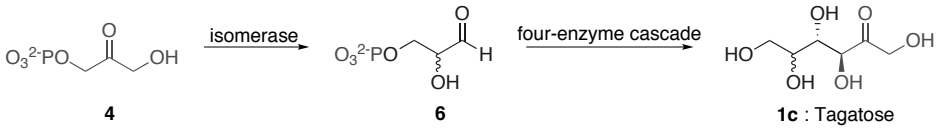
Table 2 — Four-enzyme cascade with four different aldolases and three different aldehydes.



Entry		Aldehyde	Conversion (%)[a]	Aldolase[17]	d.r.	Configuration[17]
1	**1a**	propanal	87	RAMA	14:1	(3*S*,4*R*):(3*S*,4*S*)
2		R = Et	72	RhuA	2:1	(3*R*,4*S*):(3*R*,4*R*)
3			15	TagA	8:1	(3*S*,4*S*):(3*S*,4*R*)
4			77	FucA	2:1	(3*R*,4*R*):(3*R*,4*S*)
5	**1b**	isobutanal	61	RAMA	> 20:1	(3*S*,4*R*):(3*S*,4*S*)
6		R = *i*Pr	25	RhuA	13:1	(3*R*,4*S*):(3*R*,4*R*)
7			-	TagA	-	
8			16	FucA	> 20:1	(3*R*,4*R*):(3*R*,4*S*)

a) Conversions were based on the aldehyde and determined with HPLC on aliquots of the reaction mixture. The diastereoisomeric ratio was determined based upon the peak areas in the HPLC profile and the assignments of the stereochemistry on the reported specificity of these aldolases in literature.[17]

Tagatose-1,6-diphosphate aldolase (TagA) showed higher diastereoselectivity (entry 3), but significantly lower conversions, due to the lower activity of the aldolase (entry 3). The presence of an isomerase could explain the low activity of the aldolase. The isomerase converted DHAP (**4**) into glyceraldehyde-3-phosphate (**6**) and the latter underwent an aldol reaction with DHAP as well resulting in product **1c** which was confirmed with HPLC by comparison with reference compound tagatose (**1c**, Scheme 4). This caused a competition between propanal and glyceraldehyde-3-phosphate in favor of the latter one, which is the natural substrate for the enzyme.[18]



Scheme 4 — Reaction with an isomerase present in cascade reaction.

3.3 — Conclusion

A convenient procedure was developed for the production of carbohydrates in a highly enantio- and diastereoselective manner using an efficient one-pot four-enzyme-catalytic cascade. This cascade starts from inexpensive reagents and proceeds without the need for protecting groups. The primary step is chemoenzymatic phosphorylation of glycerol and subsequent oxidation to the energy rich phosphate ester DHAP at the expense of PP_i. In the same pot, the formed DHAP is coupled to a variety of aldehydes by DHAP-dependent aldolases allowing the synthesis of all four possible stereoisomers. Dephosphorylation to the final aldol product occurs once PP_i is fully consumed.

3.4 — Acknowledgements

Louis Hartog, Dr. Teunie van Herk, Prof. Dr. Ron Wever and especially Lara Babich (J. H. van 't Hoff Institute for Molecular Sciences, University of Amsterdam) are gratefully acknowledged for the fruitful cooperation and contributions to the optimization of the four-enzyme cascade reaction. Pierpaolo Falcicchio (Laboratory of Microbiology, Wageningen University) is greatly acknowledged for his contribution to the expression of the complementary aldolases.

3.5 — Experimental

3.5.1 — General information

The general information is described in Section 2.5.1

3.5.2 — Enzymes

RAMA, catalase from bovine liver and GPO from *Aerococcus viridans* were purchased from Sigma Aldrich. GPO from *Pediococcus* and other microorganisms were also commercially available via Toyobo.

Recombinant *Shigella flexneri* acid phosphatase (PhoN-Sf) was expressed in *E. coli* and purified as previously described.[19] Recombinant rhamnulose-1-phosphate aldolase from the thermophilic organism *T. maritima* (pBAD/gIII plasmid provided by DSM) was expressed in *E. coli* strain TOP10 by inducing with 0.02% L-arabinose overnight at 28 °C. The resulting culture broth was centrifuged at 6000 rpm for 15 min at 4 °C and the cells were resuspended in 100 mM Bis-Tris pH 6.5, 0.1 mM $ZnCl_2$. Cell disruption was performed by sonication followed by centrifugation at 14000 rpm for 30 min at 4 °C. The thermophilic enzyme RhuA was isolated from the cell free extract by heat-shock in a 70 °C water bath for 20 min, followed by centrifugation at 14000 rpm for 30 min.

Tagatose-1,6-diphosphate aldolase from *E. coli* (pBAD plasmid supplied by DSM) was expressed in *E. coli* TOP10 cells and induced by 0.02% L-arabinose in the presence of 0.3 mM $ZnCl_2$. Cells were harvested by centrifugation at 6000 rpm for 15 min and resuspended in 20 mM Tris/HCl pH 7.5. Cell disruption was performed by sonication. From the cell-free extract, TagA was recovered by 40% ammonium sulfate precipitation and centrifugation at 13000 rpm for 30 min. The supernatant containing TagA was dialyzed overnight against 20 mM Tris/HCl pH 7.5 and loaded onto HiTrap DEAE FF 1 mL FPLC column for further purification. TagA was eluted at 200 mM NaCl and dialyzed against 20 mM Tris/acetate pH 7.5, 0.1 mM $ZnCl_2$.

Recombinant fuculose-1-phosphate (FucA) aldolase from the thermophilic bacterium *T. ethanolicus* (pBAD/gIII plasmid provided by DSM) was expressed in *E. coli* strain TOP10 by inducing with 0.02% L-arabinose overnight at 37 °C. The resulting culture was centrifuged at 7000 rpm for 15 min at 4 °C and the cells were resuspended in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1 mM $ZnCl_2$. Cell disruption was performed by French Press followed by centrifugation at 17000 rpm for 30 min at 4 °C. The thermophilic enzyme was isolated from the cell free extract by heat-shock in a 70 °C water bath for 20 min, followed by centrifugation at 17000 rpm for 30 min at 4 °C. The supernatant containing FucA was loaded onto a Q-Sepharose FF FPLC column (40 mL) and eluted with a linear gradient of NaCl (0-1 M NaCl in 20 column volumes). The purified fractions were collected and desalted using 50 mM Tris-HCl pH 7.5 and 150 mM NaCl buffer.

3.5.3 — Enzyme activity assays

Aldolase activity was assayed by measuring the decrease of DHAP concentration in a reaction in which the aldolase performed the aldol coupling in the presence of DHAP (50 mM), *rac*-glyceraldehyde (100 mM), 0.1 mM $ZnCl_2$, 50 mM acetate pH 6, 30 °C. The decrease in DHAP concentration was assayed every 5 min with a coupled-enzyme system in the presence of NADH-consuming glycerol-3-phosphate dehydrogenase by measuring the NADH concentration by UV spectroscopy. Samples of the aldolase reaction mixture were diluted and incubated in 100 mM Tris/acetate pH 7.5, 0.16 mM NADH, and 1 U/

mL glycerophosphate dehydrogenase. The absorption was analyzed at 340 nm at 20 °C (molar absorption coefficient 6.22 mM^{-1} cm^{-1}).

The K_m value of PhoN-Sf for glycerol was determined with a coupled enzyme system as described by Bergmeyer.[20] L-glycerol-3-phosphate resulting from the phosphatase reaction was assayed by coupling with glycerol-3-phosphate dehydrogenase in the presence of hydrazine and NAD^+ at pH 9.5 and 25 °C. The reaction was spectrophotometrically monitored at 340 nm.

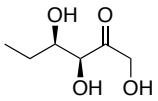
3.5.4 — Analytical methods and product characterization

The time course of the cascade reaction and the conversions were determined by HPLC using an Alltech OA 1000 organic acid column (0.65 × 30 cm). 20 µL of the reaction mixture was diluted 10-fold before injection on the HPLC. Isocratic runs were performed using 4.5 mM H_2SO_4, with a flow rate of 0.4 mL min^{-1}. The effluent was analyzed at 215 and 254 nm by refractive index detector.

3.5.5 — General procedure for the preparative scale one-pot four-enzyme cascade

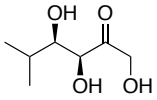
To a solution of 500 mM glycerol, 250 mM PP_i (3:2 Na_2PP_i/Na_4PP_i),[21] 20 mM sodium acetate (pH 6), and 100 mM aldehyde in water, 10 U/mL catalase from bovine liver, 5 U/mL RAMA, and 50 U/mL GPO were added. The reactions were started by adding 3 U/mL PhoN-Sf and incubated at 20 °C under mild shaking until completion. Dephosphorylation to the end product was completed after 24 h and 4 g of silica gel was added. The reaction slurry was concentrated under reduced pressure, and the silica gel was poured on top of a silica gel column and eluted with EtOAc/MeOH 19:1. The pure fractions were collected and concentrated under reduced pressure to give product **1** as a light-yellow oil.

3.5.6 — 5,6-Dideoxy-D-*threo*-2-hexulose (1a)



This compound was synthesized via method 3.5.5 from propnanal (58 mg, 1.0 mmol) and resulted in ***1a*** *(102 mg, 0.69 mmol) in 69% isolated yield.* ^{1}H NMR (CD_3OD, 300 MHz) δ 4.53 (d, *J* = 19.2 Hz, 1H), 4.42 (d, *J* = 19.2 Hz, 1H), 4.14 (d, *J* = 2.4 Hz, 1H), 3.78 (dt, *J* = 2.4, 6.0 Hz, 1H), 1.65-1.50 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (CD_3OD, 75 MHz) δ 212.0, 77.1, 73.2, 66.0, 25.3, 8.7; $[\alpha]_D^{20}$ = –17.7 (*c* = 0.22, MeOH); υ_{max} (film) cm^{-1} 3397, 1723; LRMS (FAB) *m/z* calcd for $C_6H_{12}O_4$: 148.07, found 149.08 $[M+H]^+$, 171.09 $[M+Na]^+$. The analytical data were in accordance with previously reported results.[15]

3.5.7 — 5,6-Dideoxy-5-methyl-D-*threo*-2-hexulose (1b)



This compound was synthesized via method 3.5.5 from isobutanal (72 mg, 1.0 mmol) and resulted in ***1b*** *(64 mg, 0.40 mmol) in 40% isolated yield.* ^{1}H NMR (CD_3OD, 300 MHz) δ 4.53 (d, *J* = 19.2 Hz, 1H), 4.44 (d, *J* = 19.2 Hz, 1H), 4.31 (d, *J* = 1.8 Hz, 1H), 3.45 (dd, *J* = 2.0, 9.1 Hz, 1H), 1.90 (ddd, *J* = 2.3, 6.7, 13.4 Hz, 1H), 1.01 (d, *J* = 6.7 Hz, 3H), 0.93 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (CD_3OD, 75 MHz) δ 212.6, 77.4, 75.8, 65.9, 29.8, 17.8, 17.6; $[\alpha]_D^{20}$ = –12.2 (*c* = 0.16, MeOH); υ_{max} (film) cm^{-1} 3396, 1723; LRMS (FAB) *m/z* calcd for $C_7H_{14}O_4$: 162.08, found 163.10 $[M+H]^+$, 185.12 $[M+Na]^+$.

3.6 — References

1. a) Bruggink, A.; Schoevaart, R. and Kieboom, T. *Org. Process Res. Dev.* **2003**, *7*, 622–640; b) *Multi-step enzyme catalysis: Biotransformations and chemoenzymatic synthesis* (Ed. García-Junceda, E.), Wiley-VCH: Weinheim, **2008**.
2. Sheldon, R. A. *Chem. Commun*. **2008**, 3352–3365.
3. Dean, S. M.; Greenberg, W. A. and Wong, C.-H. *Adv. Synth. Catal*. **2007**, *349*, 1308–1320.
4. a) Ichikawa, Y.; Igarashi, Y.; Ichikawa, M. and Suhara, Y. *J. Am. Chem. Soc*. **1998**, *120*, 3007–3018; b) *Iminosugars: from synthesis to therapeutic applications* (Eds. Compain, P.; Martin, O. R.), Wiley-VCH: Weinheim, **2007**; c) Asano, N. *Cell. Mol. Life Sci*. **2009**, *66*, 1479–1492.
5. a) Movassaghi, M. and Jacobsen, E. N. *Science* **2002**, *298*, 1904–1905; b) Ko, S. Y.; Lee, A. W. M.; Masamune, S.; Reed, L. A.; Sharpless, K. B. and Walker, F. J. *Science* **1983**, *220*, 949–951.
6. Northrup, A. B. and MacMillan, D. W. C. *Science* **2004**, *305*, 1752–1755.
7. a) Gijsen, H. J. M. and Wong, C.-H. *J. Am. Chem. Soc*. **1995**, *117*, 7585–7591; b) Koeller, K. M. and Wong, C.-H. *Chem. Rev*. **2000**, *100*, 4465–4494; c) Clapés, P.; Fessner, W.-D.; Sprenger, G. A. and Samland, A. K. *Current Opin. Chem. Biol*. **2010**, *14*, 154–167.
8. a) Bolt, A.; Berry, A. and Nelson, A. *Arch. Biochem. Biophys*. **2008**, *474*, 318–330; b) Silvestri, M. G.; Desantis, G.; Mitchell, M. and Wong, C.-H. *Top. Stereochem*. **2003**, *23*, 267-342; c) Schumperli, M.; Pellaux, R. and Panke, S. *Appl. Microbiol. Biotechnol*. **2007**, *75*, 33–45.
9. Crans, D. C. and Whitesides, G. M. *J. Am. Chem. Soc*. **1985**, *107*, 7019–7026.
10. Fessner, W.-D. and Sinerius, G. *Angew. Chem. Int. Ed*. **1994**, *33*, 209–212.
11. a) Eyrisch, O.; Sinerius, G. and Fessner, W.-D. *Carbohydr. Res.* **1993**, *238*, 287-306; b) Wong, C.-H. and Whitesides, G. M. *J. Org. Chem.* **1983**, *48*, 3199–3205.
12. Schoevaart, R.; van Rantwijk, F. and Sheldon, R. A. *J. Org. Chem.* **2000**, *65*, 6940–6943.
13. van Herk, T.; Hartog, A. F.; Schoemaker, H. E. and Wever, R. *J. Org. Chem.* **2006**, *71*, 6244–6247.
14. van Herk, T.; Hartog, A. F.; van der Burg, A. M. and Wever, R. *Adv. Synth. Catal*. **2005**, *347*, 1155–1162.
15. Bednarski, M. D.; Simon, E. S.; Bischofberger, N.; Fessner, W.-D.; Kim, M. J.; Lees, W.; Saito, T.; Waldmann, H. and Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627–635.
16. Clapés, P. and Carrabou, X. *Adv. Synth. Catal.* **2011**, *353*, 2263–2283.
17. Faber, K. *Biotransformations in Organic Chemistry,* 5th ed.; Springer-Verlag: Berlin, 2004; pp 277–284.

18. Hall, D. R.; Bond, C. S.; Leonard, G. A.; Watt, I.; Berry, A. and Hunter, W. N. *J. Biol. Chem*. **2002**, *277*, 22018–22024.

19. Tanaka, N.; van Herk, T. and Wever, R. *Org. Biomol. Chem.* **2003**, *1*, 2833-2839.

20. Bergmeyer, H. U.; Gawehn, K. M.; Grassl, M. *Glucose-6-phosphate dehydrogenase from yeast, in Methods of Enzymatic Analysis*, (Bergmeijer, H. U. ed.), Verlag Chemie, Vol. 1, 1986, pp. 458–459.

21. PP_i was added as a mixture of 3.5 equiv. (350 mg) Na_2PP_i and 2 equiv. (241 mg) Na_4PP_i resulting in a buffered solution of pH 6.

Chapter 4

Synthesis of iminosugars using a one-pot enzymatic cascade

This work was published in: Lieke J. C. van Hemert,[1] Lara Babich,[1] Aleksandra Bury, Aloysius F. Hartog, Pierpaolo Falcicchio, John van der Oost, Teunie van Herk, Ron Wever and Floris P. J. T. Rutjes, Synthesis of non-natural carbohydrates from glycerol and aldehydes in a one-pot four-enzyme cascade reaction, Green Chem. **2011***, 13, 2895–2900 and Lieke J. C. van Hemert, Alexander J. J. Groenen, Luuk J. A. Wilders, Lara Babich, Ron Wever, Sander Nabuurs, and Floris P. J. T. Rutjes, Expanding the scope of one-pot four-enzyme cascade aldol reactions, manuscript in preparation.*

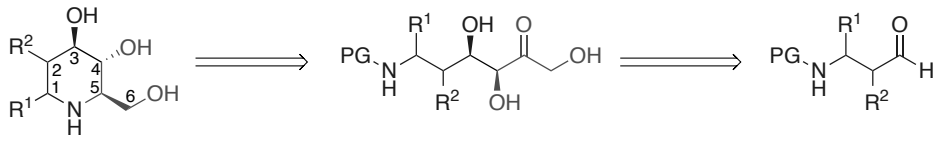
1 Both authors equally contributed to this work.

"In all things of nature there is something of the marvelous."

– Aristoteles

Abstract

Having developed a one-pot four-enzyme cascade for the synthesis of carbohydrate fragments, we envisioned that a two-step synthesis of nitrogen heterocycles would be feasible. This strategy was validated with the chemoenzymatic synthesis of two well-investigated iminosugars. Thus, after optimization D-fagomine was obtained in an overall yield of 69%. Furthermore, deoxynojirimycin (DNJ) was obtained in a similar strategy in 23% yield using a minimum of protecting groups. Subsequent derivatization of DNJ resulted in the synthesis of C-3 (di)fluorinated iminosugars. The four-enzyme cascade was also applied using the complementary aldolases RhuA, TagA and FucA to synthesize precursor epimers of D-fagomine.



4.1 — Introduction

Perhaps the first known therapeutic application of iminosugars dates from the 17th century, when Haarlem Oil was recommended for the treatment of diabetes.[1] Haarlem Oil partly consists of extracts of the leaves of *Morus alba*, a rich source of iminosugars. It was, however, not until the 1960's that scientific interest arose in the synthesis of these sugar derivatives. The first iminosugars that were isolated were derived from bacteria, such as 1-deoxynojirimycin (DNJ (**1**), Figure 1). This iminosugar was first synthesized by Paulsen in 1966,[2] ten years before it was also isolated from the plant *Mori cortex*. As mentioned in Section 1.6, DNJ acts as a sugar mimic and inhibits glycosidases[3] and glycosyltransferases[4] by mimicking the charge and hydrolysis transition state of the enzymatic reaction. The efficacy of DNJ has also been demonstrated in multiple clinical studies.[5] Glycosidases are involved in many biological and pathological processes, making them important targets for the treatment of various diseases, such as type II diabetes,[6] HIV,[7] Gaucher's disease[8] and cancer.[9] *N*-Alkylated DNJ derivatives also showed potent inhibiting properties, two of which have been marketed as miglustat (*N*-butyl, **3**, Figure 1)[10] and miglitol (*N*-C_2H_4OH, **4**).[11]

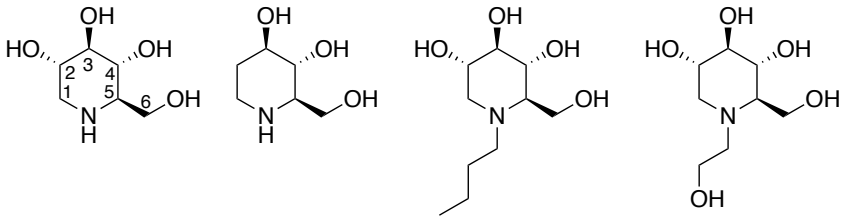
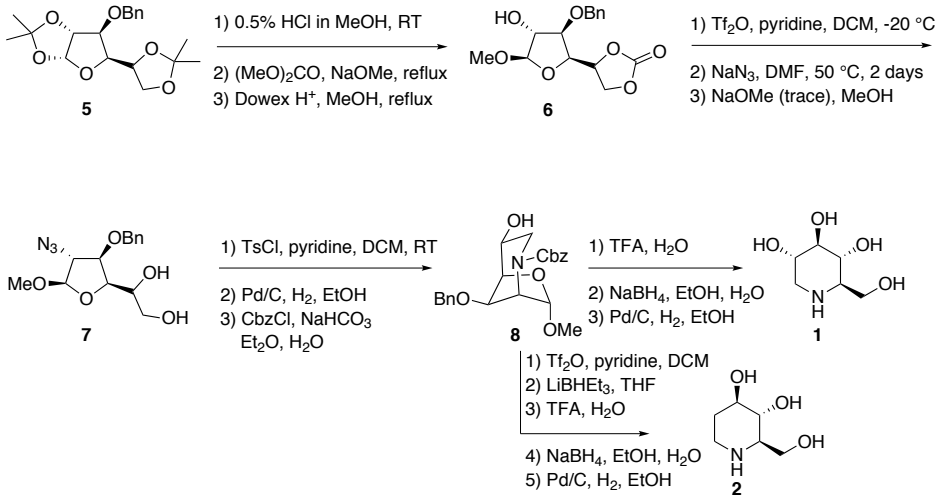


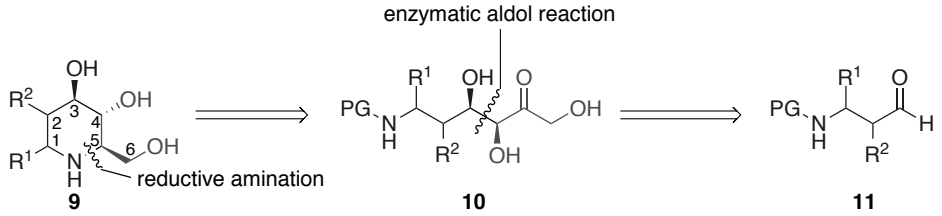
Figure 1 — Examples of iminosugars

The first iminosugar derived from a plant was D-fagomine (**2**), which was isolated from buckwheat seeds (*Fagopyrum esculentum* Moench) and characterized for the first time in 1974.[12] D-Fagomine has a potent antihyperglycemic effect in streptozocin-induced diabetic mice and is also an inhibitor of isomaltase and α- and β-galactosidases.[13,14] In 1985, a first synthesis of D-fagomine was reported providing the iminosugar from glucose in a rather lengthy procedure of 14 steps in an overall yield of 20% (Scheme 1).[15] The piperidine ring was constructed via intramolecular nucleophilic attack by nitrogen onto the primary tosylate of **7** to produce bicyclic intermediate **8**, subsequent acetal deprotection, reduction and hydrogenation resulted in D-fagomine (**2**). This route also provided access to DNJ (**1**), which was isolated in 57% overall yield in 12 steps.



Scheme 1 — Synthesis of iminosugars 1 and 2 from protected D-glucose derivative 5.

Over the years, several new pathways have been developed for the synthesis of iminosugars.[16] A first example of a chemoenzymatic approach involving aldolases and dihydroxyacetone phosphate (DHAP) was published by Effenberger *et al*. in 1988.[17] We envisioned that a straightforward route to these iminosugars would also be feasible using the one-pot four-enzyme cascade strategy described in Chapter 3. Thus, the aim of this research was to chemoenzymatically synthesize various iminosugars **9** with different substitution patterns. The heterocyclic systems **9** should be obtained via intramolecular reductive amination of aldol products **10**, which should be directly accessible through the enzymatic cascade described in the previous chapter (Scheme 2). Different stereoisomers might be obtained by using stereocomplementary aldolases, while the introduction of substituents on the C-1 and C-2 positions should rely on the presence of substituents (e.g. hydroxyl, fluoride) in the starting aldehydes **11**.

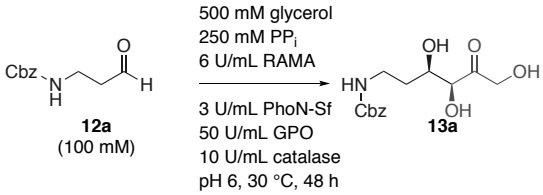


Scheme 2 — Retrosynthesis of iminosugars 9.

4.2 — Results and discussion

4.2.1 — Synthesis of D-fagomine

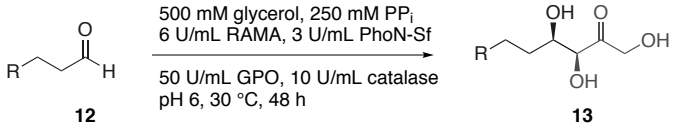
D-Fagomine was synthesized starting with commercially available Cbz-protected aminopropanal **12a** (Scheme 3). The first attempt under the standard enzymatic cascade conditions resulted in a 21% isolated yield of aldol product **13a** in a 6:1 (*syn*:*anti*) ratio of diastereoisomers.



Scheme 3 — First attempt to synthesize aldol product 13a.

Due to the low solubility of aldehyde **12a**, the cascade was also tested in the presence of cosolvents using propanal (**12b**) as the acceptor aldehyde (Table 1). In all cases, the addition of cosolvents (10%) lowered the reaction rate. Both MeCN and DMSO gave reasonable results (entries 3 and 6, 38 and 39%, respectively) with conversions in the range

Table 1 — Effect of cosolvents in the enzymatic cascade



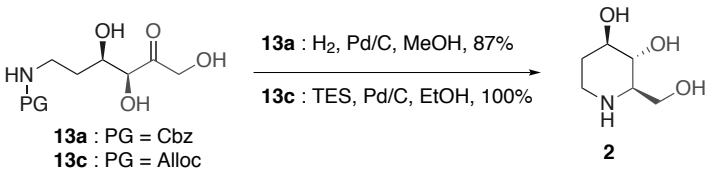
Entry	R	Aldehyde	Cosolvent	Conversion (%)[a]	d.r.[b]
1	CbzNH	**12a**	-	21	6:1
2	H	**12b**	-	51	14:1
3	H	**12b**	MeCN	38	14:1
4	H	**12b**	1,4-dioxane	18	14:1
5	H	**12b**	DMF	28	14:1
6	H	**12b**	DMSO	39	14:1
7	CbzNH	**12a**	DMSO	32	20:1
8	AllocNH	**12c**	-	75	5:1
9	AllocNH	**12c**	-	87[c]	5:1
10	AllocNH	**12c**	-	50[d]	30:1
11	AllocNH	**12c**	-	87[e] (69)[f]	11:1

a) Conversions were based on the aldehyde and determined using HPLC on aliquots of the reaction mixture; b) Ratio *syn*:*anti*; c) After 6 h an extra equivalent of PP_i was added; d) The incubation temperature was 10 °C; e) The incubation temperature was 20 °C; f) isolated yield.

of the control experiment (entry 2, 51%). The addition of DMF to the reaction mixture resulted in reasonable conversions (entry 5, 28%), while the addition of 1,4-dioxane gave a significantly lower conversion (entry 4, 18%) and more side products as observed by HLPC analysis.

In a second attempt to synthesize D-fagomine, the experiment was repeated in the presence of 10% DMSO resulting in a significant increase in yield from 21 to 32% (entry 7). Besides, a drastic improvement in diastereomeric ratio (d.r.) was observed from 6:1 to 20:1. This could be due to an increased difference in the rate of formation of both diastereoisomers caused by solvent-induced active site perturbation of the aldolase. To further optimize the solubility of the aldehyde, the Cbz-protecting group was replaced by an Alloc-group (**12c**). Gratifyingly, this more water soluble aldehyde could be subjected to the cascade conditions without using cosolvents providing a 75% conversion into the desired aldol product **13c** in a d.r. of 5:1 (entry 8). Addition of extra 250 mM of PP_i to the cascade reaction after 6 h increased the conversion to 87% (entry 9). Since it was noticed that the temperature of incubation had an effect on the diastereoselectivity of the aldolase (Section 3.2), we also investigated the effect of temperature on the cascade with the Alloc-protected aldehyde **12c**. There was a marked increase in the isomeric ratio from 5:1 to 30:1 when the temperature was lowered from 30 to 10 °C, while the conversion dropped to approximately 50% (entry 10). Optimal results in terms of isolated yield were obtained at 20 °C giving product **13b** in 69% yield (entry 11). The subsequent formation of D-fagomine (**2**) occurred via straightforward reductive amination of aldol product **13a**, which provided the target molecule as a single diastereoisomer in 87% yield (Scheme 4). Our first attempt to convert aldol product **13c** proceeded with formic acid and a catalytic amount of $Pd(PPh_3)_4$,[18] however, degradation of the product was observed. Changing to milder reaction conditions replacing formic acid by ammonium formate also did not result in the desired product. We then resided to a publication of the group of McMurry who reported a Pd/C-induced catalytic transfer hydrogenation with triethylsilane (TES) for Alloc-deprotection,[19] which allowed TES to behave as a hydrogenation reagent through *in situ* generation of H_2. With this strategy we envisioned that both deprotection and ring closure via reductive amination would proceed in one step. This led to the diastereoselective formation of D-fagomine (**2**) in quantitative yield, thereby realizing a two-step synthesis of the natural product in 69% overall yield. To prove the concept of *in situ* H_2 formation, the reaction was also tested with Pd/C and H_2, however, no product was observed. Probably the Alloc-deprotection proceeded via initial palladium-mediated formation of the π-allyl palladium intermediate, capturing of this complex with TES as a hydride donor and subsequent reductive amination via *in situ* H_2 generation. The yields we obtained are in the same range as previously reported by the

Clapés group for a two-step approach using DHA and D-fructose-6-phosphate aldolase (FSA, Section 1.6).[20]



Scheme 4 — Formation of D-fagomine (2).

Epimers of D-fagomine precursors were obtained by using aldehyde **12c** in the enzymatic cascade with the complementary aldolases (Figure 2, Table 2). Using instead of RAMA the RhuA aldolase derived from the thermophilic organism *Thermatoga maritima*, gave a moderate conversion (45%), while the isomeric ration dropped to 2:1 (entry 2). Decreasing the temperature to 10 °C considerably lowered the conversion (25%), but the isomeric ratio remained unchanged. A similar outcome was observed for FucA: the conversion was slightly higher than with RhuA, but the diastereoselectivity was worse (entry 3). The aldolase TagA gave almost no conversion and slightly better diastereoselectivity, which was also observed in case of propanal (entry 4 and Section 3.2). These results demonstrate that the synthesis of epimers of D-fagomine precursors **13c** with the complementary aldolases is feasible, but also strongly depend on the substrate specificity and selectivity of the aldolases.

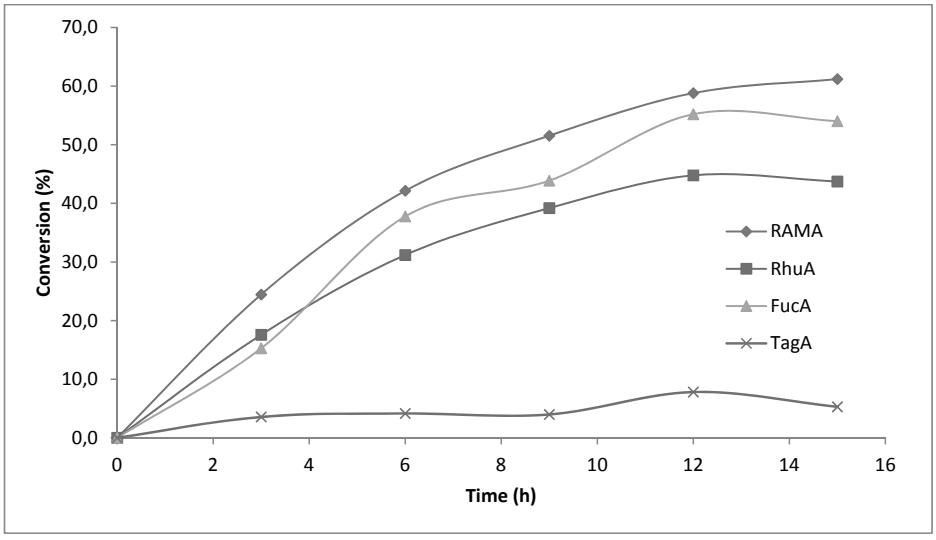
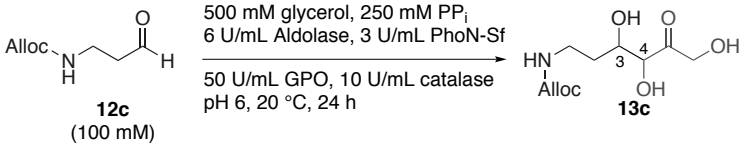


Figure 2 — Time course of the four enzyme cascade with various aldolases and *N*-Alloc-aminopropanal 12c.

Table 2 — Aldehyde 12c with stereocomplementary alsolases

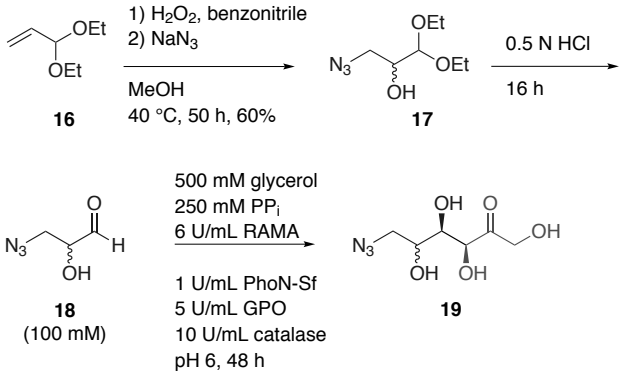


Entry	Conversion (%)[a]	Aldolase[21]	d.r.	Configuration[21]
1	87	RAMA	11:1	(3*S*,4*R*):(3*S*,4*S*)
2	45	RhuA	2:1	(3*R*,4*S*):(3*R*,4*R*)
3	54	FucA	3:2	(3*R*,4*R*):(3*R*,4*S*)
4	5.3	TagA	5:2	(3*S*,4*S*):(3*S*,4*R*)

a) Conversions were based on the aldehyde and determined using HPLC on aliquots of the reaction mixture after 24 h.

4.2.2 — Synthesis of deoxynojirimycin

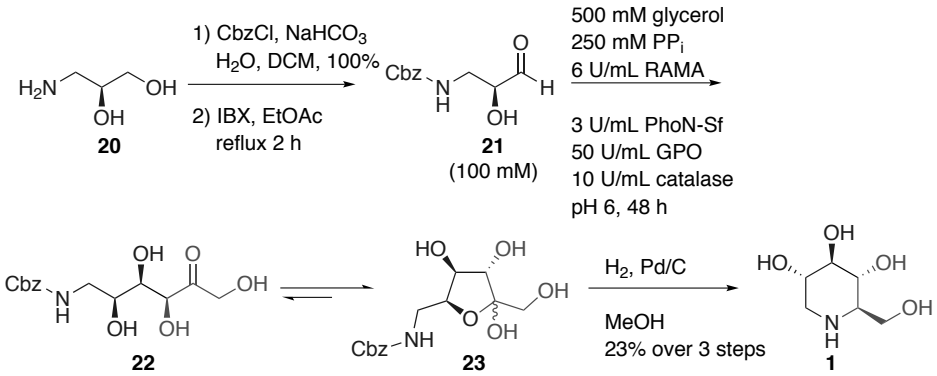
Following the same strategy DNJ (**1**) was also synthesized. To this end, a first attempt proceeded via aldol reaction of *in situ* generated DHAP and racemic azido aldehyde **18** (Scheme 5), a known substrate for the two-enzyme cascade reaction with DHA.[22] Aldehyde **18** was synthesized from acetal-protected acrolein **16** in two steps.[23] Unfortunately, subjecting this aldehyde to the conditions of the enzymatic cascade reaction resulted in a rather slow formation of aldol product **19**, never approaching conversions that were previously reached in the two-enzyme cascade with DHA.[22] Multiple attempts showed decomposition of aldehyde **18** under these conditions and concomitant inhibition of GPO.



Scheme 5 — Four-enzyme cascade synthesis of DNJ precursor 19.

Therefore another strategy was pursued which previously had been successfully applied using FSA by Clapés *et al*.[24] Enantiopure aldehyde **21** was prepared from the corresponding alcohol **20** by Cbz-protection and subsequent IBX oxidation (Scheme 6). In our hands, however, aldehyde **21** was obtained as a mixture of products, in particular since

the IBX oxidation did not proceed in a clean manner. *N*-Alloc-protection of the alcohol and subsequent oxidation did not result in the desired aldehyde as no clean conversions were observed when applying the same IBX oxidation (not shown). Nevertheless, *N*-Cbz-aldehyde **21** was incubated without purification with glycerol and the four enzymes at pH 6. To enhance the solubility of the aldehyde, 10% of DMSO was added to the mixture. The reaction was followed by HPLC and after 48 h the salts were precipitated in methanol and the crude product was purified with column purification to obtain a mixture of **22** and the ring-closed isomer **23** in a in a d.r. of 16:1 (based on HPLC peaks),[24] which was hydrogenated to obtain DNJ (**1**) in 23% yield over three steps as a single diastereoisomer.



Scheme 6 — Chemoenzymatic synthesis of DNJ (1).

4.2.3 — Synthesis of fluorinated iminosugars

The introduction of fluoride substituents on the iminosugar skeleton was also investigated. A study by Andersen *et al.* showed that C-2 (**24** and **28**), C-3 (**25**) and C-6 (**26** and **27**) monofluorination of DNJ impairs its inhibitory properties (Figure 3).[25] In addition, geminal difluorination of DNJ at C-3 (**29**) created an inhibitor with tenfold higher activity against α-glucosidase than Miglitol (**4**, Figure 1) with low cellular cytotoxicity.[26] Especially the latter results, prompted us to adapt our strategy to be able to prepare such fluorinated iminosugars.

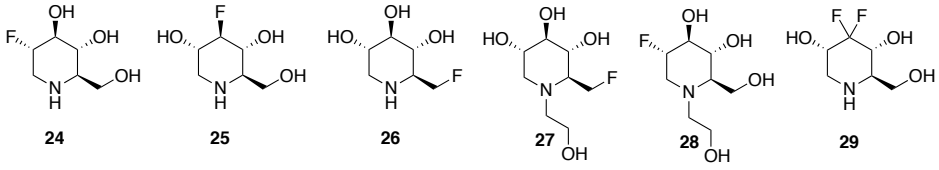
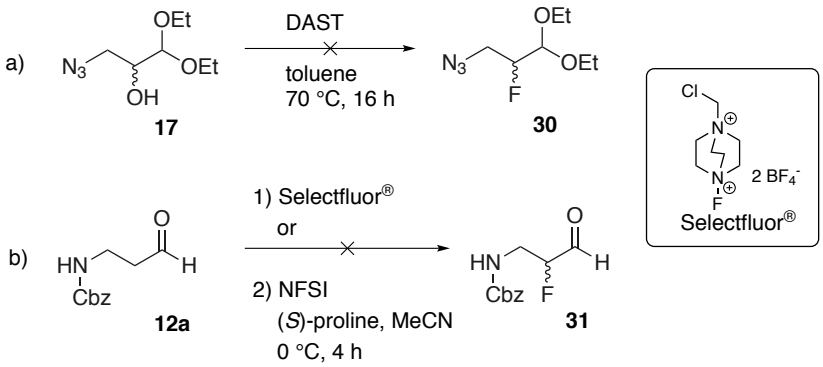


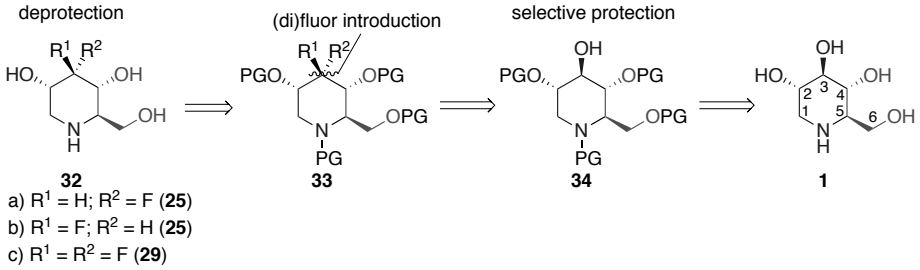
Figure 3 — Fluorinated derivatives of DNJ (1) and Miglitol (8).

The introduction of fluoride substituents was initially pursued by using fluorinated aldehydes in the cascade reaction. Hence, we aimed to synthesize such aldehydes by nucleophilic substitution of the C-2–hydroxy group of acetal protected aldehyde **17** with DAST (Scheme 7, route a).[27] Formation of fluorinated product **30** was confirmed by TLC and IR, however, isolation was unsuccessful. An alternative route was developed based on organocatalyzed α-substitution of aldehydes as described by MacMillan *et al*. using Selectfluor® or *N*-fluorobenzenesulfonamide (NFSI) as electrophilic fluor source (route b).[28] In case of Selectfluor®, the desired product was never observed and multiple products were formed. Lowering the temperature did not result in the desired product either. Therefore the reaction was repeated with NFSI, which is a milder reagent than Selectfluor®,[29] but again failed to produce the desired product **31**.



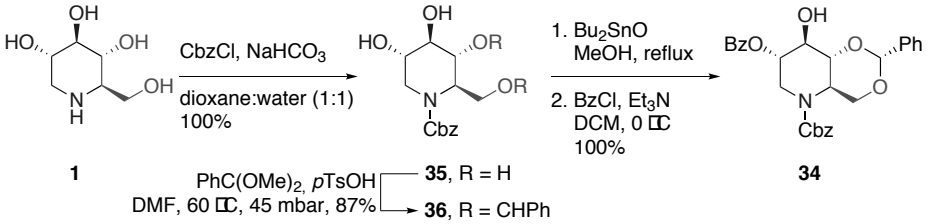
Scheme 7 — Attempted synthesis of fluoro aldehydes 30 and 31.

Since this approach was unsuccessful, we decided to follow another strategy and introduce the fluoride atoms after iminosugar formation via nucleophilic substitution of the hydroxy group on the C-3 position of the DNJ ring (Scheme 8). Despite the fact that such modifications have been claimed in patent literature,[30] the relevance of the resulting products inspired us to establish reliable procedures to access these compounds and optimize the required pathway. The fluorinated iminosugars **32** should thus be obtained via selective fluorination on C-3 and subsequent deprotection of **33**. To introduce fluorides on C-3, the other hydroxy groups of DNJ (**1**) had to be orthogonally protected, as well as the endocyclic nitrogen atom.



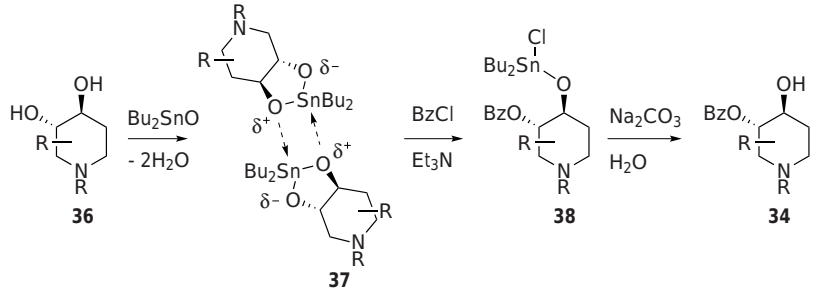
Scheme 8 – Retrosynthesis of fluoride introduction on C-3 of DNJ.

The first step involved Cbz-protection of the nitrogen atom using CbzCl under Schotten-Bauman conditions (Scheme 9). The C-4 and C-6 alcohols were subsequently protected via acetal formation with benzaldehyde dimethyl acetal under acidic conditions, proceeding in excellent overall yield.



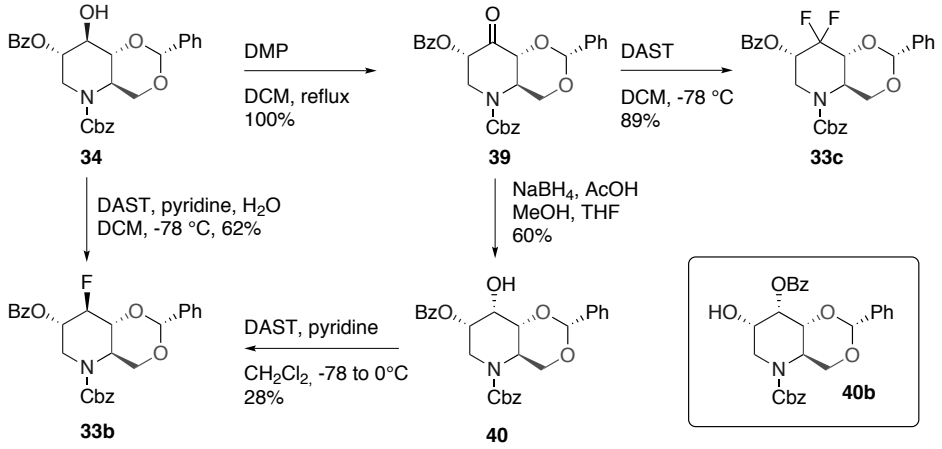
Scheme 9 – Synthesis of protected DNJ 34.

Selective C-2 hydroxy protection was accomplished by treating diol **36** with dibutyltin oxide in refluxing methanol, affording the corresponding cyclic dibutyltin ether as a dimer (Scheme 10). The tin complex exists in a trigonal bipyramidal conformation, in which both C-2 oxygens occupy an apical position and are not involved in the Sn_2O_2 parallelogram. While the C-3 oxygens were deactivated by electron donation to tin, the stannylene dimer complex enhanced the nucleophilicity of the apically bound C-2 oxygens by electron channeling from tin resulting in activation of these atoms.[31] Treating this complex with benzoyl chloride and triethylamine afforded solely C-2 benzoyl-protected alcohol **34** in quantitative yield.[32]



Scheme 10 — Introduction of the benzoyl group on C-2.

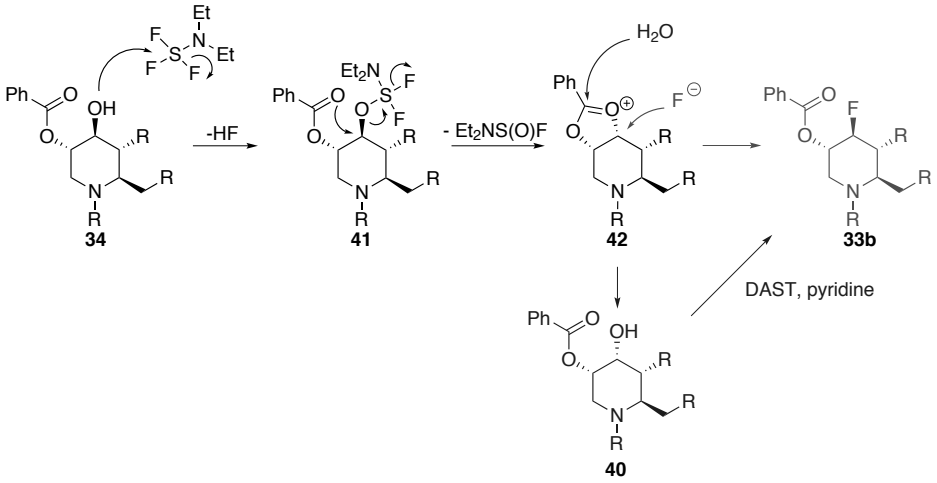
To introduce a fluoride atom in the equatorial position, C-3 alcohol **34** had to be inverted via oxidation and subsequent reduction of the ketone (Scheme 11). First attempts under Swern oxidation conditions did not result in desired ketone **39**, and the same negative result was obtained with IBX. Fortunately, oxidation using Dess-Martin periodinane (DMP) afforded ketone **39** in quantitative yield. It was reduced with LS-Selectride to obtain the axial C-3 alcohol **40** besides an equimolar amount of the C-2–C-3 benzoyl-migrated product (**40b**). Apparently, the basic reduction conditions allowed migration of the benzoyl group over the *cis*-substituted vicinal diol. The reaction was repeated with sodium borohydride under mildly acid conditions to circumvent the migration, yielding the axial alcohol **40** in 60% yield. Formation of the equatorial alcohol was also observed, however, this product was not isolated. Treating alcohol **40** with DAST and pyridine afforded the equatorial fluoride **33b** in 28% yield.[30]



Scheme 11 — Synthesis of DNJ fluoro-derivatives 33b and c.

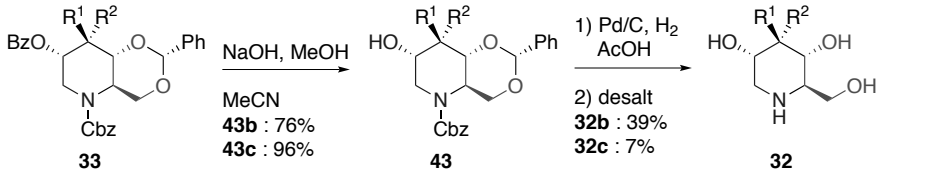
To synthesize the axial C-3 fluoride, alcohol **34** was treated with DAST and pyridine, but surprisingly, equatorial fluoride **33b** was obtained, which was unequivocally confirmed

by ^{1}H-NMR. Although the H-3/H-4–H-5 coupling constants (6.0 and 8.5 Hz, respectively) are not in the range of normal diaxial couplings (11-13 Hz), a 2D-NOESY experiment showed no spatial couplings between these protons, proving their diaxial relationship. The first reactions were performed under anhydrous conditions affording the equatorial fluoride in rather poor yields (0-34%), even after adding 5 equivalents of DAST. However, when the DCM layer was saturated with water prior to the reaction, the yield doubled to 62%. We postulate that the benzoyl group participated in the DAST reaction via intramolecular nucleophilic attack on the activated alcohol, creating the stabilized 5-membered dioxycarbenium intermediate **42** (Scheme 12). Presumably, water present in the reaction reacted with DAST to increase the concentration of fluoride in solution driving the reaction towards product **33b** (Scheme 12, in red). Interestingly, axial C-3 alcohol **40** was obtained as a byproduct in yields varying between 22 and 43%. The formation of this byproduct could be explained by attack of water on the electrophilic tertiary carbon of the 5-membered ring intermediate (Scheme 12, in blue), which upon reaction with DAST resulted in product **33b**.



Scheme 12 — Formation of the equatorial fluoride with DAST.

The difluorinated compound was also synthesized by treating ketone **39** with excess DAST to afford geminal difluoride **33c** in 89% yield. In the next step, the C-2 alcohol was saponified with sodium hydroxide and methanol in excellent yield, after which subsequent hydrogenation gave full conversion into the acetate salts **32** after double deprotection (Scheme 13). Attempts to desalt these iminosugars for analytical purposes via ion exchange column chromatography drastically decreased the yield to 39 and 7% yield for **32b** and **32c**, respectively.



Scheme 13 — Deprotection of fluoro-derivatives 33.

4.3 — Conclusion

Based on the four-enzyme-catalytic cascade, a two-step strategy to yield iminosugars was successfully demonstrated. Upon optimization, D-fagomine was obtained in an overall yield of 69%. A chemoenzymatic synthesis of DNJ was also realized with this strategy and derivatization afterwards resulted in the corresponding C-3 (di)fluorinated iminosugars. The four-enzyme-catalytic cascade was also applied on the complementary aldolases RhuA, TagA and FucA to synthesize epimeric precursors of D-fagomine. The results, however, demonstrated that the synthesis of the four diastereoisomers is strongly dependent on the substrate specificity and stereoselectivity of the aldolases. Nevertheless this approach may lead to the simple and inexpensive synthesis of a variety of non-natural heterocycles that presently can only be chemically produced at high cost.

4.4 — Acknowledgements

Louis Hartog, Dr. Teunie van Herk, Prof. Dr. Ron Wever and especially Lara Babich (J. H. van 't Hoff Institute for Molecular Sciences, University of Amsterdam) are gratefully acknowledged for the fruitful cooperation and contributions to the optimization of the four-enzyme cascade reaction. Pierpaolo Falcicchio (Laboratory of Microbiology, Wageningen University) is greatly acknowledged for his contribution to the expression of the complementary aldolases. Sybrin Schröder is gratefully acknowledged for his contribution to the derivatization of DNJ.

4.5 — Experimental

4.5.1 — General information

The general information is described in Section 2.5.1

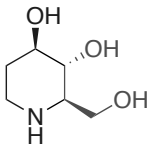
4.5.2 — Enzymes

The general information of the enzymes is described in Section 3.5.2

4.5.2 — Analytical methods and product characterization

The time-course of the cascade reaction and the conversions were determined by HPLC using an Alltech OA 1000 organic acid column (0.65 × 30 cm). 20 µL of the reaction mixture was diluted 10-fold before injection on the HPLC. Isocratic runs were performed using 4.5 mM H_2SO_4, with a flow rate of 0.4 mL min^{-1}. The effluent was UV-analyzed at 215 and 254 nm and by refractive index detector.

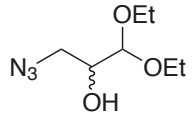
4.5.3 — D-Fagomine (2)



N-Alloc-aminopropanal (**12c**, 157 mg, 1.0 mmol) was dissolved in H_2O (5.47 mL). Subsequently glycerol (2.00 mL, 2.5 M, 5.0 mmol) and PP_i (591 mg, 2.5 mmol)[33] were added, followed by GPO (1.00 mL, 50 U/mL), catalase from bovine liver (20 µL, 10 U/mL) and RAMA (161 µL, 6 U/mL). The reactions were initiated by adding PhoN-Sf (600 µL, 3 U/mL) and incubated until completion at 20 °C under mild shaking. Silica gel (2 g) was added to the reaction mixture and it was concentrated under reduced pressure. The product was obtained by flash chromatography (EtOAc:MeOH 19:1), giving product **13c** (170 mg, 69 mmol) in 69% yield.

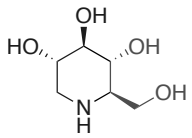
(3*S*,4*R*)-6-[(Allyloxycarbonyl)amino]-5,6-dideoxy-2-hexulose (**13c**, 20 mg, 0.081 mmol) was dissolved in EtOH (10 mL) and triethylsilane (0.16 ml, 0.97 mmol) was added, followed by the addition of Pd/C (10 mg, 0.094 mmol). The reaction mixture was stirred overnight at room temperature and filtered over Celite. The filtrate was concentrated *in vacuo* and purified with solid phase extraction, giving D-fagomine (**2**, 12 mg, 0.082 mmol, 100%). ^{1}H NMR (CD_3OD, 400 MHz) δ 3.87 (dd, *J* = 10.9, 3.1 Hz, 1H), 3.63-3.55 (m, 1H), 3.42-3.38 (m, 1H), 3.11 (t, *J* = 9.0 Hz, 1H), 3.02 (ddd, *J* = 12.7, 4.6, 2.4 Hz, 1H), 2.66-2.58 (m, 2H), 2.48-2.41 (m, 1H), 1.97-1.90 (m, 1H), 1.54-1.43 (m, 1H); ^{13}C NMR (CD_3OD, 75 MHz) δ 73.3, 73.1, 61.4, 61.2, 42.6, 32.8; $[\alpha]_D^{20}$ = +6.6 (*c* = 0.60, MeOH); LRMS (ESI) *m/z* calcd for $C_6H_{13}NO_3$: 147.09, found 148.10 $[M+H]^+$. The analytical data were in accordance with previously reported results.[15]

4.5.4 — 3-Azido-2-hydroxypropanal diethyl acetal (17)



To a stirred suspension of $KHCO_3$ (2.26 g, 22.5 mmol) in methanol (75 mL) acrolein diethyl acetal (**16**, 22.5 mL, 148 mmol), benzonitrile (15 mL) and H_2O_2 (16 mL, 30% solution in water, 156.5 mmol) were added. The solution was warmed to 40 °C, and after 4 and 20 h another 5 mL of the H_2O_2 solution was added. The solution was allowed to react for an additional 25 h at 40 °C, cooled to RT and stirred for 25 h. Next, sodium azide (24.5 g, 0.31 mol) was added and the pH was adjusted and kept at 7.5 with 1 M H_2SO_4, while the reaction mixture was stirred at 30 °C for 20 h. It was concentrated under reduced pressure, water (80 mL) was added to the residue, and it was extracted with DCM (3 × 80 mL). The organic layer was washed with brine (250 mL), dried over Na_2SO_4 and evaporated under reduced pressure. Heptane (50 mL) was added to the residue to precipitate the benzamide, which was filtered off and the filtrate was evaporated under reduced pressure. The crude product was purified with silica gel column chromatography (heptane:diethyl ether 9:1) to yield 3-azido-2-hydroxypropanal diethyl acetal (**17**, 16.7 g, 88.5 mmol, 60%) as a yellow oil. ^{1}H NMR (CD_3OD, 300 MHz) δ 4.47-4.52 (m, 1H), 3.77-3.74 (m, 3H), 3.61-3.59 (m, 2H), 3.48-3.47 (m, 1H), 3.35-3.33 (m, 1H), 2.52 (s, 1H), 1.27-1.22 (m, 6H); ^{13}C NMR (CD_3OD, 75 MHz): δ 102.8, 71.0, 63.2, 52.2, 13.8; υ_{max} (film) cm^{-1} 3435, 2098. The analytical data were in accordance with previously reported results.[22a]

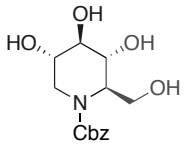
4.5.5 — Deoxynojirimycin (1)



To a solution of *N*-Cbz-3-amino-1,2-dihydroxypropane (**20**, 0.225 g, 1.00 mmol) in EtOAc (15 mL) was added IBX (0.560 g, 2.00 mmol) and the reaction mixture was heated to reflux for 6 h. The formed precipitate was filtered off and DMSO was added (1 mL). The reaction mixture was concentrated under reduced pressure at 20 °C to remove EtOAc and the crude product was used without further purification. Subsequently glycerol (2.00 mL, 2.5 M, 5.0 mmol) and PP_i (591 mg, 2.5 mmol)[33] were added, followed by the addition of GPO (1.00 mL, 50 U/mL), catalase from bovine liver (20 µL, 10 U/mL) and RAMA (161 µL, 6 U/mL). The reactions were initiated by adding PhoN-Sf (600 µL, 3 U/mL) and incubated until completion at 20 °C under mild shaking. Silica gel (2 g) was added to the reaction mixture, after which it was concentrated under reduced pressure. The product was purified by flash chromatography (EtOAc:MeOH 19:1) giving aldol product **22** (41 mg) as a colorless oil. The crude product was dissolved in EtOH (5 mL), Pd/C (10 mg) was added and the mixture was stirred overnight under a H_2 atmosphere. The mixture was filtered over Celite, the filtrate was concentrated *in vacuo*, giving DNJ (**1**, 37 mg, 0.23 mmol, 23%) as a colorless oil. ^{1}H NMR (CD_3OD, 400 MHz) 4.61

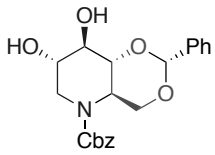
(br s, 1H), 3.85 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.61 (dd, *J* = 11.0, 6.5 Hz, 1H), 3.45-3.40 (m, 1H), 3.24-3.15 (m, 2H), 3.11 (dd, *J* = 12.0, 5.0 Hz, 1H), 2.50-2.42 (m, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 79.3, 72.0, 71.2, 61.7, 61.5, 49.7; $[\alpha]_D^{20}$ = +44.3 (*c* = 0.23, MeOH); υ_{max} (film) cm^{-1} 3314, 2892; LRMS (ESI) *m/z* calcd for $C_6H_{13}NO_4$: 163.08, found 164.09 $[M+H]^+$. The analytical data were in accordance with previously reported results.[15]

4.5.6 — *N*-Cbz-1-Deoxynojirimycin (35)



1-Deoxynojirimycin (**1**, 500 mg, 3.06 mmol) was dissolved in water (10 mL), dioxane (10 mL) and $NaHCO_3$ (296 mg, 3.52 mmol) were added, followed by dropwise addition of CbzCl (575 mg, 3.37 mmol). The mixture was stirred at RT for 75 min and neutralized with 10% aqueous citric acid (8 mL). Dioxane was evaporated, the water phase was saturated with NaCl and extracted with EtOAc (8 × 25 mL). The combined organic layers were dried with Na_2SO_4, filtrated and concentrated to give product **35** as a yellow oil (1.12 g, 3.06 mmol, 100%). R_f 0.44 (EtOAc:MeOH:H_2O 85:10:5); ^{1}H NMR (CD_3OD, 500 MHz): δ 7.40-7.26 (m, 5H), 5.11 (s, 2H), 4.20 (br s, 1H), 3.98 (br d, *J* = 14.0 Hz, 1H), 3.86 (dd, *J* = 11.5, 6.5 Hz, 1H), 3.74 (m, 2H), 3.67 (m, 2H), 3.42 (br d, *J* = 14.0 Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz): δ 176.8, 173.4, 158.5, 138.0, 129.4, 128.9, 128.6, 74.1, 71.0, 70.1, 68.3, 61.2, 61.1, 43.8; $[\alpha]_D^{20}$ –14.4 (*c* = 0.20, MeOH); υ_{max} (film) cm^{-1} 3382, 2360, 2339, 1717; HRMS (ESI) *m/z* calcd for $C_{15}H_{19}NO_6$ 297.1212, found 298.1291 $[M+H]^+$. The analytical data were in accordance with previously reported results.[30]

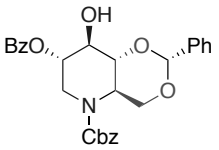
4.5.7 — *N*-Cbz-4,6-*O*-Benzylidene-1-deoxynojirimycin (36)



To a solution of *N*-Cbz-1-deoxynojirimycin (**35**, 3.07 mmol, 913 mg) in DMF (25 mL) were added benzaldehyde dimethyl acetal (606 mg, 3.98 mmol) and *p*-toluene sulfonic acid (56.6 mg, 11 mol%) and the mixture was stirred under reduced pressure (45 mbar) at 60 °C. After 14 h, more benzaldehyde dimethyl acetal was added (303 mg, 1.99 mmol). The reaction was stirred overnight under reduced pressure, cooled to RT and poured into ice water (20 mL) containing $NaHCO_3$ (150 mg). The white precipitate was filtered off, washed with cold water, redissolved in DCM, dried with Na_2SO_4, filtered and concentrated. The water filtrate was stored at 5 °C overnight and the precipitate was filtered off, dried and recrystallized in hot isopropanol/heptane (1:1). The precipitate was filtered off, washed with cold heptanes, dried in high vacuum overnight to give product **36** as a white powder (1.03 g, 2.67 mmol, 87%). R_f 0.69 (EtOAc/MeOH, 9:1); ^{1}H NMR (CD_3OD, 500 MHz): δ 7.52-7.49 (m, 2H), 7.40-7.30 (m, 8H), 5.58 (s, 1H), 5.11 (d, *J* = 2.0 Hz, 2H), 4.73 (dd, *J* = 11.5, 7.0 Hz, 1H), 4.35 (t, *J* = 10.5 Hz, 1H), 4.12 (dd, *J*

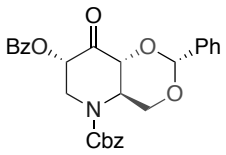
= 13.5, 9.5 Hz, 1H), 3.61 (dd, *J* = 10.0, 8.5 Hz, 1H), 3.55-3.47 (complex m, 2H), 3.48-3.29 (m, 2H), 2.94 (dd, *J* = 13.5, 9.0 Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz): δ 156.7, 139.3, 137.8, 129.9, 129.6, 129.3, 129.1, 129.0, 127.5, 102.8, 81.9, 77.5, 71.5, 70.6, 68.5, 55.9, 50.3; $[\alpha]_D^{20}$ +11.4 (*c* = 0.20, DCM); υ_{max} (film) cm^{-1} 3354, 1694, 1147, 1089, 750, 698 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{21}H_{23}NO_6$: 385.1525, found 386.1604 [M+H]$^+$. The analytical data were in accordance with previously reported results.[30]

4.5.8 — *N*-Cbz-2-*O*-Benzoyl-4,6-*O*-benzylidene-1-deoxynojirimycin (34)



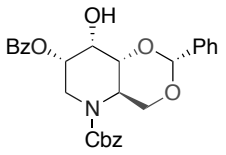
A mixture of *N*-Cbz-4,6-*O*-benzylidene-1-deoxynojirimycin (**36**, 1.45 g, 3.76 mmol) and dibutyltin oxide (1.03 g, 4.14 mmol) in methanol (60 mL) was refluxed for 2 h. The methanol was evaporated under reduced pressure and toluene (2 × 50 mL) was added and removed under vacuum. The residue was dissolved in DCM (75 mL) and triethylamine (0.63 mL, 4.51 mmol) was added. The mixture was cooled to 0 °C, and benzoyl chloride (0.480 mL, 4.14 mmol) was added. After 30 min the red colored mixture was allowed to warm to RT, and after 1.5 h stirring at RT the mixture was diluted with DCM (50 mL) and quenched with saturated aqueous Na_2CO_3 (25 mL). The organic layer was separated and the aqueous layer was extracted with DCM (3 × 50 mL). The combined organic layers were washed subsequently with water (100 mL), 1 N HCl (100 mL) and water (100 mL), dried with Na_2SO_4, filtrated and concentrated. The product was recrystallized from DCM/hexanes (1:1) to give piperidine **34** as a white powder (1.89 g, 3.76 mmol, 100%). R_f 0.54 (heptane:EtOAc 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.02-7.99 (m, 2H), 7.61-7.56 (m, 1H), 7.52-7.48 (m, 2H), 7.46-7.41 (m, 2H), 7.40-7.28 (m, 8H), 5.61 (s, 1H), 5.12 (d, *J* = 3.0 Hz, 2H), 5.12-5.08 (m, 1H), 4.87 (dd, *J* = 4.5, 11.5 Hz, 1H), 4.22 (t, *J* = 11.0 Hz, 1H), 4.18 (dd, *J* = 4.0, 14.0 Hz, 1H), 4.00 (ddd, *J* = 2.5, 6.0, 9.0 Hz, 1H), 3.85 (dd, *J* = 1.0, 9.5 Hz, 1H), 3.51 (ddd, *J* = 4.5, 10.0 Hz, 1H), 3.46 (dd, *J* = 7.5, 13.5 Hz, 1H), 2.82 (d, *J* = 2.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.9, 155.4, 137.1, 135.9, 133.4, 129.8, 129.4, 129.3, 128.6, 128.5, 128.4, 128.3, 128.1, 126.3, 102.0, 80.1, 74.2, 73.3, 69.5, 67.7, 53.1, 45.2; $[\alpha]_D^{20}$ −2.0 (*c* = 0.20, DCM); υ_{max} (film) cm^{-1} 3447, 1713, 1267, 1093, 698 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{28}H_{27}NO_7$: 489.1788, found 490.1866 [M+H]$^+$. The analytical data were in accordance with previously reported results.[30]

4.5.9 — *N*-Cbz-2-*O*-Benzoyl-4,6-*O*-benzylidene-3-keto-1-deoxynojirimycin (39)



To a solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-1-deoxynojirimycin (**34**, 100 mg, 0.204 mmol) in DCM (10 mL) was added Dess-Martin periodinane (173 mg, 0.409 mmol). The reaction was refluxed for 16 h, after which the precipitate was filtered off and the organic layer was washed with saturated $Na_2S_2O_3$/$NaHCO_3$ (1:1, 2 × 40 mL). The organic phase was washed with brine (20 mL), dried (Na_2SO_4), filtrated and concentrated under reduced pressure to obtain product **39** as a yellow oil in quantitative yield (99 mg). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.06-8.03 (m, 2H), 7.62-7.58 (m, 1H), 7.53-7.50 (m, 2H), 7.47-7.43 (m, 2H), 7.38-7.33 (m, 8H), 5.64 (s, 1H), 5.51 (ddd, *J* = 1.0, 6.0, 15.5 Hz, 1H), 5.20 (d, *J* = 12.5 Hz, 1H), 5.15 (d, *J* = 12.0 Hz, 1H), 4.83 (dd, *J* = 4.0, 11.0 Hz, 1H), 4.64 (dd, *J* = 6.0, 14.0 Hz, 1H), 4.61 (dd, *J* = 1.0, 10.5 Hz, 1H), 4.58 (t, *J* = 10.5 Hz, 1H), 3.70 (dt, *J* = 10.5 Hz, 1H), 3.57 (dd, *J* = 9.5, 14.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 195.3, 165.1, 154.9, 136.7, 135.6, 133.8, 130.2, 129.5, 128.9, 128.9, 128.9, 128.7, 128.6, 128.5, 128.5, 126.5, 101.9, 80.9, 72.5, 70.0, 68.4, 56.8, 48.8; $[\alpha]_D^{20}$ -10.3 (*c* = 0.2, DCM); υ_{max} (film) cm^{-1} 1719, 1262, 1096, 1024, 700 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{28}H_{25}NO_7$: 487.1631, found 488.1709 $[M+H]^+$. The analytical data were in accordance with previously reported results.[30]

4.5.10 — *N*-Cbz-2-*O*-Benzoyl-4,6-*O*-benzylidene-1,5-dideoxy-1,5-imino-D-allitol (40)

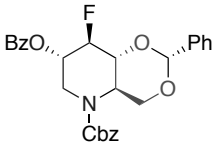


Method A – A solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-3-keto-1-deoxynojirimycin (**39**, 100 mg, 0.205 mmol) in methanol (0.5 mL) and THF (4 mL) was cooled to –5 °C on an ice bath and acetic acid (0.012 mL, 0.205 mmol) was added followed by sodiumborohydride (7.76 mg, 0.205 mmol). The mixture was stirred for 10 min, after which another equivalent of sodiumboro hydride (7.76 mg, 0.205 mmol) was added and stirred for an additional 1 min. The reaction was quenched with saturated NH_4Cl (1 mL) and diluted with water to dissolve the precipitate. The solution was extracted with EtOAc (3 × 5 mL), the organic layers were dried over Na_2SO_4, filtrated and concentrated. A mixture of C-3 epimers was obtained, with very little benzoyl migrated product. The compounds were separated by flash column chromatography (DCM:EtOAc 98:2). Product **40** was obtained after crystallization (DCM:heptane 1:1) as a white solid (60 mg, 0.123 mmol, 60%).

Method B - To a solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-3-keto-1-deoxynojirimycin (**39**, 200 mg, 0.409 mmol) in anhydrous DCM (12 mL), pyridine (0.069 mL, 0.858 mmol) was added under an argon atmosphere. The mixture was cooled to –78

°C, and DAST (0.167 mL, 1.27 mmol) was added dropwise over 5 min. The mixture was heated to reflux for 14 h, after which another two equivalents of DAST (0.108 mL, 0.817 mmol) were added at –78 °C. The mixture was refluxed for an additional 16 h, cooled to RT and quenched with saturated $NaHCO_3$ (10 mL). The mixture was extracted with DCM (3 × 15 mL) and washed with 1 N HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL) and brine (20 mL). The organic layer was dried with Na_2SO_4, filtrated and concentrated. The crude product was purified via flash column chromatography (heptane:EtOAc 9:1) to give product **40** as a white solid (84 mg, 0.172 mmol, 42%). R_f 0.58 (heptane:EtOAc 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.09-8.06 (m, 2H), 7.60-7.56 (m, 1H), 7.50-7.31 (m, 12H), 5.66 (s, 1H), 5.16 (d, *J* = 12.0 Hz, 1H), 5.11 (d, *J* = 12.0 Hz, 1H), 5.08 (m, 1H), 4.89 (dd, *J* = 11.5, 4.5 Hz, 1H), 4.57 (t, *J* = 11.0 Hz, 1H), 4.51 (br s, 1H), 4.31 (dd, *J* = 12.5, 5.0 Hz, 1H), 3.86 (dd, *J* = 10.0, 2.5 Hz, 1H), 3.78 (dt, *J* = 10.5, 4.5 Hz. 1H), 3.45 (dd, *J* = 13.0, 12.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.6, 155.0, 137.3, 136.1, 133.5, 130.0, 129.7, 129.4, 128.8, 128.6, 128.5, 128.4, 128.3, 126.2, 101.5, 69.7, 69.1, 67.8, 67.8, 50.7, 43.7; $[\alpha]_D^{20}$ –14.3 (*c* = 0.20, DCM); υ_{max} (film) cm^{-1} 3453, 1713, 1270, 1211, 1107 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{28}H_{27}NO_7$: 489.1788, found 490.1870 $[M+H]^+$. The analytical data were in accordance with previously reported results.[30]

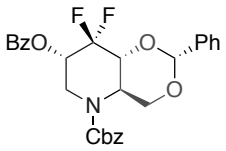
4.5.11 — *N*-Cbz-2-*O*-Benzoyl-4,6-*O*-benzylidene-1,3-dideoxy-3-fluoronojirimycin (33b)



To a solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-1-deoxynojirimycin (**34**, 50 mg, 0.102 mmol) in DCM saturated with water (7 mL) in a round-bottomed flask equipped with a reflux condenser was added pyridine (0.017 mL, 0.214 mmol). The mixture was cooled to –78 °C, followed by dropwise addition of DAST (0.042 mL, 0.317 mmol) over 5 min. After 24 h of refluxing another amount of DAST (0.042 mL, 0.317 mmol) was added at –78 °C and the reaction mixture was heated to reflux for an additional 24 h. The mixture was diluted with EtOAc (20 mL) and quenched with water (25 mL), followed by the extraction of the reaction mixture with EtOAc (3 × 25 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (100 mL), 1 N HCl (100 mL), saturated aqueous $NaHCO_3$ (100 mL) and brine (100 mL). The organic layer was dried with Na_2SO_4, filtrated, concentrated and purified with column chromatography (100% DCM). The product was recrystallized from DCM:heptane (1:1) to give piperidine **33b** as white needles (31 mg, 0.0632 mmol, 62%). R_f 0.60 (heptane:EtOAc 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.02-7.99 (m, 2h), 7.61-7.57 (m, 1H), 7.53-7.50 (m, 2H), 7.47-7.43 (m, 2H), 7.40-7.30 (m, 8H), 5.63 (s, 1H), 5.31 (dddd, *J* = 16.5, 8.0, 5.5, 4.0 Hz, 1H), 5.09 (s, 2H), 4.89 (ddd, *J* = 11.0, 4.0, 2.0 Hz, 1H), 4.80 (ddd, *J* = 51.0, 8.5, 6.0 Hz, 1H), 4.28 (t, *J* = 11.0 Hz, 1H), 4.22 (ddd, *J* = 14.0, 3.5, 2.0 Hz, 1H), 4.10 (ddd, *J* = 19.0, 10.5, 8.5 Hz, 1H), 3.51

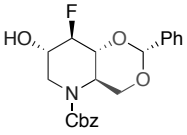
(ddd, *J* = 10.5, 10.0, 4.5 Hz, 1H), 3.45 (dd, *J* = 14.0, 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.2, 155.4, 137.0, 135.8, 133.7, 129.9, 129.4, 129.2, 128.8, 128.7, 128.5, 128.5, 128.2, 126.3, 101.7, 92.5 (d, $J_{C3,F}$ = 186.9 Hz), 78.5 (d, *J* = 19.5 Hz), 70.7 (d, *J* = 23.5 Hz), 69.7, 68.0, 52.6 (d, $J_{C5,F}$ = 7.3 Hz), 45.2 (d, $J_{C1,F}$ = 6.0 Hz); $[\alpha]_D^{20}$ –1.2 (*c* = 0.2, DCM); υ_{max} (film) cm^{-1} 1715, 1089, 1014, 698 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{28}H_{26}FNO_6$: 491.1744, found 492.1829 $[M+H]^+$. The analytical data were in accordance with previously reported results.[30]

4.5.12 — *N*-Cbz-2-*O*-Benzoyl-4,6-*O*-benzylidene-1,3-dideoxy-3,3-difluoronojirimycin (33c)



To a solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-3-keto-1-deoxynojirimycin (**39**, 100 mg, 0.205 mmol) in anhydrous DCM (5 mL) under argon atmosphere DAST (0.136 mL, 1.03 mmol) was added at 78 °C. The mixture was warmed to RT and stirred for 30 h. DCM (10 mL) was added and the mixture was washed with water (10 mL). The layers were separated and the water layer was extracted with DCM (3 × 10 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ (30 mL), 1 N HCl (30 mL), saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL). The organic layer was dried with Na_2SO_4, filtrated and concentrated. The product was redissolved in dioxane and lyophilized, affording product **33c** as a white solid (93 mg, 0.182 mmol, 89%). R_f 0.60 (heptane:EtOAc 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.11-8.07 (m, 2H), 7.64-7.59 (m, 1H), 7.54-7.51 (m, 2H), 7.50-7.44 (m, 2H), 7.41-7.32 (m, 8H), 5.63 (s, 1H), 5.31 (m, 1H), 5.17 (d, *J* = 12.0 Hz, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 4.86 (dd, *J* = 11.5, 2.5 Hz, 1H), 4.56 (t, *J* = 11.0 Hz, 1H), 4.52 (ddd, *J* = 13.0, 5.0, 3.0 Hz, 1H), 4.03 (ddd, *J* = 18.0, 10.5, 3.5 Hz, 1H), 3.61 (dt, *J* = 10.5, 5.0 Hz, 1H), 3.20 (dd, *J* = 11.5, 1.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.1, 154.6, 136.7, 135.7, 134.0, 130.2, 129.5, 128.9, 128.8, 128.7, 128.6, 128.5, 127.4 (d, $J_{C3,F}$ = 243.75 Hz), 101.8, 77.7, 69.3, 68.2 (t, *J* = 19.3 Hz), 53.5 (d, $J_{C5,F}$ = 6.3 Hz), 45.9 (d, $J_{C1,F}$ = 5.9 Hz); ^{19}F NMR ($CDCl_3$, 470 MHz): δ -119.72 (d, *J* = 241.6 Hz), -135.20 (dt, *J* = 239.7, 17.4 Hz); $[\alpha]_D^{20}$ –15.8 (*c* = 0.2, DCM); υ_{max} (film) cm^{-1} 1713, 1264, 1103, 698 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{28}H_{25}F_2NO_6$: 509.1650, found 510.1718 $[M+H]^+$.

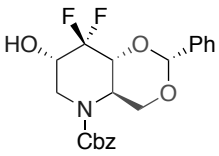
4.5.13 — *N*-Cbz-4,6-*O*-Benzylidene-1,3-dideoxy-3-fluoronojirimycin (43b)



To a solution of *N*-Cbz-2-*O*-benzoyl-4,6-*O*-benzylidene-1,3-dideoxy-3-fluoronojirimycin (**33c**, 31.9 mg, 0.0651 mmol) in acetonitrile (2 mL) was added a solution of sodium hydroxide (3.1 mg, 0.078 mmol) in methanol (2 mL) and the mixture was stirred at RT for 1 h. It was diluted with EtOAc (10 mL), 1 N HCl (5 mL) was added, which was followed by an extraction with EtOAc (3 × 10 mL). The organic layers were

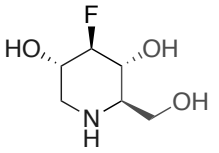
washed with brine (30 mL), dried over Na_2SO_4, filtrated and concentrated. The product was recrystallized from DCM:heptane (1:1) to give product **43b** as a white solid (19 mg, 0.0487 mmol, 76%). R_f 0.47 (heptane:EtOAc 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.52-7.48 (m, 2H), 7.41-7.33 (m, 8H), 5.57 (s, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 5.10 (d, *J* = 12.0 Hz, 1H), 4.83 (ddd, *J* = 11.5, 4.5, 2.0 Hz, 1H), 4.55-4.38 (m, 2H), 4.32 (dt, *J* = 13.5, 5.0 Hz, 1H), 3.89 (m, 2H), 3.31 (dt, *J* = 10.0, 4.5 Hz, 1H), 2.89 (dd, *J* = 13.5, 10.0 Hz, 1H), 2.32 (d, *J* = 3.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 154.9, 137.1, 135.9, 129.4, 128.8, 128.6, 128.5, 128.4, 126.3, 101.5, 96.2 (d, $J_{C3,F}$ = 182.5 Hz), 78.3 (d, *J* = 17.5 Hz), 77.7, 69.5, 69.0, 68.8, 68.0, 54.4 (d, $J_{C5,F}$ = 7.2 Hz), 48.4 (d, $J_{C1,F}$ = 7.7 Hz); ^{19}F NMR ($CDCl_3$, 470 MHz): δ -194.14; $[\alpha]_D^{20}$ +8.2 (*c* = 0.2, DCM); υ_{max} (film) cm^{-1} 3416, 1076, 1215, 1152, 1007, 756 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{21}H_{22}FNO_5$: 387.1482, found 388.1567 $[M+H]^+$. The analytical data were in accordance with previously reported results.[30]

4.5.14 — *N*-Cbz-4,6-*O*-Benzylidene-1,3-dideoxy-3,3-difluoronojirimycin (43c)



*Same procedure as for N-Cbz-4,6-O-benzylidene-1,3-dideoxy-3-fluoronojirimycin (**43b**).* Product **43c** was obtained as a pale yellow solid (64 mg, 96%). R_f 0.45 (heptane:EtOAc, 1:1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.53-7.49 (m, 2H), 7.42-7.34 (m, 8H), 5.59 (s, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 5.10 (d, *J* = 12.5 Hz, 1H), 4.85 (ddd, *J* = 11.5, 4.5, 2.0 Hz, 1H), 4.52 (t, *J* = 11.0 Hz, 1H), 4.41 (ddd, *J* = 14.0, 5.5, 4.0 Hz, 1H), 3.90-3.78 (m, 2H), 3.50 (dt, *J* = 10.0, 4.0 Hz, 1H), 2.95 (t, *J* = 12.0 Hz, 1H), 2.19 (br s, 1H); ^{13}C NMR (CD_3OD, 125 MHz): δ 154.8, 137.4, 136.1, 128.7, 128.2, 128.0, 127.9, 127.7, 126.0, 117.7 (t, $J_{C3,F}$ = 246.9 Hz), 101.2, 76.8 (t, *J* = 19.1 Hz), 68.9, 67.4 (t, *J* = 20.1 Hz), 67.4, 53.1 (d, $J_{C5,F}$ = 6.3 Hz), 48.0 (d, $J_{C1,F}$ = 6.3 Hz); ^{19}F NMR ($CDCl_3$, 470 MHz): δ -121.45 (d, *J* = 235.9 Hz), -139.15 (dt, *J* = 236.4, 18.3 Hz); $[\alpha]_D^{20}$ +7.8 (*c* = 0.2, DCM); υ_{max} (film) cm^{-1} 3351, 1738, 1365, 1216 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{21}H_{21}FNO_5$: 405.1388, found 406.1479 $[M+H]^+$.

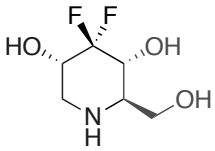
4.5.15 — 1,3-Dideoxy-3-fluoronojirimycin (32b)



To a solution of *N*-Cbz-4,6-*O*-benzylidene-1,3-dideoxy-3-fluoronojirimycin (**43b**, 40 mg, 0.103 mmol) in glacial acetic acid (3 mL) was added Pd/C (22 mg, 0.206 mmol) and the solution was stirred under a hydrogen atmosphere for 78 h at RT. The solution was filtered over Celite, concentrated and dried on a vacuum pump to obtain the acetate salt. The product was dissolved in water and Serdolit CG-400 I (OH type) was added. The suspension was stirred overnight, filtrated over Celite and the filtrate was lyophilized. This procedure was repeated two times to fully desalt the product, which was purified by preparative TLC ($CHCl_3$:MeOH:NH_4OH 4:3:1) to give

product **32b** as a white solid (6.6 mg, 0.0402 mmol, 39%). R_f 0.59 ($CHCl_3$:MeOH:NH_4OH 4:3:1); ^1H NMR (D_2O, 500 MHz): δ 4.36 (dt, *J* = 53.5, 9.0 Hz, 1H), 3.88-3.80 (m, 1H), 3.76 (dd, *J* = 11.5, 5.5 Hz, 1H), 3.63 (dt, *J* = 13.0, 10.0 Hz, 1H), 3.22-3.14 (m, 1H), 2.66 (br s, 1H), 2.57 (t, *J* = 11.5 Hz, 1H); ^{13}C NMR (D_2O, 125 MHz): δ 99.0 (d, $J_{C3,F}$ = 178.3 Hz), 69.6 (d, *J* = 16.9 Hz), 69.2 (d, *J* = 16.5 Hz), 60.7, 59.3 (d, $J_{C5,F}$ = 6.6 Hz), 47.7 (d, $J_{C1,F}$ = 8.0 Hz). ^{19}F NMR (D_2O, 470 MHz): δ -152.54.The analytical data were in accordance with previously reported results.[34]

4.5.16 — 1,3-Dideoxy-3,3-difluoronojirimycin (32c)



*Same procedure as for 1,3-dideoxy-3-fluoronojirimycin (**32b**).* Product **32c** was obtained as a white solid (3 mg, 7%). R_f 0.35 ($CHCl_3$/MeOH/NH_4OH, 4:3:1); ^1H NMR (CD_3OD, 500MHz): δ 3.76 (ddd, *J* = 11.0, 2.5, 1.5 Hz, 1H), 3.74-3.65 (m, 1H), 3.68 (dd, *J* = 11.0, 5.0 Hz, 1H), 3.52 (ddd, *J* = 20.5, 10.0, 4.0 Hz, 1H), 3.10 (ddd, *J* = 12.5, 5.5, 4.0 Hz, 1H), 2.61 (m, 2H); ^{13}C NMR (CD_3OD, 125 MHz): δ 120.4 (t, $J_{C3,F}$ = 248.8 Hz), 68.8 (t, *J* = 32.5), 68.3 (t, *J* = 19.4 Hz), 68.0 (d, $J_{C5,F}$ = 7.5 Hz), 59.6 (d, $J_{C1,F}$ = 6.8 Hz), 58.2, 41.8. The analytical data were in accordance with previously reported results.[26]

4.6 — References

1. *Iminosugars: from Synthesis to Therapeutic Applications*, Compain, P. and Martin O. R. (Eds.), Wiley-VCH: Weinheim, 2007.
2. Paulsen, H.; Sangster, I. and Heyns, K. *Chem. Ber*. **1967**, *100*, 802–815.
3. a) Ganem, B. *Acc. Chem. Res.* **1996**, *29*, 340–347; (b) Heightman, T. D. and Vasella, A. T. *Angew. Chem. Int. Ed.* **1999**, *38*, 750–770; (c) Lillelund, V. H.; Jensen, H. H.; Liang, X. and Bols, M. *Chem. Rev*. **2002**, *102*, 515–553; (d) Greimel, P.; Spreitz, J.; Stutz, A. E. and Wrodnigg, T. M. *Curr. Top. Med.Chem*. **2003**, *3*, 513–523.
4. Compain, P. and Martin, O. R. *Curr. Top. Med. Chem.* **2003**, *3*, 541–560.
5. a) Fischl, M. A.; Resniek, L.; Coombs, R.; Kremer, A. B.; Pottage, J. C. Jr; Fass, R. J.; Fife, K. H.; Powderly, W. G.; Collier, A. C.; Aspinall, R. L.; Smith, S. L.; Kowalski, K. G. and Wallemark, C.-B. *J. Acquir. Immune Defic. Syndr*. **1994**, *7*, 139–147; b) Tierney M.; Pottage J.; Kessler H.; Fischl M.; Richman D.; Merigan T.; Powderly W.; Smith S.; Karim A. and Sherman J. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 1995, 10, 549–553; A review on the synthesis, biological activity and current treatments with DNJ: c)Hughes, A. B. and Rudge, A. J. *Nat. Prod. Rep*. **1994**, *11*, 135–162.
6. a) Rhinehart, B. L.; Robinson, K. M.; Payne, A. J.; Wheatly, M. E.; Fisher, J. L.; Liu, P. S. and Cheng, W. *Life Sci*. **1987**, *41*, 2325–2331; b) Johnson, P. S.; Lebovitz, H. E.; Coniff, R. F.; Simonson, D. C.; Raskin, P. and Munera, C. L. J. *Clin. Endocrinol. Metab*. **1998**, *83*, 1515–1522.
7. Sunkara, P. S.; Bowlin, T. L.; Liu, P. S. and Sjoerdsma, A. *Biochem. Biophys. Res. Commun*. **1987**, *148*, 206–210.

8. Lieberman, R. L.; Wustman, B. A.; Huertas, P.; Powe, A. C.; Pine, C. W.; Khanna, R.; Schlossmacher, M. G.; Ringe, D. and Petsko, G. A. *Nature Chem. Biol.* **2006**, *3*, 101–107.
9. Ostrander, G. K.; Scribner, N. K. and Rohrschneider, L. R. *Cancer Res*. **1988**, *48*, 1091–1094.
10. Elstein, D.; Hollak, C.; Aerts, J. M. F. G.; van Weely, S.; Maas, M.; Cox, T. M., Lachmann, R. H.; Hrebicek, M.; Platt, F. M.; Butters, T. D.; Dwek, R. A. and Zimran, A. *J. Inherit. Metab. Dis*. **2004**, *27*, 757–766.
11. Scott, L. J. and Spencer, C. M. *Drugs* **2000**, *59*, 521–549.
12. Isobe, M. and Sakamura, S. *Agr. Biol. Chem*. **1974**, *38*, 1111–1112..
13. Werner, L.;Kniežo, L. and Dvořáková, H. *Tetrahedron Lett.* **2007**, *48*, 609–612 and references therein.
14. Gómez, L.; Molinar-Toribio, E.; Ángeles Calvo-Torras, M.; Adelantado, C.; Juana, M. E.; Planas, J. M.; Cañas, X.; Lozano, C.; Pumarol, S.; Clapés, P. and Torres, J. L. *Brit. J. Nutr*. **2011**, DOI: 10.1017/S0007114511005009.
15. Fleet, G. W. J. and Smith, P. W. *Tetrahedron Lett.* **1985**, *26*, 1469–1472.
16. For reviews on iminosugar synthesis, see: a) Clapés, P., Sprenger, G. A. and Joglar, J. Novel Strategies in Aldolase-Catalyzed Synthesis of Iminosugars, in *Modern Biocatalysis: Stereoselective and Environmentally Friendly Reactions*, Fessner, W.-D. and Anthonsen, T. Ed.; Wiley-VCH: Weinheim, 2009, 299–311 and references therein; b) Winchester, B. G. *Tetrahedron: Asymmetry* **2009**, *20*, 645–651 and references therein; d) M. Sugiyama, Z. Hong, P.-H. Liang, S. M. Dean, L. J. Whalen, W. A. Greenberg and C.-H. Wong, *J. Am. Chem. Soc*. **2007**, *129*, 14811–14817 and references therein.
17. Ziegler, T.; Straub, A. and Effenberger, F. *Angew. Chem.Int. Ed*. **1998**, 5, 716–717.
18. Tsuji, J. *Pure Appl. Chem*. **1986**, *58*, 869–878 and references therein.
19. Mandl, P. K. and McMurray, J. S. *J. Org. Chem*. **2007**, *72*, 6599–6601.
20. Castillo, A.; Calveras, J.; Casas, J.; Mitjans, M.; Pilar Vinardell, M.; Parella, T.; Inoue, T.; Sprenger, G. A.; Joglar, J. and Clapés, P. *Org. Lett*. **2006**, *8*, 6067–6070.
21. Faber K. *Biotransformations in Organic Chemistry*, 5th ed., Springer-Verlag: Berlin, **2004**, Section 2.4, 211–225.
22. a) Van Herk, T. *Bacterial class A acid phosphatases as versatile tools in organic synthesis*, **2008**, PhD thesis, http://dare.uva.nl/en/record/267354; b) Von der Osten, C. H.; Sinskey, A. J.; Barbas III, C. F.; Pederson, R. L.; Wang, Y. F. and Wong, C.-H. *J. Am. Chem. Soc.* **1989**, *111*, 3924-3927.
23. Durrwachter, J. R. and Wong, C.-H. *J. Org. Chem.*, **1988**, *53*, 4175–4181.
24. Concia, A. L.; Lozano, C.; Castillo, J. A.; Parella, T.; Joglar, J. and Clapés, P. *Chem. Eur. J*. **2009**, *15*, 3808–3816.
25. a) Andersen, S. M.; Ebner, M.; Ekhart, C. W.; Gradnig, G.; Legler, G.; Lundt, I.; Stütz, A. E.; Withers, S. G. and Wrodnigg, T. *Carbohydrate Research* **1997**, *301*, 155–166; b) Prell, E.; Korb, C.; Kluge, R.; Ströhl, D. and Csuk, R. *Arch. Pharm. Chem. Life Sci*. **2010**, *10*, 583–589; c) Prell, E. and Csuk, R. *Bioorg. Med. Chem. Lett*. **2009**, *19*, 5673–5674.
26. Csuk, R.; Prell, E.; Korb, C.; Kluge, R. and Ströhl, D. *Tetrahedron* **2010**, *66*, 467–472.
27. Resnati, G. and DesMarteau, D. D. *J. Org. Chem*. **1991**, *56*, 4925–4929.
28. Brown, S. P.; Brochu, M. P.; Sinz, C. J. and MacMillan, D.W.C. *J. Am. Chem. Soc*. **2003**, *125*, 10808–10809.
29. Differding, E. and Rüegg, G. M. *Tetrahedron Lett.* **1991**, *32*, 3815–3518.
30. a) Getman, D. P. and De Crescenzo, G. A. EP0410953 A2, **1991**; b) Khanna, I. K.; Mueller, R. A. and Weiler, R. M. US5342951 A1, **1994**.

31. David, S. and Hanessian, S. *Tetrahedron* **1985**, *41*, 643–663.
32. Martinelli, M. J.; Nayyar, N. K.; Moher, E. D.; Dhokte, U. P.; Pawlak, J. M. and Vaidyanathan, R. *Org. Lett.* **1999**, *1*, 447-450.
33. PPi was added as a mixture of 3.5 equiv. (350 mg) Na_2PP_i and 2 equiv. (241 mg) Na_4PP_i resulting in a buffered solution of pH 6.
34. Lee, C.-K.; Jiang, H.; Koh L. L. and Xu, Y. *Carbohydr. Res.* **1993**, *239*, 309–315.

Chapter 5

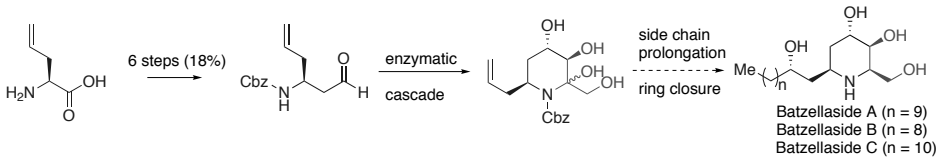
A chemoenzymatic strategy towards batzellasides A-C

Part of this work is published in Lieke J. C. van Hemert, Alexander J. J. Groenen, Luuk J. A. Wilders, Lara Babich, Ron Wever, Sander Nabuurs, and Floris P. J. T. Rutjes, Expanding the scope of one-pot four-enzyme cascade aldol reactions, manuscript in preparation.

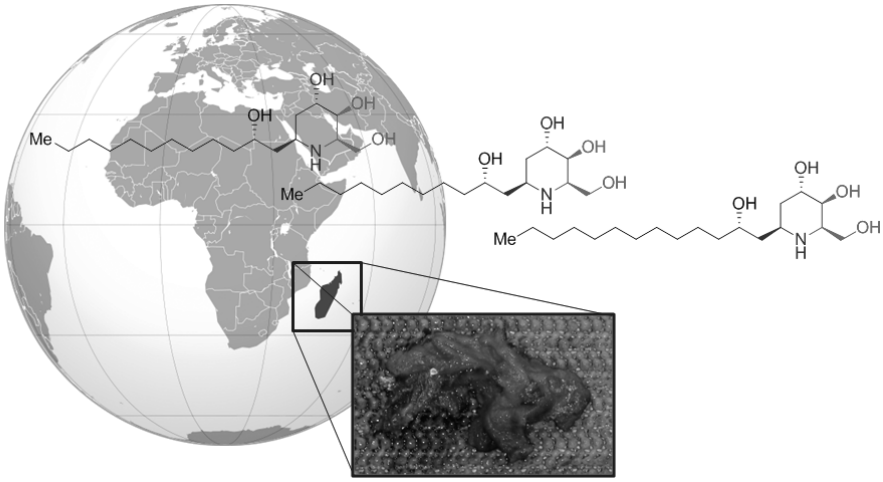
"A jug fills drop by drop."
– Buddha

Abstract

The batzellasides form a new class of C-alkylated iminosugars originally isolated from Batzella sponges collected off the west coast of Madagascar. These compounds showed interesting biological activities and therefore a synthetic route towards these compounds was developed.



Key in the approach was the chemoenzymatic coupling of the aldehyde with DHAP to form the aldol product. This coupling involved a four-enzyme cascade reaction with RhuA WT and provided the hemiaminal in 51% isolated yield as a mixture of isomers at the 2-position. Furthermore, preliminary feasibility studies for conversion into the target natural product have been carried out.



5.1 — Introduction

Since the discovery of nojirimycin (**1**, Figure 1), an antibiotic obtained from several *Streptomyces* species in the 1960s, many iminosugars have been isolated from various species, including plant and microbial sources.

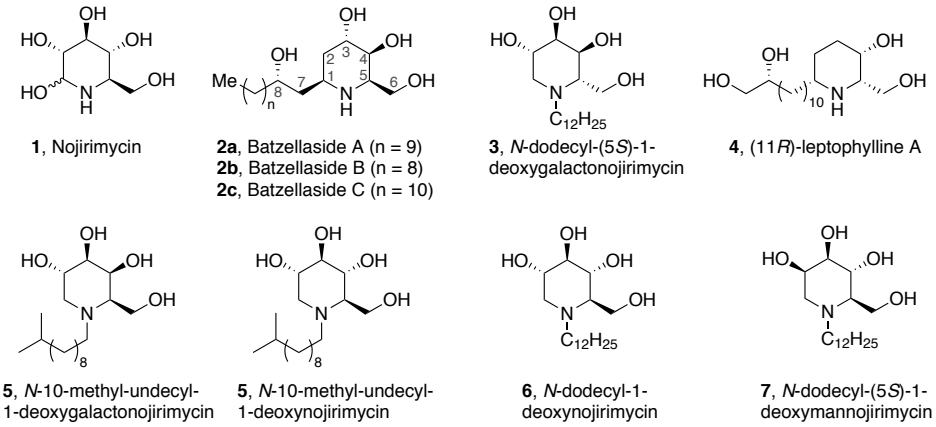
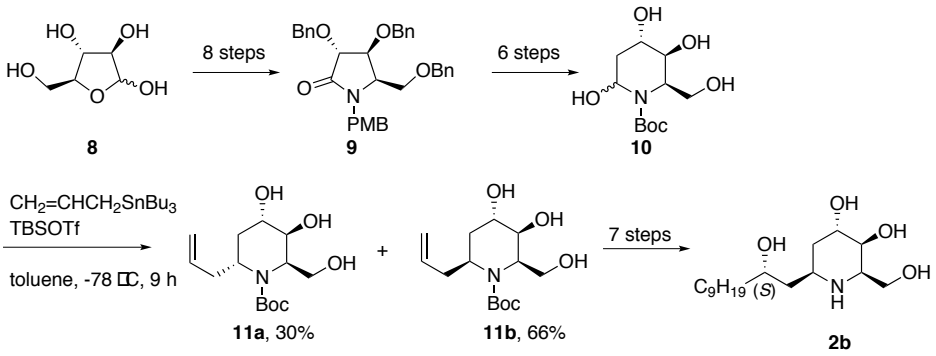


Figure 1 — Examples of iminosugars isolated from *Batzella* sp.

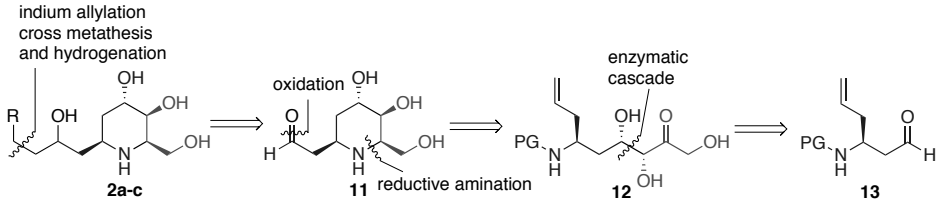
In 2005 Crews *et al.* collected sponges off the coast from Madagascar from the *Batzella* species, and discovered that they were the first marine species known to produce a variety of iminosugars (Figure 1).[1] The fractions containing iminosugars were able to inhibit the growth of *Staphylococcus epidermidis* with MIC values of ≤6.3 µg/mL. Some of the active iminosugars were isolated, characterized and named batzellasides A, B and C (**2a–c**).1 These characterizations were conducted by NMR analysis with which most stereocenters could be determined, except for the hydroxyl group attached to C8.[2] The absolute configuration of the latter stereocenter was elucidated in the beginning of 2011 by the group of Yoda through total synthesis (Scheme 1).[3]



Scheme 1 — Total synthesis of batzellaside B (2b) starting from L-arabinose.

In their approach, commercially available L-arabinose was converted in 22 steps into batzellaside B (**2b**) in an overall yield of 3.9%. Key step was the introduction of the allyl side chain via Lewis acid-mediated allylation (**10** to **11**). The absolute configuration of C8 was determined to be *S* by Mosher ester analysis of a synthetic intermediate, which was prepared through a separate route.

Independently, we were simultaneously working on a synthesis of this molecule as well. It was our goal to use the newly developed enzymatic cascade to develop a chemo-enzymatic total synthesis approach with a minimum of protecting group interconversions (Scheme 2).



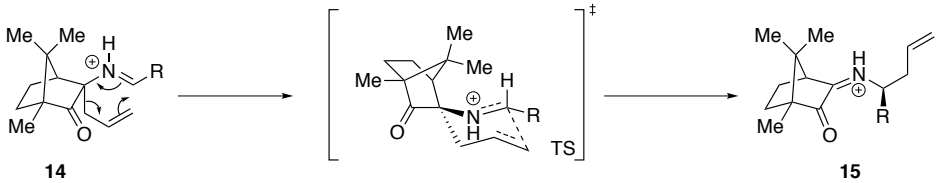
Scheme 2 — Retrosynthetic approach towards batzellasides A–C (2a–c).

Batzellasides A-C might be synthesized via indium-mediated allylation of aldehyde **11**, followed by cross-metathesis and hydrogenation to introduce the carbon chain with various lengths. Iminosugar **11** should be accessible via oxidative cleavage and reductive amination of trihydroxy aminoketone **12**. The latter product was to be synthesized in the enzymatic cascade using DHAP, aldehyde **13** and RhuA, to obtain the required stereochemistry (Section 3.2). The RhuAs available came from *Thermatoga maritima* (WT) and *Escherichia coli* (WT and mutant N29D). Since little was known about the substrate specificity of RhuA, it was difficult to predict whether the aldehyde would be accepted by the enzyme. Therefore we chose to pursue a strategy for the synthesis of aldehyde **13** that would allow us to easily vary the protecting group.

5.2 — Results and discussion

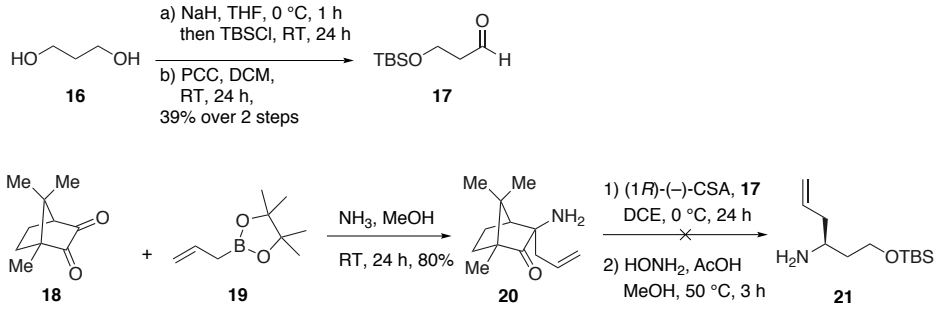
5.2.1 — Synthesis of aldehyde 13 via azonia-Cope rearrangement

A first attempt to synthesize aldehyde **13** proceeded via a method described by Kobayashi *et al*.[4] In this method, the chiral center of the aldehyde was introduced in a single step via a camphorsulfonic acid (CSA) mediated azonia–Cope rearrangement as depicted in Scheme 3 (**14** to **15**). The auxiliary CSA moiety in combination with the chairlike transition state causes the rearrangement to proceed in a highly diastereomeric fashion.



Scheme 3 — Introduction of the chiral center via an azonia–Cope rearrangement.

Such an approach would require aldehyde **17**, which was prepared via mono TBS-protection of 1,3-propanediol[5] and subsequent oxidation with PCC to afford the target aldehyde in 39% yield over two steps (Scheme 4).

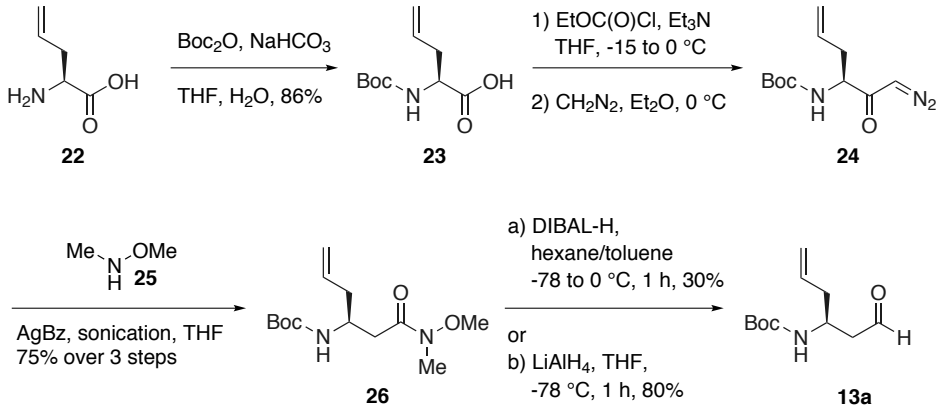


Scheme 4 — Intended synthesis of aldehyde 13 via an azonia-cope rearrangement.

To induce a diastereoselective azonia-Cope rearrangement, (1*R*)-camphorquinone (**18**) was used as the chiral auxiliary. The least hindered ketone was amino-allylated with ammonia and allylboronic acid pinacol ester (**19**) to give α-aminoketone **20** in good yield and diastereoselectivity (de >99%, ^{1}H- and ^{13}C-NMR analysis indicated the formation of a single diastereoisomer).4 After mixing amine **20** with aldehyde **17** and CSA, the azonia-Cope rearrangement proceeded via a chair like transition state in favor of formation of the most substituted imine **15** (Scheme 3).4 According to mass spectrometry the formation of **15** was successful, which was confirmed by ^{1}H-NMR experiments that showed disappearance of the imine proton of intermediate **14**. Unfortunately, subsequent release of **21** upon quenching with $HONH_2$ in AcOH and methanol appeared unsuccessful. These attempts led us to pursue an alternative strategy.

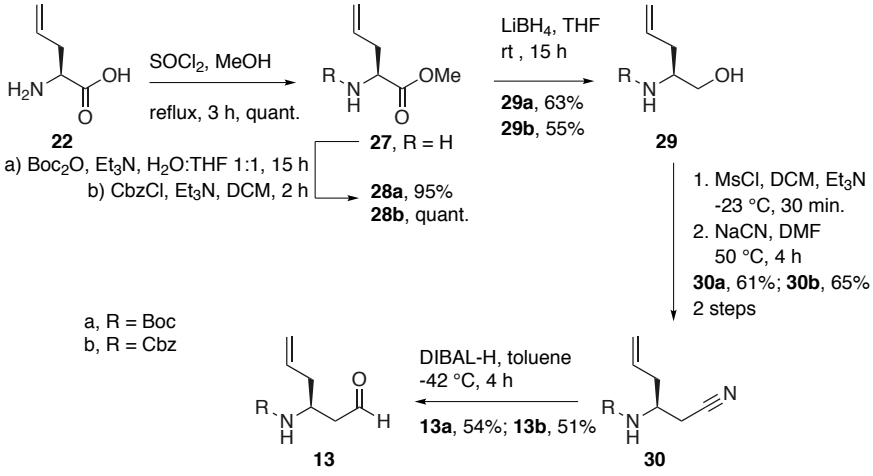
5.2.2 — Synthesis of aldehyde 13 via the Arndt-Eistert sequence

The second route towards aldehyde **13** started with commercially available (*S*)-allylglycine (**22**). Insertion of a methylene group using the Arndt-Eistert sequence should provide the desired aldehyde **13a** (Scheme 5).[6]



Scheme 5 — Synthesis of aldehyde 13a via Arndt–Eistert sequence

In the first step, (*S*)-allylglycine (**22**) was Boc-protected in 86% yield. Next, the Arndt-Eistert sequence started with activation of the acid moiety as a mixed anhydride. Subsequently, diazoketone **24** was formed by careful addition of freshly distilled diazomethane in diethylether. Next, a Wolff rearrangement of α-diazoketone **24** in the presence of *N*-methoxymethylamine (**25**) resulted in formation of the Weinreb amide **26** in an overall yield of 75% over three steps. In the final step, reduction of Weinreb amide **26** produced the desired aldehyde **13a** in moderate to good yields. The reduction with $LiAlH_4$ was superior to DIBAL-H giving yields of aldehyde **13a** of 80 and 30%, respectively. Drawback of this approach was the use of potentially explosive diazomethane, which use was limited to the size of the dedicated equipment and thus restricted to relatively small scale synthesis.[7]

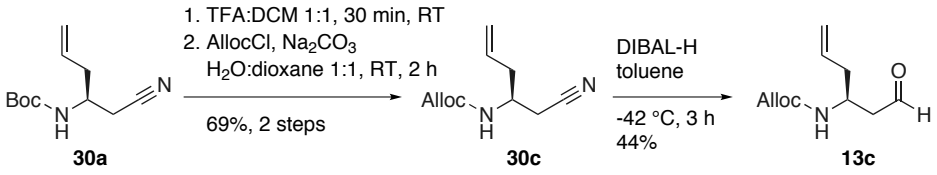


Scheme 6 — Synthesis of aldehyde 13a-b via cyanide substitution.

5.2.3 — Synthesis of aldehyde 13 via cyanide substitution

In order to arrive at a scalable synthesis, the additionally required methylene was also introduced via cyanide substitution (Scheme 6).

In the first step, commercially available (*S*)-allylglycine (**22**) was converted into the corresponding methyl ester HCl salt **27** in quantitative yield. Secondly, the free amine was protected with a Boc or Cbz protecting group giving the corresponding carbamates **28a** and **b** in 95% and 100% yield, respectively. Subsequent reduction with $LiBH_4$ led to the corresponding alcohols (63% for **29a**, 55% for **29b**), which were converted into the corresponding mesylates and substituted by NaCN in 61% (**30a**) and 65% yield (**30b**) over two steps. The desired aldehydes **13a** and **13b** were obtained via reduction of nitriles **30a** and **13b** with DIBAL-H in 54% (**31a**) and 51% yield (**31b**), respectively. This resulted in an overall yield of 20% for the Boc- (**13a**) and 18% for the Cbz-protected aldehyde (**13b**) over six steps. The scalable character of this sequence was demonstrated by the synthesis of ca. 5 g of aldehyde **13b**.

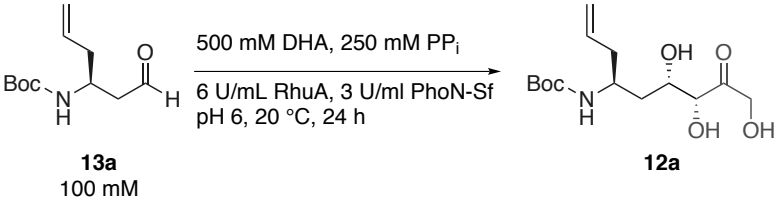


Scheme 7 — Synthesis of Alloc-protected aldehyde 13c.

The Alloc-protected aldehyde **13c** was also prepared, which proceeded through deprotection of Boc-protected cyanide **30a** with TFA, followed by standard Alloc protection using AllocCl (69% yield, Scheme 7). Subsequent reduction of the cyanide resulted in the desired aldehyde **13c** in 44% yield.

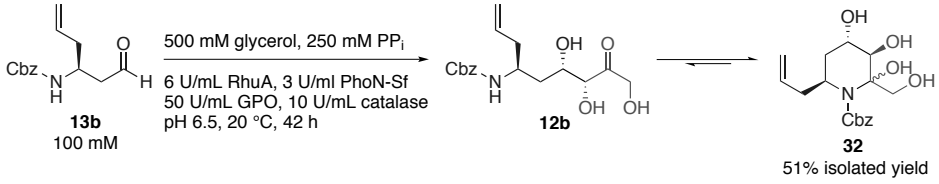
5.2.4 — Initial results with DHAP or DHA/borate and RhuA

In order to obtain some initial information on the substrate scope of RhuA, Boc-protected aldehyde **13a** was subjected to the standard conditions of the two-enzyme cascade using DHA, PhoN-Sf and RhuA from the thermophillic organism *Thermatoga maritima* on 1 and 10 mL scale (Scheme 8).



Scheme 8 — Two-enzyme cascade with aldehyde 13a and RhuA.

The first attempt to synthesize **12a** resulted in a 7% isolated yield using 100 mM aldehyde, 500 mM DHA and 250 mM PP_i with 6 U/mL RhuA and 3 U/mL PhoN-Sf on preparative scale (10 mL). These results could, however, not be reproduced despite multiple attempts to scale up and isolate this product. According to HPLC analysis, the phosphorylation of DHA proceeded smoothly, but no consumption of aldehyde **13a** took place. A test reaction with propanal, an excellent substrate for RhuA (Section 3.2), did not result in the desired product either, indicating deactivation of the aldolase over time. A possible explanation could be leaching of zinc from the active site of the enzyme. The Clapés group has prepared RhuA aldolases, both wild type (WT) and mutants, from *E. coli* and replaced the zinc with cobalt, to make the enzyme more stable.[8] Aldehydes **13a-c** (65 mM) were tested with RhuA from *E. Coli* WT and its N29D mutant in the presence of DHAP (100 mM) at neutral conditions (pH = 6.9), at 20 °C for 48 h. Parallel to these reactions, the aldehydes were tested with DHA (100 mM) in a borate buffer (200 mM, pH = 7). Wong *et al*. demonstrated that borate could mimic the phosphate ester of DHAP in aldolase-catalyzed reactions.[9] After analysis of the HPLC spectra, the reaction with DHAP and RhuA WT gave the most promising results with Cbz as a protecting group, because it showed the highest and cleanest conversions into desired aldol product **12b** (Figure 2). HPLC analysis also indicated the formation of a second product, which might be the other diastereoisomer (Section 3.2) or the cyclic aldol product **32** (Scheme 9). Motivated by this result, the one-pot four-enzyme cascade was conducted on a larger scale with aldehyde **13b** and RhuA WT to isolate the product for full characterization.



Scheme 9 : Enzymatic cascade with aldehyde **13b** and RhuA WT.

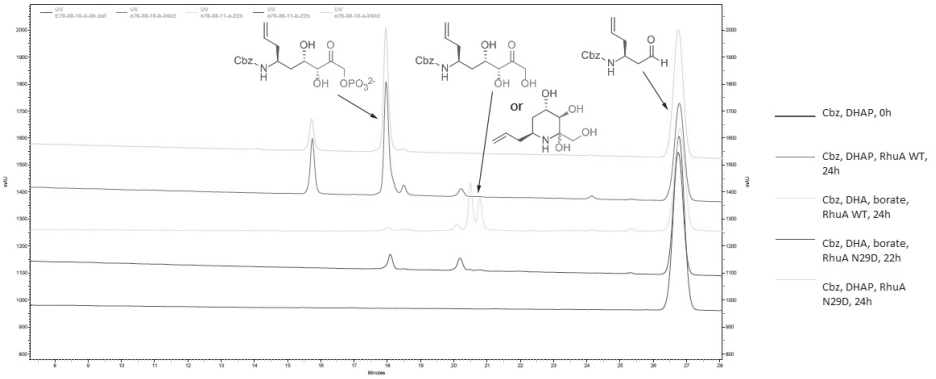


Figure 2 — HPLC analysis of screening reactions with aldehyde 13b and DHAP or DHA/borate.[1]
[1]These reactions were analyzed on a Varian HPLC with RP C-18 Bruker-Michrom at Biotransformation and Bioactive Molecules at the Catalonia Institute for Advanced Chemistry.

5.2.5 — Enzymatic cascade reaction with aldehyde 13b

Cbz-protected aldehyde **13b** (100 mM) was subjected to RhuA WT (6 U/mL) and *in situ* generated DHAP from glycerol (500 mM), PP_i (250 mM), PhoN-Sf (3 U/mL) and GPO (50 U/mL). Catalase (10 U/mL) was added to convert the formed hydrogen peroxide into water and oxygen. The reaction was performed in 1 mL at 20 °C at a pH of 6.5 over 24 h (Scheme 9). Every two hours the reaction mixture was analyzed with HPLC (Figure 3).

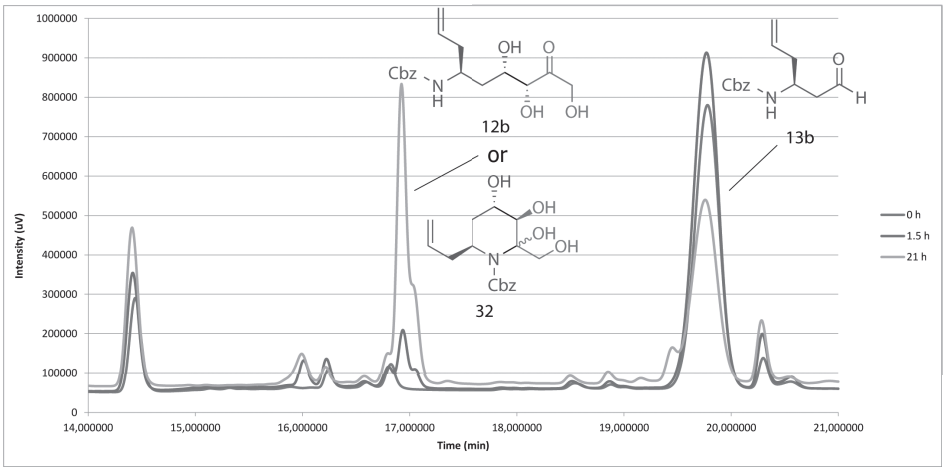


Figure 3 — HPLC analysis during the enzymatic cascade reaction with RhuA WT and 12b.[1]
1 These measurements were conducted on a Shimadzu HPLC with a RP C-18 column from Grace, which led to different retention times and the peaks of 12b and 32 were not separated as compared to Figure 2.

After 42 hours, the reaction was stopped at 65% conversion, which after work-up led to a 51% isolated yield based on recovered aldehyde. TLC analysis of the product showed

multiple products, which could indicate an equilibrium between the open aldol product **12b** and the closed hemiaminal **32**. ^{1}H-NMR analysis in CD_3OD solely revealed a 1:1 mixture of diastereoisomers of the cyclic product (Figure 4).

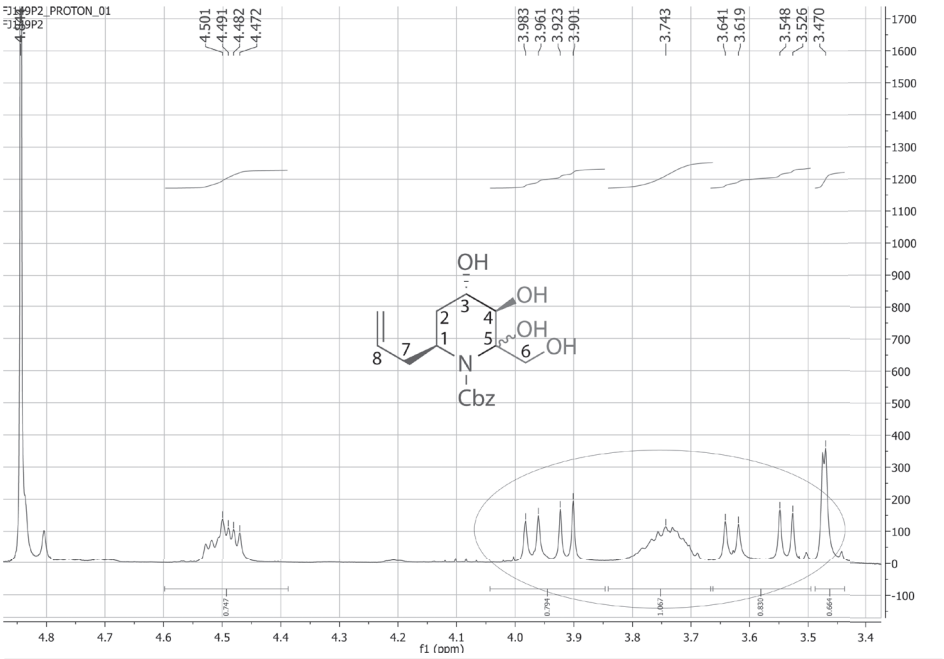
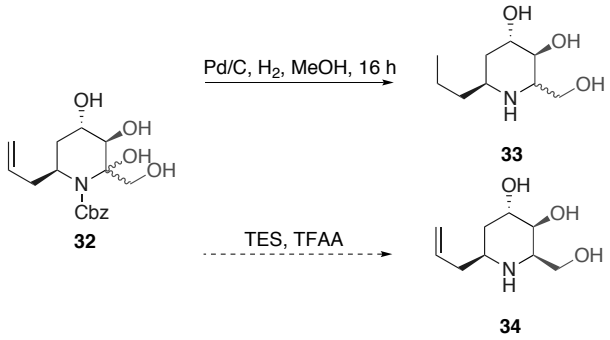


Figure 4 — Relevant part of the ^{1}H-NMR spectrum of hemiaminal 32.

All previous cascade products ^{1}H-NMR showed a distinct AB system around 4.5 ppm and portrayed the protons of the methylene group stemming from DHA (C6 and Section 3.5). The product from the cascade with aldehyde **13b**, however, showed two AB systems at 3.96 and 3.63 ppm (J = 8.8 Hz, 2H C6-I) and 3.93 and 3.48 ppm (9.0 Hz, 2H C6-II), indicating the formation of two diastereoisomers in a 1:1 ratio, which suggests formation of hemiaminal **32**.

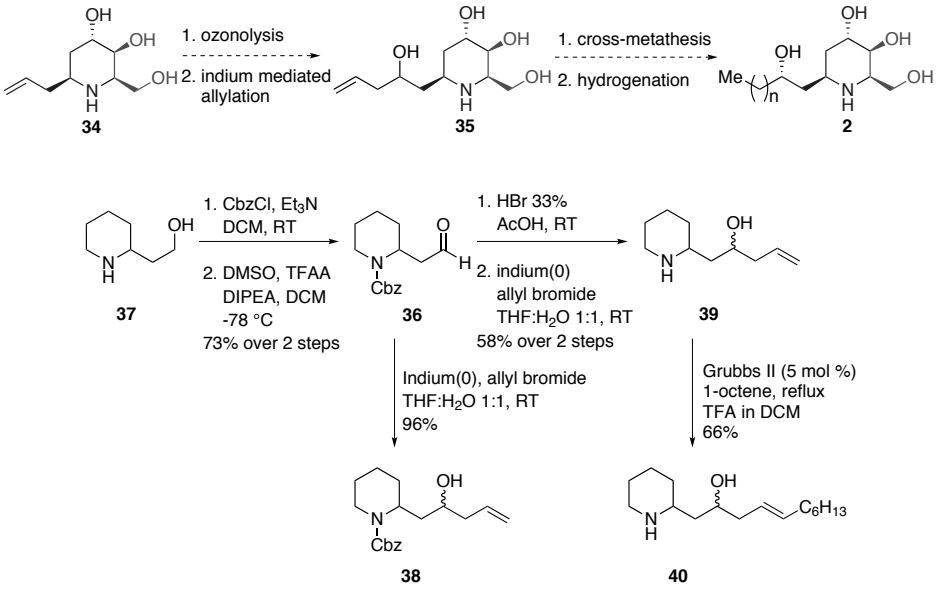
The next steps, which would also provide further proof of the structure of **32**, involved nitrogen deprotection and reductive amination of the hemiaminal product to furnish cyclic product **33** (Scheme 10). The first attempt to remove the Cbz-protecting group was performed with 33% HBr in acetic acid, but unfortunately degradation of the product was observed. Therefore the reaction was repeated in the presence of catalytic Pd/C under a hydrogen atmosphere, however this would also hydrogenate the unsaturated side chain (Scheme 10). The desired product was observed with mass spectrometry ([M+H] = 190, **33**), but isolation and purification of the product were unsuccessful. We postulated that the formation of **34** could be realized via a reductive amination with trietylsilane and trifluoroacetic acid.[12]



Scheme 10 : Hydrogenation of hemiaminal **32**.

5.2.6 — Strategy for the final steps towards batzellasides A–C

Simultaneous to the cascade reaction, a strategy was developed for follow up chemistry towards the batzellasides from the projected intermediate iminosugar **34** (Scheme 11). After formation of the cyclic product **34**, the side chain might be introduced via ozonolysis of the double bond (either with or without a protecting group on the nitrogen), followed by indium-mediated allylation[13] of the resulting aldehyde to produce iminosugar **35**. Introduction of the various side chains would occur via cross-metathesis and hydrogenation of the double bond. The feasibility of the latter steps was probed on model compound **36**.



Scheme 11 — Final steps towards batzellaside 2.

Model compound **36** was synthesized via *N*-protection and Swern-Moffatt oxidation of commercially available amino alcohol **37** in 76% yield over two steps (Scheme 11). Aldehyde **36** was directly allylated to give allyl alcohol **38** via an indium-mediated Barbier type reaction with allyl bromide in excellent yield (1:1 mixture of diastereoisomers).[13] An extra benefit of this approach is the use of water as solvent, which is favorable for the solubility of the highly polar iminosugars.

An ideal route would proceed without the protecting group, so that the allylation was also tested on the unprotected piperidine ring. Cbz-protected aminoaldehyde **36** was deprotected with HBr (33%) in acetic acid to obtain the deprotected amine as the HBr salt. Next, the indium-mediated allylation yielded allyl alcohol **39** in 58% as a 1:1 mixture of diastereoisomers over two steps. Finally, the side chain was introduced via cross-metathesis in the presence of the Grubbs-II catalyst. The reaction was initially performed on olefin **39** under neutral conditions, but this caused the free amine to coordinate to the catalyst and no reaction took place. Therefore the reaction was conducted in the presence of 1 equivalent of TFA to provide alkene **40** in 66% yield. Due to the difficulty to synthesize iminosugar **34**, these model reactions were not performed on the intermediate that leads to the batzellasides A–C. Nevertheless, the reactions might be applied in the synthesis of one of the batzellasides once iminosugar **34** becomes available.

5.3 — Conclusion

A chemoenzymatic approach to the naturally occurring batzellasides was pursued. The required precursor aldehyde was obtained via a straightforward synthesis starting from commercially available (*S*)-allylglycine using a cyanide substitution to introduce the aldehyde carbon atom in an overall yield of 18%. The four enzyme cascade reaction was performed on the aldehyde at pH 6.5 using RhuA WT to provide the desired aldol product as the corresponding cyclic hemiaminal in 51% isolated yield. Unfortunately, attempts to convert the hemiaminal via reductive amination into the desired iminosugar were unsuccessful. To probe the feasibility of the final steps, 2-piperidylacetaldehyde was used as a model compound, on which the side chain was introduced in excellent yields via indium-catalyzed allylation and subsequent cross-metathesis.

5.4 — Acknowledgements

Prof. Pere Clapés and Dr. Xavier Garrabou (Biotransformation and Bioactive Molecules, Catalonia Institute for Advanced Chemistry (CSIC), Barcelona, Spain) are greatly acknowledged for their help with RhuA aldolase and donation of the enzyme and plasmid.

Bram van Weerdenburg, Danny van Kol and Freek Janssen are kindly acknowledged for their synthesis efforts described in this chapter. Frank Haasjes is acknowledged for his contributions to the synthesis of the aldehyde. Lara Babich (J. H. van 't Hoff Institute for Molecular Sciences, University of Amsterdam) and Pierpaolo Falcicchio (Laboratory of Microbiology, Wageningen University) are kindly acknowledged for their cooperation and contributions to this chapter.

5.5 — Experimental

5.5.1 — General information

For the general information see Section 2.5.1

5.5.2 — Enzymes

For recombinant *Shigella flexneri* acid phosphatase (PhoN-Sf) , catalase from bovine liver, L-glycerol-3-phosphate oxidase and recombinant rhamnulose-1-phosphate aldolase from the thermophilic organisms *T. maritima* is referred to Section 3.5.2.

RhuA N29D mutant was received from the Clapés group:[8] the plasmids were transformed into *E. coli* strain M-15 [pREP-4] (QIAGEN). Cells were grown at 37°C in 5L of an LB medium containing ampicillin (100 mgL^{-1}) and kanamycin (25 mgL^{-1}) up to an optical density of 0.6 at 600 nm.[14] For protein expression, the temperature was lowered to 30°C to avoid inclusion bodies formation and isopropyl-b-d-thiogalactoside (IPTG) was added to a final concentration of 50 mM. After additional 4 h cells were harvested, phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0 and lysed using a Cell Disrupter (Constant Systems). Cellular debris was removed by centrifugation at 12,000 g for 10 min. The clear supernatant was collected and purified by affinity chromatography on an FPLC system (Amersham biosciences). The crude supernatant was applied to a cooled HR 16/40 column (GE Healthcare), containing affinity beads (50 mL) and was washed with start buffer (150 mL). The protein was eluted with an aqueous buffered solution (pH 8.0) that contained disodium hydrogen phosphate (50 mM), NaCl (300 mM) and imidazole (300 mM), at a flow rate of 3 $mLmin^{-1}$. $CoCl_2$ (up to 1 mM) was added to the eluted protein, which was incubated for 15 min. Addition of $(NH_4)_2SO_4$ (0.4 g per mL of liquid) caused protein precipitation. The resulting pellet was centrifuged at 12,000 g for 10 min, suspended in $(NH_4)_2SO_4$ (50 mL, 0.4 gmL^{-1}) and centrifuged again. The pellet was finally suspended in $(NH_4)_2SO_4$ (50 mL, 0.4 gmL^{-1}) and stored at 4°C.[14]

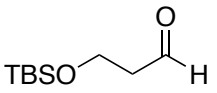
5.5.3 — Analytical methods and product characterization

The time course of the cascade reaction and the conversions were determined by HPLC that contained an Alltech OA 1000 organic acid column (0.65 × 30 cm) or C18 RP column. 20 µL of the reaction mixture was diluted 10-fold with MilliQ before injection on the HPLC. Isocratic runs were performed using 4.5 mM H_2SO_4, with a flow rate of 0.4 mL min^{-1} (OA 1000 organic acid column) or a gradient of 0 to 80% MeCN in MilliQ with 0.1% TFA (C18 RP column). The effluent was analyzed at 215 and 254 nm by UV detector and by refractive index detector.

5.5.4 — General procedure for the preparative scale one-pot four-enzyme cascade

To a solution of 500 mM glycerol, 250 mM PP_i (7:4 Na_2PP_i/Na_4PP_i),[15] and 100 mM aldehyde in water, 10 U/mL catalase from bovine liver, 6 U/mL RuA WT, and 50 U/mL GPO were added. The reactions were started by adding 3 U/mL PhoN-Sf and incubated at 20 °C under mild shaking until completion. Dephosphorylation to the end product was completed after 24 h and 4 g of silica gel was added. The reaction slurry was concentrated under reduced pressure, and the silica gel was poured on top of a silica gel column (EtOAc:MeOH 19:1). The pure fractions were collected and concentrated under reduced pressure to give product **1** as a light-yellow oil.

5.5.5 — ((*tert*-Butyldimethylsilyl)oxy)propanal (17)

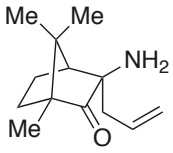


1,3-Propanediol (**16**, 4.01 g, 52.0 mmol) was dissolved in THF (dry, 250 mL) and cooled to 0 °C before NaH (3.51 g of 60 w% in mineral oil, 52.1 mmol) was added. After 1 h of stirring at 0 °C, TBSCl (7.80 g, 52.1 mmol) was added. The reaction mixture was stirred overnight at RT, quenched with water (150 mL) and diluted with DCM (200 mL). The separated water layer was extracted with DCM (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtrated and concentrated *in vacuo*. The product was purified with silica gel column chromatography (EtOAc: heptane 1:9 → 1:2) to give the mono-protected diol in 70% (6.91 g) yield.5

((*tert*-Butyldimethylsilyl)oxy)propanol (100 mg, 0.521 mmol) was dissolved in DCM (5 mL) and PCC (170 mg, 0.781 mmol) was added. The reaction mixture was stirred overnight, filtrated and the filtrate was concentrated *in vacuo*. The product was purified with silica gel column chromatography (DCM) to yield 54 mg (55%) of aldehyde **17**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.80 (t, *J* = 2.1 Hz, 1H), 3.99 (t, *J* = 6 Hz, 2H), 2.60 (dt, *J* = 6.0 Hz, 2.1 Hz,

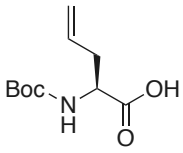
2H), 0.81 (s, 9H), 0.07 (s, 6H); υ_{max}(neat) cm^{-1} 2953, 2928, 2856, 1714, 1470, 1254, 1103. The analytical data were in accordance with previously reported results.[16]

5.5.6 — (1*R*,3*R*,4*S*)-3-Allyl-3-amino-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (20)



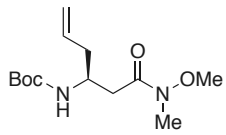
To a solution of (1*R*)-(–)-camphorquinone (**18**, 1.33 g, 8.01 mmol) in methanolic ammonia (ca. 7.0 M, 12 mL), allylboronic acid pinacol ester (**19**, 2.02 g, 12.0 mmol) was added dropwise at room temperature and stirred for 24 hours. 3.0 M aqueous HCl was added slowly to adjust the pH to 1. After stirring for 30 min, the aqueous layer was washed with DCM (3 x 50 mL), made alkaline with 6.0 M NaOH (pH ca. 10), and extracted with DCM (3 × 50 mL). The combined organic layers were dried (Na_2SO_4), filtrated and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (heptane:EtOAc 3:1) to yield amine **15** (1.32 g, 80%). $[\alpha]_D^{20}$ = +66.8, υ_{max}(neat) cm^{-1} 3386, 2973, 1741, 1637, 1454, 919, 883, 675; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.85 (ddd, *J* = 14.2 Hz, 7.2 Hz, 3.9 Hz, 1H), 5.24-5.12 (m, 2H), 2.21-2.30 (m, 1H), 2.12 (dd, *J* = 14.6 Hz, 7.9 Hz, 1H), 1.96-1.83 (m, 2H), 1.71-1.65 (m, 2H), 1.51-1.45 (m, 2H), 1.09 (s, 3H), 1.02 (s, 3H), 0.93 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 222.8, 132.8, 118.8, 61.4, 57.9, 52.1, 46.2, 42.0, 30.4, 22.8, 22.5, 20.4, 9.3. The analytical data were in accordance with previously reported results.4

5.5.7 — (*S*)-2-(*N*-Boc-amino)-4-pentenoic acid (23)



To a solution of (*S*)-2-amino-4-pentenoic acid (**22**, 2.00 g, 17.4 mmol) in a mixture of THF/H_2O (1:1, 100 mL), $NaHCO_3$ (4.43 g, 52.7 mmol) and Boc_2O (4.60 g, 21.1 mmol) were added successively at 0 °C. After 30 min, the reaction mixture was warmed to RT and stirred overnight. The solution was extracted with Et_2O (300 mL) and the aqueous layer was acidified to pH 4 by careful addition of half saturated citric acid at 0 °C, followed by extraction with EtOAc (3 × 100mL). The combined organic fractions were dried and concentrated *in vacuo* to yield (*S*)-2-(*N*-Boc-amino)-4-pentenoic acid (**23**, 3.44 g, 15.2 mmol, 86%) as a colorless oil. $[\alpha]_D^{20}$ = +12.1 (*c* = 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.56 (br s, 1H), 6.23 (br s, 1H), 5.90-5.70 (m, 1H), 5.19-5.06 (m, 3H), 4.45-4.35 (m, 1H), 2.70-2.45 (m, 2H), 1.45 (s, 9H); 176.1, 155.0, 131.6, 118.9, 65.4, 52.3, 35.9, 27.8; υ_{max}(neat) cm^{-1} 3398, 2977, 1697; HRMS (ESI) *m/z* calcd for $C_{11}H_{20}NO_4$: 229.1392, found 230.1387 $[M+H]^+$. The analytical data were in accordance with previously reported results.[17]

5.5.8 — (*S*)-3-(*N*-Boc-amino)-*N*-methoxy-*N*-methyl-5-hexenamide (26)

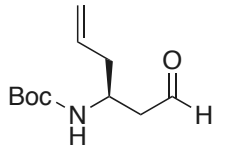


The compound was prepared according to the method described by Briand et al.[18] Solution **A**: a solution of *N,O*-dimethylhydroxylamine (**25**, 0.45 g, 4.61 mmol) and Et_3N (0.62 g, 6.14 mmol) in THF (30 mL) was stirred overnight and filtrated, followed by the addition of Et_3N (0.642 mL, 4.61 mmol).

Solution **B**: to a solution of pentenoic acid **23** (0.331 g, 1.54 mmol) in THF (12 mL) were added Et_3N (0.214 mL, 1.54 mmol) and ethyl chloroformate (0.222 mL, 1.70 mmol) at -15 °C. After 15 min, the solution was allowed to warm to RT.

To a solution of Diazald (1.65 g, 7.68 mmol) in Et_2O (30 mL), a solution of KOH (0.31 g, 5.53 mmol) in EtOH (10 mL) was added slowly at 0 °C. After 5 min, diazomethane in Et_2O was distilled off at 0 °C, followed by the addition of solution **B**. The solution was stirred at RT until a homogeneous mixture was formed. Upon completion, it was quenched with H_2O (20 mL), and the organic phase was successively washed with saturated aqueous $NaHCO_3$ (70 mL), saturated aqueous NH_4Cl (70 mL) and brine (70 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Solution **A** was added to the residue, followed by the addition of silver benzoate (0.17 g, 0.75 mmol). The mixture was placed in a sonication bath for 30 min. The resulting mixture was concentrated and purified via column chromatography (EtOAc:heptane 1:1) to yield Weinreb amide **26** (0.25 g, 0.92 mmol, 60%) as a yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.78 (ddt, *J* = 17.2, 10.2, 7.1 Hz, 1H), 5.41 (d, *J* = 4.0 Hz, 1H), 5.16-4.96 (m, 2H), 4.12 (q, *J* = 7.1 Hz, 1H), 4.00 (tdd, *J* = 6.9, 6.4, 3.7 Hz, 1H), 3.67 (s, 3H), 3.17 (s, 3H), 2.70-2.58 (m, 1H), 2.48-2.32 (m, 1H), 1.74-1.58 (m, 1H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.0, 154.9, 134.3, 117.1, 60.7, 46.7, 38.4, 34.9, 31.4, 27.9, 25.9; $[\alpha]_D$ = -8.3 (c = 1.0, CH_3OH); υ_{max}(neat)cm^{-1} 3343, 2974, 1708; LRMS (ESI) *m/z* calcd for $C_{13}H_{24}N_2O_4$: 272.17, found 273.1 $[M+H]^+$, 295.2 $[M+Na]^+$

5.5.9 — *N*-Boc-(*S*)-3-amino-5-hexenal (13a)

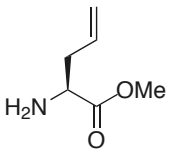


*Compound **13a** was prepared according to the method described by Silverman et al.*[19]

To a solution of Weinreb amide **26** (0.25 g, 0.91 mmol) in THF (dry, 20 mL), was added $LiAlH_4$ (0.038 g, 0.96 mmol) at -78 °C under an inert atmosphere while stirring vigorously. The dry-ice bath was removed after 30 min and the reaction mixture was stirred an additional 30 min at RT. It was quenched subsequently with 38 µL water, 38 µL 15 % aqueous NaOH and 90 µL water. The reaction mixture was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The resulting residue was dissolved in EtOAc (50 mL) and washed successively with cold aqueous HCl (1 M, 3 × 10 mL), saturated aqueous $NaHCO_3$ (2 × 50 mL) and brine (50 mL).

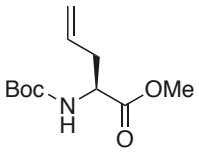
The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure to yield aldehyde **13a** (0.15 g, 0.66 mmol, 73%) as a colorless oil. R_f 0.50 (EtOAc:heptane 1:1); $[\alpha]_D^{20}$ = -8.4 (c = 1.00, DCM), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.76 (m, 1H), 5.75 (m, 1H), 5.02-5.19 (m, 2H), 4.71 (s, 1H), 4.12 (m, 1H), 2.62 (m, 2H), 2.32 (t, *J* = 6.9 Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 200.4, 154.7, 133.2, 118.0, 47.6, 45.5, 38.6, 27.9; υ_{max} (film) cm^{-1}: 3334, 2984, 1683, 1493, 1363, 1251, 1164, 1043, 1000, 914, 767. HRMS (ESI) *m/z* calcd for $C_{11}H_{20}NO_3$: 213.1443, found 214.1431 $[M+H]^+$.

5.5.10 — Methyl (*S*)-2-amino-4-pentenoate (27)



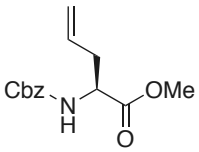
To a solution of (*S*)-2-amino-4-pentenoic acid (**22**, 5.02 g, 43.6 mmol) in methanol (125 mL) was added dropwise thionyl chloride (4 mL, 54.8 mmol) over a period of 5 minutes at 0 °C. The reaction mixture was heated to reflux for 3 hours and concentrated *in vacuo* to obtain (*S*)-methyl-2-amino-4-pentenoate **27** in quantitative yield (7.66 g, 43.6 mmol) as a white solid (HCl salt) after co-evaporation with toluene (50 mL). R_f 0.44 (MeOH:DCM 1:9); ^{1}H NMR (CD_3OD, 300 MHz) δ 8.82 (s, 2H), 5.86-5.72 (m, 1H), 5.33-5.26 (m, 2H), 4.17 (t, *J* = 6.3 Hz, 1H), 3.84 (s, 3H), 2.77-2.64 (m, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 170.3, 131.6, 121.6, 53.6, 53.5, 35.7; $[\alpha]_D^{20}$ = +28.4 (*c* = 0.10, $CHCl_3$); υ_{max} (film) cm^{-1} 2999, 2857, 1739; HRMS (ESI) *m/z* calcd for $C_6H_{12}NO_2$: 129.0868, found 130.0883 $[M+H]^+$. The analytical data were in accordance with previously reported results.[20]

5.5.11 — Methyl *N*-Boc-(*S*)-2-amino-4-pentenoate (28a)



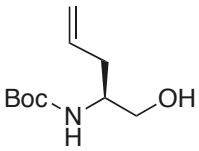
To a solution of **27** (560 mg, 4.34 mmol) in THF/H_2O (25 mL, 1:1) was added $NaHCO_3$ (1.10 g, 13.1 mmol) and di-*tert*-butyl dicarbonate (1.14 g, 5.22 mmol) consecutively at 0 °C. After 30 minutes the ice bath was removed and the reaction was stirred overnight at RT. The solution was extracted with EtOAc (3 × 25 mL). The organic layers were combined, dried (Na_2SO_4) and concentrated *in vacuo* to afford **28a** (835 mg, 84%) as a colorless oil. R_f 0.84 (MeOH:DCM 1:9); ^{1}H NMR (CD_3OD, 300 MHz) δ 5.84-5.70 (m, 1H), 5.15-5.09 (m, 2H), 4.20-4.15 (m, 1H), 3.71 (s, 3H), 2.56-2.47 (m, 1H), 2.45-2.35 (m, 1H), 1.44 (s, 9H); ^{13}C NMR (CD_3OD, 75 MHz) δ 174.2, 157.9, 134.5, 118.6, 80.6, 54.9, 52.6, 37.1, 28.7. $[\alpha]_D^{20}$ = -9.1 (*c* 1.0, CH_3OH); υ_{max} (film) cm^{-1} 3385, 2980, 2359, 1715, 1065, 777; HRMS (ESI) *m/z* calcd for $C_{11}H_{20}NO_4$: 229.1392, found 230.1387 $[M+H]^+$. The analytical data were in accordance with previously reported results.[21]

5.5.12 — Methyl *N*-Cbz-(*S*)-2-amino-4-pentenoate (28b)



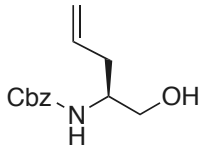
To a solution of (*S*)-methyl-2-amino-4-pentenoate (**41**, 4.42 g, 26.7 mmol) in 200 mL THF/H_2O (1:1) were added successively sodium bicarbonate (8.78 g, 105 mmol) and CbzCl (4.19 mL, 29.4 mmol) at 0 °C. After 30 min the ice bath was removed and the reaction was stirred for 16 hours at RT. The reaction was quenched with aqueous HCl (1 M, 30 mL) and extracted with DCM (3 × 20 mL). The combined organic layers were dried and concentrated *in vacuo* to afford ester **28b** in quantitative yield (7.44 g, 26.7 mmol) as a colorless oil. R_f 0.75 (EtOAc:heptane 1:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.35 (m, 5H), 5.68 (m, 1H), 5.15-5.09 (m, 4H), 4.45 (q, 1H), 3.74 (s, 3H), 2.55 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.3, 155.8, 129.5, 129.0, 129.0, 128.7, 128.3, 128.2, 119.5, 67.0, 53.4, 36.9; $[\alpha]_D^{20}$ = +9.1 (c = 1.0, DCM); υ_{max} (film) cm^{-1} 3341, 2952, 2359, 1718; HRMS (ESI) *m/z* calcd for $C_{14}H_{17}NO_4$: 163.1236, found: 164.1244 $[M+H]^+$. The analytical data were in accordance with previously reported results.[22]

5.5.13 — *N*-Boc-(*S*)-2-amino-4-pentenol (29a)



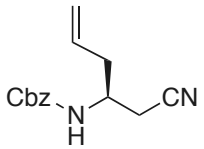
To a solution of ester **28a** (10.5 g, 45.6 mmol) in THF (250 mL) was added $LiBH_4$ (2.49 g, 114 mmol) portion wise at −5 °C. The mixture was allowed to warm to RT and stirred overnight. After completion, the reaction mixture was cooled to 0 °C and carefully quenched with aqueous saturated $KHCO_3$. The organic phase was washed with aqueous saturated Na_2SO_4 (2 × 100 mL), brine (100 mL). And subsequently dried with Na_2SO_4 and concentrated *in vacuo* to yield alcohol **29a** (6.08 g, 30.2 mmol, 63 %) as a colorless oil. R_f 0.21 (EtOAc:heptane 1:2); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.73 (ddt, *J* = 17.2, 10.1, 7.1 Hz, 1H), 5.08 (dd, *J* = 3.3, 1.4 Hz, 2H), 5.06-4.99 (m, 1H), 4.93 (d, *J* = 7.4 Hz, 1H), 3.72-3.47 (m, 3H), 3.40 (s, 1H), 2.39-2.05 (m, 2H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.8, 133.8, 117.3, 79.1, 64.1, 51.6, 35.4, 27.9; $[\alpha]_D^{20}$ = +3.7 (c = 1.0, MeOH); υ_{max} (film) cm^{-1} 3344, 1685; LRMS (ESI) *m/z* calcd for $C_{10}H_{19}NO_3$: 201.14, found 202.0 $[M+H]^+$, 224.1 $[M+Na]^+$. The analytical data were in accordance with previously reported results.[21, 23]

5.5.14 — *N*-Cbz-(*S*)-2-amino-4-pentenol (29b)



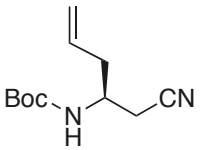
This compound was synthesized via method 5.5.13 from ***28b*** *(7.44 g, 28.3 mmol) and resulted in* ***29b*** *(4.20 g, 17.85 mmol) in 63% isolated yield*. R_f 0.40 (EtOAc:heptane 1:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.41-7.32 (m, 5H), 5.80 (ddt, *J* = 17.4, 10.2, 7.2 Hz, 1H), 5.14 (ddd, *J* = 9.5, 3.6, 1.4 Hz, 4H), 4.96 (s, 1H), 3.87-3.59 (m, 3H), 2.33 (td, *J* = 14.1, 7.0 Hz, 3H), 1.64 (s, 1H).; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 134.0, 128.7, 128.4, 128.3, 118.6, 67.0, 65.2, 52.7, 35.9; $[\alpha]_D^{20}$ = +24.0 (c = 1.0, DCM); υ_{max} (film) cm^{-1} 3400, 2359, 1696; HRMS (ESI) *m/z* calcd for $C_6H_{11}NO_2$: 235.1208, found 236.1301 $[M+H]^+$. The characterization was in accordance with previously reported results.[21]

5.5.15 — *N*-Cbz-(*S*)-3-amino-5-hexenenitrile (30b)



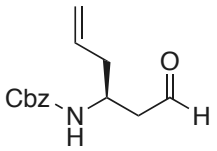
The compound was prepared according to the method described by Beyermann et al.[24] To a solution of *N*-Cbz-(*S*)-2-amino-4-pentenol (**29b**, 3.87 g, 16.5 mmol) in dry DCM (150 mL) was added triethylamine (3.80 mL, 27.3 mmol) and methanesulfonyl chloride (1.70 mL, 21.8 mmol) at 50 °C. After one hour the reaction mixture was warmed to RT and diluted with heptane (100 mL), and successively washed with saturated aqueous $KHCO_3$ (100 mL), brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo* to yield the mesylate as an off-white solid. This was dissolved in DMF (150 mL) and NaCN (0.95 g, 19.4 mmol) was added. The mixture was heated for 4 hours at 50 °C. After completion water (250 mL) was added to the mixture and the organic phase was extracted with Et_2O (2 × 250 mL). The combined organic layers were washed with water (250 mL), brine (250 mL), dried (Na_2SO_4) and concentrated *in vacuo* to yield *N*-Cbz-(*S*)-3-amino-5-hexenenitrile (**30b**, 2.07 g, 8.46 mmol, 51% yield) in two steps as a colorless oil. R_f 0.70 (EtOAc:heptane 1:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.41-7.30 (m, 5H), 5.26-5.17 (m, 2H), 5.11 (s, 2H), 4.92 (s, 1H), 3.95 (dd, *J* = 11.9, 5.4 Hz, 1H), 2.69 (ddd, *J* = 20.9, 16.8, 4.9 Hz, 2H), 2.42 (t, *J* = 7.1 Hz, 2H).; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 136.1, 132.3, 128.8, 128.5, 128.3, 120.1, 117.1, 67.3, 47.3, 37.77, 23.08; $[\alpha]_D^{20}$ = -10.3 (c = 1.0, DCM); υ_{max} (film) cm^{-1}, 2857, 2240, 1739; HRMS (ESI) m/z calcd for $C_{14}H_{16}N_2O_2$: 244.1297 found 245.1290 $[M+H]^+$.

5.5.16 — *N*-Boc-(*S*)-3-amino-5-hexenenitrile (30a)



*This compound was synthesized via method 5.5.15 from **29a** (6.08g, 30.2mmol) and resulted in **30a** (1.96g, 9.33mmol) in 30% isolated yield*. R_f 0.54 (EtOAc:heptane 1:1); ^{1}H NMR (300 MHz) δ 5.76-5.70 (m, 1 H), 5.25-5.19 (m, 2 H), 4.67 (s, 1 H), 3.89 (s, 1 H), 2.78-2.70 (m, 1 H), 2.57 (dd, *J* = 4.2 Hz, 1H), 2.40 (t, *J* = 7.1 Hz, 1H), 1.45 (s, 9 H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.1, 132.6, 119.8, 117.3, 80.4, 46.8, 37.9, 28.4, 23.1; $[\alpha]_D^{20}$ = -20.2 (c = 1.1, DCM); υ_{max} (film) cm^{-1}, 3364, 2242, 1678; LRMS (ESI) *m/z* calcd for $C_{11}H_{18}N_2O_2$: 210.14 found 211.1 $[M+H]^+$, 233.1 $[M+Na]^+$.

5.5.17 — *N*-Cbz-(*S*)-3-amino-5-hexenal (13b)

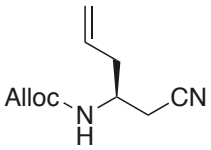


The compound was prepared according to the method described by Ibuka et al.[25]

To a solution of *N*-Cbz-(*S*)-3-amino-5-hexenenitrile (**30b**, 391 mg, 1.60 mmol) in dry toluene (50 mL) was added DIBAL-H (2 mL, 2.00 mmol) at -42 °C over a period of 3 hours. After full conversion, saturated aqueous citric acid (10 mL) was added dropwise over a period of 20 minutes and the reaction mixture was allowed to warm up to RT and subsequently extracted with Et_2O (3 × 30 mL). The water layer was neutralized with saturated $NaHCO_3$ and extracted with Et_2O (3 × 30 mL). The organic layer was washed with saturated $NaHCO_3$ (1 × 40 mL), dried with Na_2SO_4, filtrated and concentrated *in vacuo*. After column purification *N*-Cbz-(*S*)-3-amino-5-hexenenitrile (**12b**, 215 mg, 0.869 mmol) was obtained in 54% yield as a white solid. R_f 0.60 (EtOAc:heptane 1:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.75 (s, 1H), 7.45-7.26 (m, 5H), 5.87-5.62 (m, 1H), 5.21-5.02 (m, 2H), 4.96 (s, 1H), 2.83-2.65 (m, 2H), 2.35 (t, *J* = 4.6 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 200.9, 155.9, 133.6, 128.7, 128.3, 128.2, 119.3, 67.0, 47.9, 46.6, 39.1; $[\alpha]_D^{20}$ = -24.5 (c = 0.75, DCM); υ_{max} (film) cm^{-1} 2938, 2361, 1747; HRMS (ESI) *m/z* calcd for $C_{14}H_{17}NO_3$: 247.12867, found 248.12846 $[M+H]^+$.

*N-Boc-(S)-3-amino-5-hexenal (**13a**) was obtained in the same fashion from N-Boc-(S)-3-amino-5-hexenenitrile (**30a**, 0.250 mg, 1.19 mmol) and resulted in the desired product in 54% yield (138 mg, 0.649 mmol).*

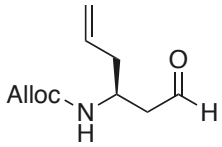
5.5.18 — *N*-Alloc-(*S*)-3-amino-5-hexenenitrile (30c)



To a solution of *N*-Boc-(*S*)-3-amino-5-hexenenitrile (**30a**, 268 mg, 1.28 mmol) in DCM (10 mL), was added dropwise TFA (10 mL, 1.28 mmol) at 0 °C. The reaction mixture was warmed to RT after 30 minutes and stirred for 2 h and was subsequently concentrated *in vacuo* to yield (*S*)-3-amino-5-hexenenitrile TFA salt (290 mg, 1.30 mmol, 100%) as a white solid.

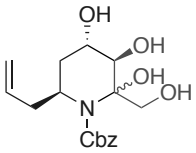
(*S*)-3-Amino-5-hexenenitrile TFA salt (150 mg, 0.670 mmol) was dissolved in water (30 mL) and sodium carbonate (355 mg, 3.35 mmol) was added while stirring. The resulting solution was cooled to 5 °C and allyl chloroformate (0.143 mL, 1.34 mmol) was added slowly as a cooled solution in 1,4-dioxane (30 mL). The resulting mixture was stirred at 0 °C for 1.5 h and allowed to warm to RT. Water (50 mL) was added and the aqueous layer was extracted with EtOAc (2 × 50 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (2 × 75 mL). The combined aqueous layers were acidified to pH 1 with 2 M aqueous HCl and extracted with EtOAc (3 × 75 mL). The combined organic layers were dried (Na_2SO_4) and concentrated *in vacuo*. The resulting oil was purified with column chromatography (EtOAc:heptane 1:1) to obtain *N*-Alloc-(*S*)-3-amino-5-hexenenitrile (**30c**, 90 mg, 69%). R_f 0.45 (EtOAc:heptane 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.91 (m, 1 H), 5.72 (m, 1 H) , 5.25 (m, 4 H), 4.88 (m, 1 H), 4.57 (d, *J* = 5.5 Hz, 2H), 3.94 (m, 1 H), 2.77 (dd, *J* = 5.0, 16.3 Hz, 1H), 2.59 (dd, *J* = 4.3, 16.8 Hz, 1H), 2.42 (t, 2 H).

5.5.19 — *N*-Alloc-(*S*)-3-amino-5-hexenenitrile (13c)



This compound was synthesized via method 5.5.17 from ***30c*** *(80.0 mg, 0.412 mmol) and resulted in* ***13a*** *(35.1 mg, 0.181 mmol) in 44% isolated yield*. R_f 0.40 (EtOAc:heptane, 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.76 (s, 1 H), 5.95-5.89 (m, 1 H), 5.81-5.72 (m, 1 H), 5.30-5.15 (m, 4 H), 4.99-4.85 (m, 1 H), 4.54 (d, *J* = 4.9 Hz, 2H), 4.20-4.12 (m, 1 H), 2.72-2.63 (m, 2 H), 2.42-2.29 (m, 2H).

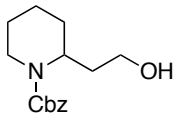
5.5.20 — *N*-Cbz-(3*R*,4*S*,6*S*)-6-allyl-2-(hydroxymethyl)piperidine-2,3,4-triol (32)



To a solution of glycerol (1.00 mL, 500 mM), PP_i (296 mg, 250 mM, 3:2 Na_2PP_i/Na_4PP_i) was added 1M aqueous NaOH solution until pH 6.5. Then aldehyde **13b** (192 mg, 500 µL, 1 M) in DMSO was added after which catalase (20 µL, 10 U/mL) from bovine liver, RhuA (2892 µL, 6 U/mL) and GPO (884 µL, 50 U/mL) were added. The total volume of the reaction was set with MilliQ to 4.43 mL and PhoN-Sf (667 µL, 3

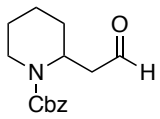
U/mL) was added, after which the reaction mixture was incubated at 20 °C for 25 h while the reaction mixture was gently shaken. The reaction mixture was analyzed with HPLC every 2 h and after 25 h it was precipitated in MeOH (50 mL), filtered and the filtrate was concentrated *in vacuo*. The crude product was purified using column chromatography (EtOAc:MeOH 19:1). A conversion of 65% was obtained, based on recovered starting material. 85.1 mg of product **32** was isolated (multiple spots on TLC (EtOAc:DCM 1:2), which is 51% yield based on product mass). R_f 0.5-0.6 (EtOAc:MeOH 19:1); ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.30 (m, 5 H), 5.74 (m, 1 H), 5.22-4.95 (m, 4 H), 4.47 (m, 1 H), 3.74 (m, 1 H), 3.96 and 3.63 (*J* = 8.75 Hz, 2 H C6-I), 3.93 and 3.48 ppm (9.02 Hz, 2 H C6-II), 3.47 (m, 1 H), 2.22 (m, 2 H), 1.71 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 157.5, 156.89, 138.4, 138.2, 129.4, 129.3, 129.3, 128.7, 128.6, 128.5, 122.1, 117.9, 117.6, 115.3, 97.8, 96.6, 75.3, 72.5, 70.9, 66.9, 66.6, 65.8. υ_{max} (film) cm^{-1} 3329, 3069, 2927, 2359, 2330, 1695, 1534, 1262, 1233, 1056, 698. LRMS (ESI) *m/z* calcd for $C_{17}H_{23}NO_6$: 337.15 found 338.16.

5.5.21 — 2-(*N*-Cbz-2-piperidinyl)ethanol (37)



2-(2-Piperidinyl)ethanol (10.1 g, 78.0 mmol) was dissolved in DCM (250 mL) and triethylamine (10.8 mL, 78 mmol) was added, followed by the dropwise addition of CbzCl (1.55 mL, 109 mmol) over a period of 20 min at 0 °C. The reaction mixture was stirred overnight at RT and washed subsequently with aqueous saturated $NaHCO_3$ (500 mL) water (250 mL) and brine (250 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified using flash chromatography (EtOAc:heptane 1:2 + 1% NEt_3) to afford 2-(*N*-Cbz-2-piperidinyl)ethanol (**37**, 18.2 g, 69.1 mmol, 89% yield) as an oil. R_f 0.45 (EtOAc:heptane 1:2 + 1% NEt_3); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.42-7.30 (m, 5 H), 5.15 (br s, 2 H), 4.55-4.41 (m, 1 H), 4.20-4.01 (m, 1 H), 3.56-3.39 (m, 3 H), 2.77 (t, *J* = 12.7 Hz, 1 H), 1.97-1.46 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 156.9, 136.6, 128.7, 128.2, 128.0, 67.5, 58.6, 47.0, 39.4, 32.5, 29.3, 25.6, 19.2; υ_{max} (film) cm^{-1}: 3383, 3062, 3030, 2935, 2862, 1690, 1423, 1263, 1051, 697. HRMS (ESI) *m/z* calcd for $C_{15}H_{22}NO_3$: 263.3321, found 264.3373 $[M+H]^+$. The characterization was in accordance with previously reported results.[26]

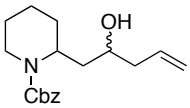
5.5.22 — 2-(*N*-Cbz-2-piperidinyl)acetaldehyde (36)



To a cooled solution (–78 °C) of DMSO (3 mL, 42.2 mmol) in DCM (100 mL) was added slowly a solution of 2,2,2-trifluoroacetic anhydride (3.5 mL, 24.8 mmol) in DCM (100 mL). After 10 minutes a solution of 2-(*N*-Cbz-2-piperidinyl) ethanol (**37**, 2.04 g, 7.74 mmol) in DCM (50 mL) was added over 15 minutes. The reaction was quenched with DIPEA (11.0 mL, 63.0

mmol) in DCM (50 mL). After 10 minutes, the reaction temperature was allowed to warm up to RT. The reaction mixture was washed with water (100 mL), aqueous HCl (100 mL, 0.1 M), saturated aqueous $NaHCO_3$ (100 mL) water (100 mL) and brine (100 mL). The organic layer was dried and concentrated *in vacuo* to afford 2-(*N*-Cbz-2-piperidinyl) acetaldehyde (**36**, 1.65 g, 6.31 mmol, 82% yield) after column chromatography as an oil. R_f 0.50 (EtOAc:heptane 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.73 (s, 1H), 7.42-7.29 (m, 5 H), 5.14 (s, 2 H), 5.05-4.89 (m, 1 H), 4.09 (d, *J* = 13.5 Hz, 1 H), 2.87 (t, *J* = 13.4 Hz, 1 H), 2.85-2.57 (m, 2 H), 1.82-1.36 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 200.3, 155.2, 136.8, 128.7, 128.2, 128.0, 67.0, 46.3, 44.7, 39.5, 28.6, 25.3, 19.0; υ_{max} (film) cm^{-1}: 3380, 2935, 2861, 1687, 1421, 1259, 1173 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{15}H_{20}NO_3$: 261.14432, found 262.14331 $[M+H]^+$. The characterization was in accordance with previously reported results.[26]

5.5.23 — 1-(*N*-Cbz-2-piperidinyl)-4-penten-2-ol (38)

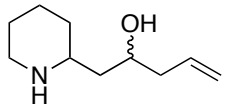


To a suspension of indium powder (**28**, 201 mg, 1.75 mmol) in ethanol:water 1:1 (30 mL) was added 3-bromoprop-1-ene (0.144 mL, 1.66 mmol). The solution was stirred for 30 min at RT followed by the addition of 2-(*N*-Cbz-2-piperidinyl) acetaldehyde (**36**, 146 mg, 0.561 mmol. The reaction was stirred overnight and the solid indium was filtered off. Ethanol was evaporated *in vacuo* and the residue was dissolved in DCM (50 mL). The organic layer was washed with brine (50 mL), dried over Na_2SO_4 and evaporated to dryness. The resulting oil was purified using column chromatography to obtain 1-(*N*-Cbz-2-piperidinyl)-4-penten-2-ol (**38**) as a 1:1 mixture of diastereoisomers: **A** (79.2 mg, 0.260 mmol, 46%) and **B** (88.0 mg, 0.290 mmol, 52%).

A: R_f 0.45 (EtOAc:heptane 1:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.45-7.29 (m, 5 H), 5.88-5.76 (m, 1 H), 5.23-5.05 (m, 2 H), 5.12-5.02 (m, 2 H), 4.59-4.46 (m, 1 H), 4.10-3.98 (m, 1 H), 3.55-3.39 (m, 1 H), 2.86 (t, *J* = 13.7 Hz, 1H), 2.30-2.18 (m, 2 H), 2.11-1.99 (m, 1 H), 1.81-1.51 (m, 7 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 157.0, 138.2, 135.2, 129.5, 129.3, 129.0, 125.1, 117.4, 69.5, 68.1, 49.9, 37.5, 30.1, 29.8, 29.5, 19.6; υ_{max} (film) cm^{-1}: 3372, 2932, 2855, 1666, 1425, 1259, 1173.

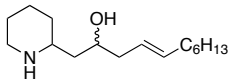
B: R_f 0.30 (EtOAc:heptane 1:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.43-7.31 (m, 5 H), 5.89-5.78 (m, 1 H), 5.15-5.09 (m, 2 H), 5.07-4.99 (m, 2 H), 4.55-4.47 (m, 1 H), 4.07-4.02 (m, 1 H), 3.65-3.51 (m, 1H), 2.96 (t, *J* = 13.7 Hz, 1H), 2.27-2.20 (m, 2 H), 1.86-1.51 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 157.1, 138.2, 136.2, 129.5, 129.0, 128.8, 117.4, 69.5, 68.1, 49.7, 42.8, 40.7, 37.4, 29.2, 26.7, 19.8; υ_{max} (film) cm^{-1}: 3385, 2927, 2855, 1671, 1423, 1260, 1170. The characterization was in accordance with previously reported results.[27]

5.5.24 — 1-(2-Piperidinyl)-4-penten-2-ol (39)



1-(*N*-Cbz-2-piperidinyl)-4-penten-2-ol (**38**, 300 mg, 1.15 mmol) was added to a stirred solution of HBr in acetic acid (33%, 30 mL). After 1 hour the solution was concentrated *in vacuo* and co-evaporated with toluene (3 × 30 mL). To a suspension of indium powder (245 mg, 2.13 mmol) in ethanol:water (1:1, 30 mL) was added 3-bromo-1-propene (0.200 mL, 2.32 mmol). The solution was stirred for 30 min at RT, followed by the addition of 2-(2-piperidineyl)acetaldehyde HBr salt (148 mg, 0.711 mmol). The reaction was stirred for 16 h and the solid indium was filtered off. Ethanol was evaporated *in vacuo*, followed by subsequent addition of aqueous saturated $NaHCO_3$ until pH 7 and extraction with EtOAc (4 × 20 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness. The resulting oil was purified using a sulfonic acid column to obtain 1-(2-piperidinyl)-4-penten-2-ol (**39**, 66.8 mg, 0.404 mmol, 55% over 2 steps). R_f 0.60 (DCM:MeOH, 9:1 + 1% NEt_3); ^{1}H NMR (300 MHz, CD_3OH) δ ppm 5.95-5.79 (m, 1 H), 5.14-5.06 (m, 2 H), 3.94-3.65 (m, 2 H), 2.97-2.88 (m, 2 H), 2.28-2.20 (m, 2 H), 1.95-1.69 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 135.8, 118.3, 70.0, 68.2, 59.4, 57.2, 46.2, 46.1, 43.7, 43.1, 40.3, 30.2, 30.0, 23.3, 23.4; υ_{max} (film) cm^{-1}: 3512, 2920, 2855, 1412, 1164; HRMS (ESI) *m/z* calcd for $C_{10}H_{20}NO$: 169.27190, found: 170.27967 $[M+H]^+$.

5.5.25 — (*E*)-1-(2-Piperidinyl)-4-undecen-2-ol (40)



To a stirred solution of 1-octene (1.00 mL, 6.42 mmol) in DCM (10 mL) was added Grubbs-II (20 mg, 0.024 mmol) and 1-(2-piperidinyl)-4-penten-2-ol (**39**, 50.4 mg, 0.295 mmol). The reaction mixture was heated to refluc and TFA (0.025 mL, 0.324 mmol) in DCM (2 mL) was added dropwise over a period of 1 hour, after which the reaction mixture was refluxed for an additional 16 h. After cooling to RT, aqueous saturated $NaHCO_3$ was added to the reaction mixture until pH 7 and subsequently the aqueous mixture was extracted with EtOAc (4 × 10 mL). The organic layers were dried with Na_2SO_4 and concentrated *in vacuo* to obtain (*E*)-1-(2-piperidinyl)-4-undecen-2-ol (**40**, 49.7 mg, 0.197 mmol, 66%) as a colorless oil after solid phase extraction with a sulfonic acid column. R_f 0.90 (DCM:MeOH, 9:1 + 1% NEt_3); ^{1}H NMR (300 MHz, CD_3OH) δ ppm 5.55-5.47 (m, 2 H), 3.90-3.63 (m, 2 H), 2.92-2.84 (m, 2 H), 2.32-2.17(m, 4 H), 1.81-1.43 (m, 16 H), 0.88-0.81 (m, 3 H); LRMS (ESI) *m/z* calcd for $C_{16}H_{31}NO$: 253.24 found 254.5 $[M+H]^+$.

5.6 — References

1. Segraves, N. L. and Crews, P. *J. Nat. Prod.* **2005**, *68*, 118–121.
2. McNaught, A. D. *Carbohydr. Res.* **1997**, *297*, 1–92.
3. Wierzejska, J.; Ohshima, M.; Inuzuka, T.; Sengoku, T.; Takahashi, M. and Yoda, H. *Tetrahedron Lett*. **2011**, *52*, 1173–1175.
4. Sugiura, M.;Mori, C. and Kobayashi, S. *J. Am. Chem. Soc.* **2006**, *128*, 11038–11039.
5. McDougal, P. G.; Rico, J.; Oh, Y. -I. and Condon, B. D. *J. Org. Chem.* **1986**, *51*, 3388–3390.
6. Podlech, J. and Seebach, D. *Angew. Chem. Int. Ed.* **1995**, *34*, 471–472.
7. Diazomethane is extremely flammable and explosive http://www.inchem.org/documents/icsc/icsc/eics1256.htm
8. Garrabou, X.; Joglar, J.; Parella, T.; Crehuet, R.; Bujons, J. and Clapés, P. *Adv. Synth. Catal.* **2011**, *353*, 89–99.
9. Sugiyama, M.; Hong, Z.; Whalen, L. J.; Greenberg, W. A. and Wong, C. -H *Adv. Synth. Catal*. **2006**, *348*, 2555–2559.
10. These reactions were analyzed on a Varian HPLC with RP C-18 Bruker-Michrom at Biotransformation and Bioactive Molecules at the Catalonia Institute for Advanced Chemistry.
11. These measurements were conducted on a Shimadzu HPLC with a RP C-18 column from Grace, which led to different retention times and the peaks of **12b** and **32** were not separated as compared to Figure 2.
12. Chen,B. -C.; Sundeen, J. E.; Guo, P.; Bednarz, M. S. and Zhao, R. *Tetrahedron Lett*. **2001**, *42*, 1245–1246.
13. Haddad, T. D.; Hirayama, L. C. and Singaram, B. *J. Org. Chem.* **2010**, *75*, 642–649.
14. Vidal, L.; Durany, O.; Suau, T.; Ferrer, P.; Benaiges, M. D. and Caminal, G. *J. Chem. Technol. Biotechnol.* **2003**, *78*, 1171–1179.
15. PP_i was added as a mixture of 3.5 equiv. (350 mg) Na_2PP_i and 2 equiv. (241 mg) Na_4PP_i which resulted in a buffered solution of pH 6.5.
16. Miles, W. H.; Connell, K. B.; Ulas, G.; Tuson, H. H.; Dethoff, E. A.; Mehta, V. and Thrall, A. J. *J. Org. Chem*. **2010**, *75*, 6820–6829.
17. Passarella, D.; Peretto, B.; Danieli, B.; Blasco y Yepes, R.; Cappelletti, G.; Cartelli, D.; Ronchi, C.; Snaith, J.; Fontana, G. and Borlak, J. *Eur. J. Med. Chem.* **2010**, *45*, 219–226.
18. a) Müller, A.; Vogt, C. and Sewald, N. *Synthesis* **1998**, 837–841; b) Limal, D.; Quesnel, A. and Briand J. *Tetrahedron Lett.* **1998**, *39*, 4239–4242.
19. Erdal, E. P.; Martàsek, P.; Roman, L. J. and Silverman, R. B. *Bioorg. Med. Chem.* **2007**, *15*, 6096–6108.
20. Kaul, R.; Surprenant, S. and Lubell, W. D. *J. Org. Chem*. **2005**, *70*, 3838–3844.
21. Mina, J. G.; Mosely, J. A.; Ali, H. Z.; Denny, P. W. and Steel, P. G. *Org. Biomol. Chem*. **2011**, *9*, 1823–1830.
22. Abbott, S. D.; Lane-Bell, P.; Sidhu, K. P. S. and Vederas, J. C. *J. Am. Chem. Soc.* **1994**,*116*, 6513–6520.
23. Soai, K. and Ookawa, A. *J. Org. Chem.* **1986**, *51*, 4000–4005.
24. Carpino, L. A.; Sadat-Aalaee, D. and Beyermann, M. *J. Org. Chem.* **1990**, *55*, 1673–1675.
25. Ohno, H.; Anzai, M.; Toda, A; Ohishi, S.; Fujii, N.; Tanaka, T.; Takemoto, Y. and Ibuka, T. *J. Org. Chem*. **2001**, *66*, 4904–4914.
26. Rouchaud, A. and Braekman, J.-C. *Eur. J. Org. Chem*. **2009**, *74*, 2666–2674.
27. Takahata, H.; Kubota, M. and Ikota, N. *J. Org. Chem*. **1999**, *64*, 8594–8601.

Chapter 6

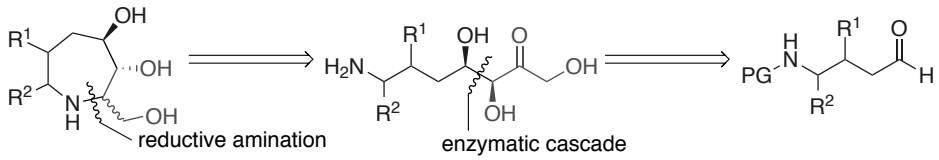
Chemoenzymatic synthesis of azepanes

Part of this work is published in Lieke J. C. van Hemert, Alexander J. J. Groenen, Luuk J. A. Wilders, Lara Babich, Ron Wever, Sander Nabuurs, and Floris P. J. T. Rutjes, Expanding the scope of one-pot four-enzyme cascade aldol reactions, manuscript in preparation.

"If we knew what it was we were doing, it would not be called research, would it?"
– Albert Einstein

Abstract

This chapter describes the application of the four-enzyme cascade in the synthesis of azepane derivatives. Trihydroxysubstituted azepanes were obtained in a four- to six-step synthesis via intramolecular reductive amination of the aldol product, which in turn was obtained in the enzymatic cascade reaction. Starting materials were aminoaldehydes, of which the nitrogen atom was typically protected as an azido- or nitro-functional group.



6.1 — Introduction

Research so far in the area of iminosugars has been mainly focused on the synthesis of five- and six-membered ring analogues that resembled the transition state of known glycosidase inhibitors.[1] In addition, some efforts have been directed at seven-membered ring iminosugars. For example, Wong *et al*. showed that seven-membered polyhydroxyiminocyclitols, in particular C_2-symmetrical tetrahydroxyiminocyclitol (**1**), can be used as common scaffold for the development of glycosidase and HIV/FIV inhibitors (Figure 1).[1a]

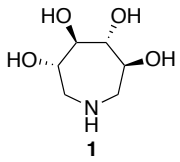
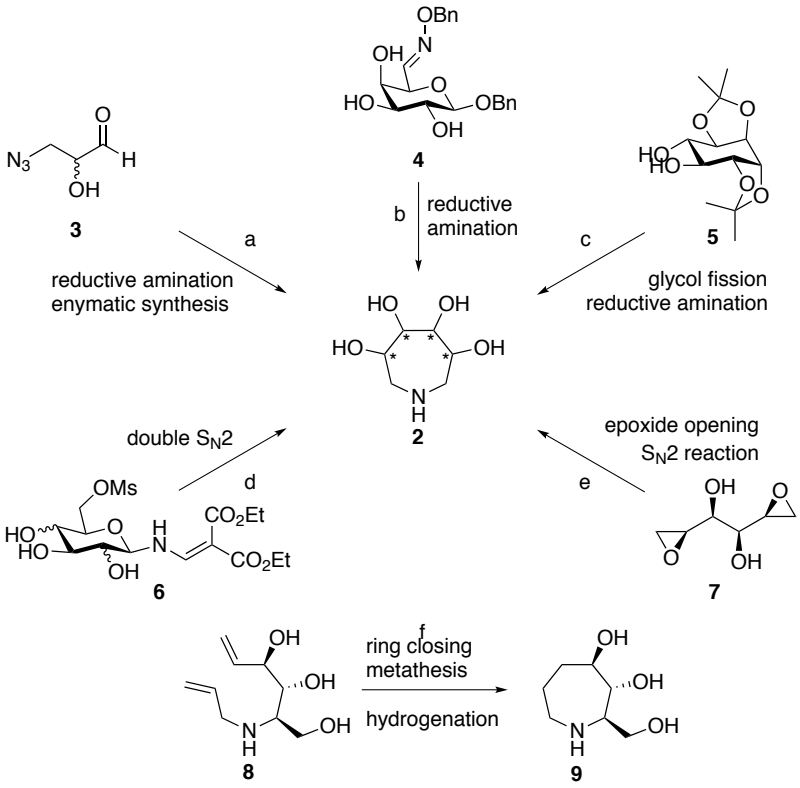


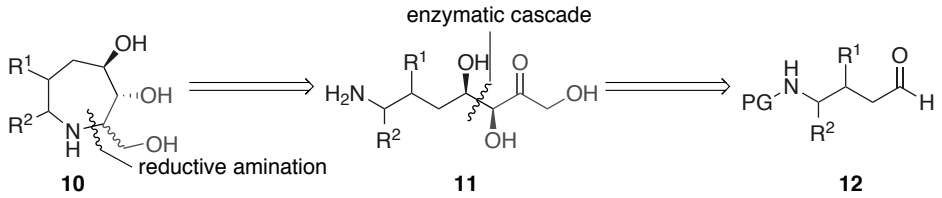
Figure 1 — C_2-symmetrical tetrahydroxyiminocyclitol.



Scheme 1 — Examples of synthetic routes to azepanes.

Polyhydroxyazepanes (seven-membered ring nitrogen heterocycles) display more flexibility as compared to their five- and six-membered analogues. They may adopt a *pseudo*-chair conformation that mimics the transition state of the enzymatic glycosidic cleavage. This could result in an improved binding to the active site of the glycosidase enzyme.[1a] Throughout the years, different polyhydroxyazepane derivatives have been synthesized and evaluated for their biological activity (Scheme 1). Synthetic routes have been developed based on enzymatic aldol reactions (a),[1a] synthesis starting from sugar derivatives (b and c),[2] epoxide openings (d and e),[3] and ring-closing metathesis (f).[4] Drawbacks of these generally elegant approaches include lengthy synthetic procedures, use of organic solvents and production of relatively large amounts of waste. These drawbacks are largely due to the fact that multiple protecting group interconversions are required to obtain azepane analogues with the desired functionality and stereochemistry.

The synthetic route described in example f results in the 7-membered analogue **9** of D-fagomine (Section 4.2.1). We envisioned that a synthetic route to the latter analogue may also proceed via the previously described two- and four-enzyme cascade reactions, requiring a minimum of protecting group manipulation. More generally, azepanes of type **10** might be obtained via reductive amination of the corresponding aldol precursor **11** (Scheme 2). This aldol product might be derived from protected aminoaldehyde **12** using the enzymatic cascade reaction that was previously described (Section 3.2).



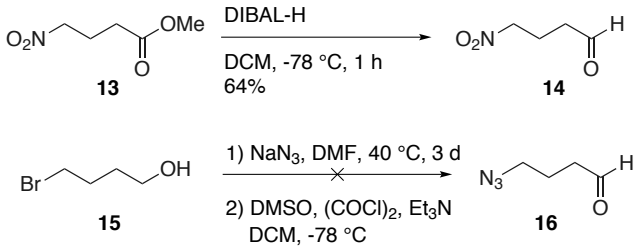
Scheme 2 — Retrosynthetic analysis of hydroxylated azepane 10

Clearly, the nucleophilic character of the amine in aldehyde **12** is not compatible with the aldehyde function.[5] Therefore, the amine had to be masked as an azide or nitro moiety, which can be reduced prior to the reductive amination to liberate the required amine functional group.

6.2 — Results and discussion

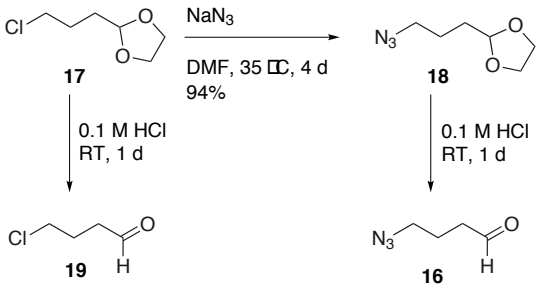
6.2.1 — Synthesis of aldehyde derivatives

We initially restricted ourselves to the synthesis of 4-nitrobutanal (**14**) and 4-azidobutanal (**16**) (Scheme 3). Nitro aldehyde **14** was obtained through DIBAL-H reduction of the corresponding commercially available methyl ester **13**, which proceeded smoothly in 64% isolated yield.



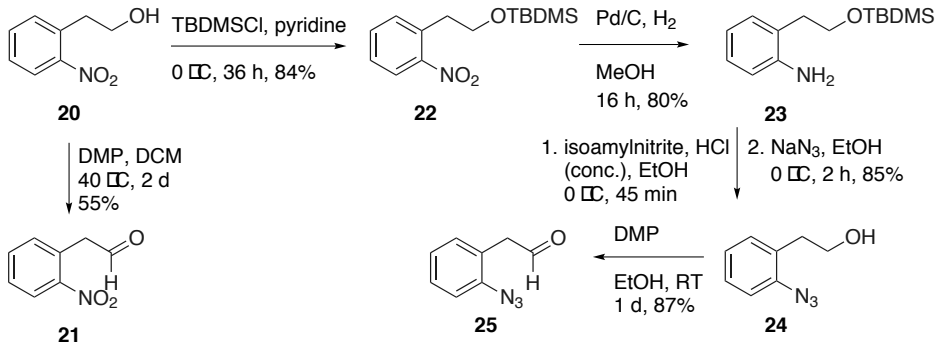
Scheme 3 — Synthesis of 4-nitro- and 4-azidobutanal.

The synthesis of 4-azidobutanal (**16**) appeared more challenging. A first attempt involved nucleophilic substitution of bromide **15** with sodium azide.[6] Subsequently, the azido alcohol was oxidized to the corresponding aldehyde **16** under Swern conditions. TLC analysis indicated a clear spot-to-spot conversion, however no aldehyde was obtained after work-up. An alternative route proceeded via ozonolysis of 6-bromohexene, but also did not result in the desired aldehyde. To circumvent the problem of isolation and possible degradation, commercially available chloro acetal **17** was used as the starting point. On the one hand, substitution with sodium azide provided acetal **18**, which could be hydrolyzed *in situ* to aldehyde **16** without further work-up and isolation (Scheme 4). On the other hand, this strategy also provided 4-chlorobutanal (**19**), which may additionally serve as a substrate for the enzymatic cascade.



Scheme 4 — Synthesis of azido aldehyde 16.

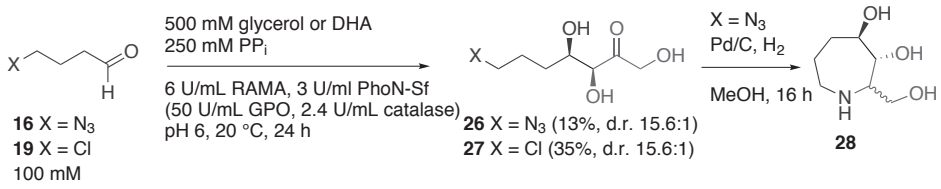
2-(2-Nitrophenyl)acetaldehyde (**21**) and 2-(2-azidophenyl)acetaldehyde (**25**) were also synthesized (Scheme 5). We anticipated that the conformational restriction introduced by the aryl moiety should enhance seven-membered ring formation during the reductive amination. 2-(2-Nitrophenyl)acetaldehyde (**21**) was synthesized via Dess-Martin periodinane (DMP) oxidation of commercially available alcohol **20** in a moderate yield of 55% (Scheme 5). Azido analogue **25** was synthesized from the same starting material **20** in a four-step procedure.[7] First, alcohol **20** was protected with a *tert*-butyldimethylsilyl (TBDMS) group in 80% yield, followed by subsequent reduction of the nitro-group and diazotization with isoamyl nitrite to afford 2-(2-azidophenyl)ethanol (**24**) in 85% yield over two steps. Finally, DMP was used as a mild oxidative agent to oxidize the primary alcohol into the corresponding aldehyde, thereby providing the targeted aldehyde **25** in an overall yield of 50% over four steps.



Scheme 5 — Synthesis of phenacetaldehydes 21 and 25

6.2.2 — Two-step synthesis of azepanes via the catalytic cascade

The four aldehydes were subjected to the conditions of the enzymatic cascade reactions (Chapter 3). First, these aldehydes were tested in the two-enzyme cascade with DHA to investigate the substrate acceptance by RAMA on analytical scale (0.5 mL). When product formation was observed by HPLC, the reaction was repeated with the four-enzyme cascade and glycerol on analytical scale and eventually on preparative scale (10 mL) so that the products could be isolated and characterized.



Scheme 6 — Two-step azepane synthesis via enzymatic cascade reactions.

Aliphatic aldehydes **16** and **19** were tested first (Scheme 6). A 0.5 mL solution of 500 mM DHA, 250 mM PP_i and 100 mM aldehyde at pH 6 was incubated with RAMA (6 U/mL) and PhoN-Sf (3 U/mL) at 20 °C. It was possible to analyze the reaction by HPLC at selected time intervals to determine the ratio of PP_i to P_i, the formation of phosporylated product, dephosphorylated end product, and the amount of aldehyde converted. Figure 2 clearly indicates that aldehyde **16** was accepted by RAMA, and similar chromatograms were obtained for aldehyde **19**. Then the reaction was repeated with glycerol (500 mM), RAMA (6 U/mL), PhoN-Sf (3 U/mL), GPO (50 U/mL) and catalase (20 U/mL), which also proceeded smoothly according to HPLC analysis. The reaction was repeated at preparative scale (10 mL) and after 48 h, the aldol products were obtained in a d.r. of 15.6:1 and isolated via silica gel purification and obtained in 13 (**26**) and 35% (**27**) isolated yield.

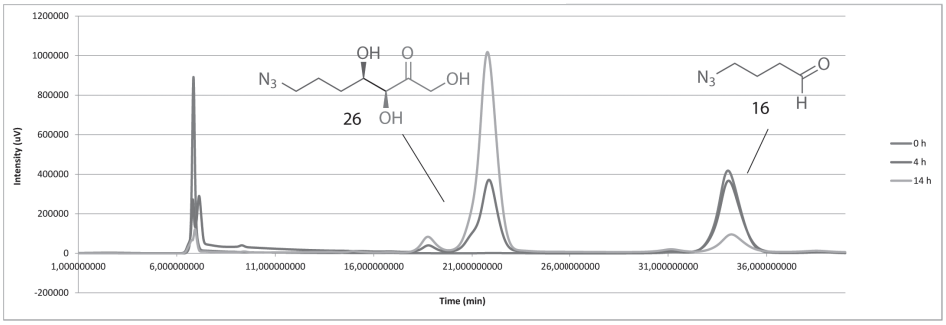
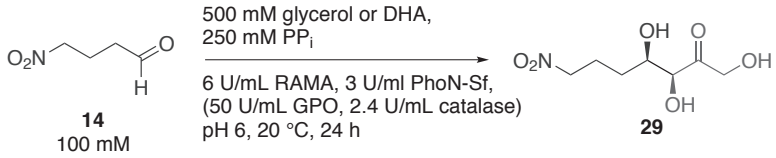


Figure 2 — Time course on HPLC, aliquots of 20 µL were 10 times diluted and measured.

These yields were surprisingly low compared to the conversions measured by HPLC. Conversions were estimated at 75-85% for the azido (**26**) and 85-95% for the chloro aldol product (**27**), based on the NMR analysis and calibration curves of the isolated products. Multiple products, however, were observed on TLC after work-up, indicating degradation of the isolated products that led to the relatively low isolated yields upon purification.

Nevertheless, aldol product **26** was submitted to the reductive amination conditions ($Pd/C, H_2$) in MeOH at room temperature, resulting in full conversion overnight to afford azepane **28** in quantitative yield. The reductive amination occurred without selectivity at the C-2 position, providing a 1:1 ratio of the corresponding diastereoisomers. Separation of the diastereoisomers has hitherto not been successful. This enzymatic route produced

the azepane in four steps, while the route described in literature required ten steps.4 Unfortunately, chlorinated aldol product **27** degraded over time, therefore the synthesis of the corresponding azepane was no longer pursued.



Scheme 7 — Aldol reaction of 4-nitrobutanal 14.

Nitrobutanal **14** was readily accepted by RAMA (Scheme 7), which was also predicted by the computational studies on RAMA described in Chapter 2. The conversion of nitrobutanal into the corresponding aldol product **29** occurred at a rate that was more than twice as high as for propanal in the four-enzyme cascade. In six hours, most of the aldehyde was consumed and the reaction was incubated overnight for optimal conversion (Figure 3).

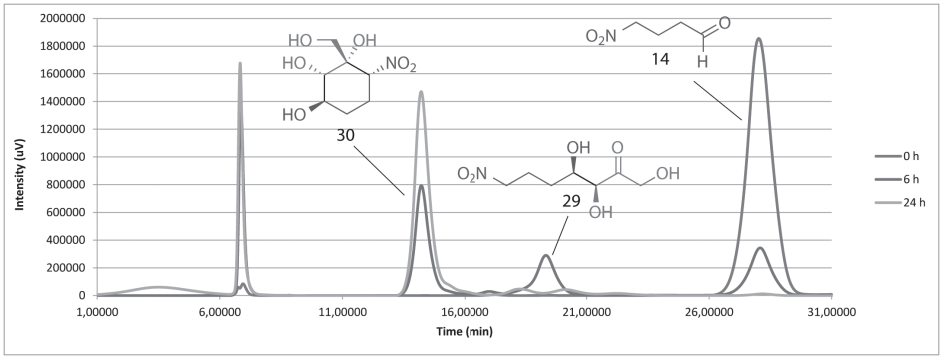
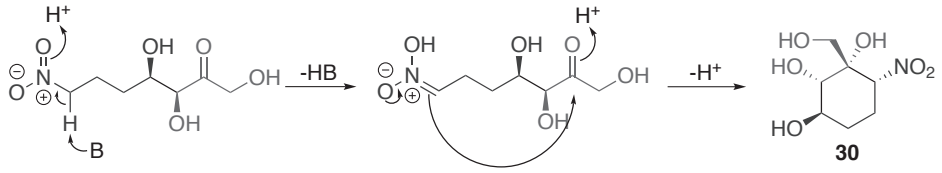


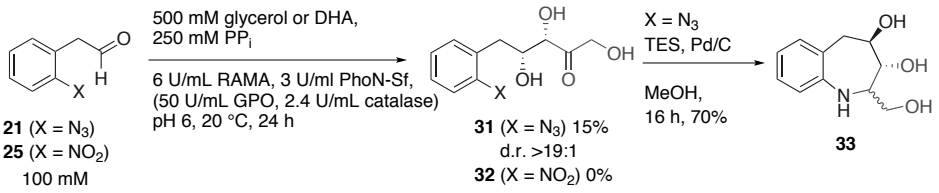
Figure 3 — HPLC chromatogram of four-enzyme cascade with 4-nitrobutanal.

However, no traces of nitroaldol product **29** were observed after work-up, instead formation of a single diastereoisomer of nitrocyclohexitol **30** was observed (Scheme 8).[8]



Scheme 8 — Intramolecular Henry reaction.

The slightly acidic conditions of the cascade reaction were sufficient for the Henry reaction to take place in 81% conversion to provide nitrocyclohexitol **30** as a single diastereoisomer in 45% isolated yield.



Scheme 9 – Synthesis of an aryl-substituted azepane.

Finally, the phenacetaldehydes **21** and **25** were submitted to the cascade reaction conditions (Scheme 9). The two-enzyme cascade reaction with DHA and nitro analogue **25** did not result in any product formation. Upon analyzing the HPLC results, we concluded that no phosphorylation had occurred, indicating that aldehyde **25** inhibited the PhoN-Sf activity and blocked the synthesis of DHAP. This inhibition was not observed with azido-substituted aldehyde **21**. Product formation was observed on analytical scale with the two-enzyme cascade reaction and DHA, however, the reaction required extra addition of DMSO to solubilize the aldehyde. With the four enzyme cascade reaction on preparative scale, 15% of aldol product **31** was isolated as a single diastereoisomer. The poor solubility thwarted accurate determination of the conversion, as no adequate calibration curve could be obtained. Next, the reductive amination was performed to produce azepane **33**. Subjection of aldol product **31** to standard hydrogenation conditions (H_2 and Pd/C in MeOH) did not result in the formation of the azepane. Therefore, the reaction was repeated in the presence of triethylsilane (TES) and Pd/C as catalyst to generate H_2 *in situ*, which produced azepane **33** in an isolated yield of 70%.[9] Again, no selectivity was observed at the C-2 carbon so that the product was isolated as a 1:1 mixture of diastereoisomers.

6.3 – Conclusion

Two trihydroxyazepane derivatives were synthesized using the four-enzyme cascade reaction. The examples involved an aliphatic aldehyde and 2-azidophenylacetaldehyde, which afforded the desired azepane derivatives in four to six steps. This route offers an alternative strategy towards azepanes in significantly less steps compared to the classical protecting group strategy synthesis from sugars.4

6.4 — Acknowledgements

Luuk Wilders is gratefully acknowledged for his contributions to this chapter. Han Peeters (J. H. van 't Hoff Institute for Molecular Sciences, University of Amsterdam) is acknowledged for the mass spectrometry analyses and Paul Schlebos for his help with the NMR analysis.

6.5 — Experimental

6.5.1 — General information

The general information is described in Section 2.5.1

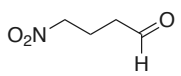
6.5.2 — Enzymes

The information of the enzymes is described in Section 3.5.2.

6.5.3 — Analytical methods and product characterization

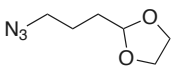
The HPLC method is described in Section 5.5.3.

6.5.4 — 4-Nitrobutanal (14)



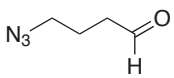
To a solution of methyl 4-nitrobutanoate (**11**, 0.401 g, 2.71 mmol) in anhydrous DCM (30 mL, –78 °C) was added DIBAL-H (3.0 mL, 3.0 mmol, 1M solution in hexanes) and the reaction was stirred for 1 hour. The temperature was increased to –40 °C and 10% aqueous potassium sodium tartrate (2 mL) was added. After stirring for 30 min, the temperature was increased to RT, and the aqueous layer was extracted with Et_2O (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL), dried over Na_2SO_4, filtrated and concentrated *in vacuo*. The crude product was purified by column chromatography (heptane:EtOAc 9:1 → 2:1) to afford **14** (204 mg, 64%) as a yellow liquid. R_f: 0.35 (heptane: EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.80 (s, 1H), 4.45 (t, *J* = 6.6 Hz, 2H), 2.67 (t, *J* = 6.9 Hz, 1H), 2.31 (t, *J* = 6.7 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.8, 73.8, 39.2, 19.1; υ_{max}(film) cm^{-1} 2840, 2734, 1720, 1435, 1383, 1277, 816, 677. The analytical data were in accordance with previously reported results.[10]

6.5.5 — 2-(3-Azidopropyl)-1,3-dioxolane (18)



A solution of 2-(3-chloropropyl)-1,3-dioxolane (**17**, 0.810 g, 5.38 mmol) in DMF (40 mL) was treated with sodium azide (0.55 g, 8.34 mmol) and stirred for 3 days at 40 °C. The conversion was analyzed by GC. 10 Equiv. of H_2O were added to the reaction mixture and the aqueous layer was extracted with EtOAc (3 × 50 mL). The combined organic fractions were dried and concentrated at 500 mbar to afford **18** as a colorless liquid (0.79 g, 93%). R_f: 0.35 (heptane: EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.89 (t, *J* = 4.1 Hz, 1H), 4.02-3.79 (m, 4H), 3.33 (t, *J* = 6.7 Hz, 2H), 1.80-1.68 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 104.3, 65.5, 51.8, 31.4, 24.0; υ_{max}(neat) cm^{-1} 2932, 2094, 1673, 1449, 1091, 609.

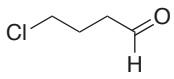
6.5.6 — 4-Azidobutanal (16)



<u>*Method 1*</u>: A solution of 2-(3-azidopropyl)-1,3-dioxolane (**18**, 0.11 g, 0.68 mmol) in 0.1 M aqueous HCl (20 mL) was stirred at RT for 2 days. The deprotection was analyzed by TLC and GC. H_2O (5 mL) was added and the reaction mixture was extracted thoroughly with Et_2O (3 × 50 mL). The organic phase was washed with brine (75 mL), dried with Na_2SO_4 and concentrated under reduced pressure (650 mbar). The crude product was purified over a small silica plug and eluted with DCM. The pure fractions were combined and concentrated *in vacuo* (100 mbar, 40 °C) to obtain **16** as a colourless liquid in quantitative yield (78 mg, 100%). The sample was stored at –80°C under argon.

<u>*Method 2*</u>: 2-(3-Azidopropyl)-1,3-dioxolane (**18**, 0.25 g, 1.6 mmol) was dissolved in 0.1M HCl as a 0.5 M solution and stirred for 16 h. Quantitative deprotection to **16** was observed by HPLC. R_f: 0.4 (heptane: EtOAc 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (t, *J* = 1.2 Hz, 1H), 3.36 (t, *J* = 6.6 Hz, 2H), 2.58 (td, *J* = 7.1, 1.2 Hz, 2H), 2.10-1.90 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.6, 50.3, 40.5, 21.2; υ_{max}(film) cm^{-1} 3441, 2936, 2874, 2087, 1721. The analytical data were in accordance with previously reported results.[11]

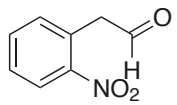
6.5.7 — 4-Chlorobutanal (19)



*This compound was synthesized via method 1 for 4-azidobutanal (**16**).*

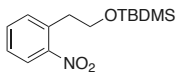
^{1}H NMR (300 MHz, $CDCl_3$) δ 9.82 (t, *J* = 1.0 Hz, 1H), 3.60 (t, *J* = 6.3 Hz, 2H), 2.67 (td, *J* = 7.0, 1.0 Hz, 2H), 2.17-2.05 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.4, 43.6, 40.4, 24.4; υ_{max}(film) cm^{-1}: 3441, 2961, 2832, 2727, 1720, 790, 732. The analytical data were in accordance with previously reported results.[12]

6.5.8 — 2-(2-Nitrophenyl)acetaldehyde (21)



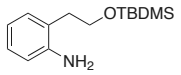
Dess-Martin periodinane (0.26 g, 0.61 mmol) was added in one portion to a stirred solution of 2-(2-nitrophenyl)ethanol (**20**, 0.51 g, 3.0 mmol) in DCM (20 mL) at RT. The reaction mixture was stirred at RT under an inert atmosphere for 2 days and two additional portions of DMP (2 × 0.26 g, 2 × 0.61 mmol) were added after 18 h and 40 h. The mixture was poured into a 1:1 mixture of saturated aqueous $NaHCO_3$ and a saturated aqueous $Na_2S_2O_3$ and was stirred vigorously for 30 min. The layers were separated and the aqueous layer was extracted with DCM (3 × 40 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (heptane:EtOAc 4:1 → 2:1) to afford **21** (0.27 g, 55%) as a yellow solid. R_f: 0.54 (heptane:EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.83 (t, *J* = 0.9 Hz, 1H), 8.12 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.62 (td, *J* = 7.5, 1.4 Hz, 1H), 7.49 (td, *J* = 8.1, 1.5 Hz, 1H), 7.31 (d, *J* = 9.0 Hz, 1H), 4.11 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.3, 148.0, 133.4, 133.0, 128.4, 128.0, 124.9, 48.0; υ_{max}(neat) cm^{-1}: 2850, 1723, 1610, 1520, 787. The analytical data were in accordance with previously reported results.7

6.5.9 — *O-t*-Butyldimethylsilane-2-(2-nitrophenyl)ethanol (22)



An excess of pyridine (5 mL) was slowly added to an ice-cooled mixture of 2-(2-nitrophenyl)ethanol (**20**, 0.501 g, 3.01 mmol) and *tert*-butyldimethylsilyl chloride (0.451 g, 2.99 mmol). The reaction mixture was stirred overnight, quenched by the addition of ice and extracted with Et_2O (3 × 20 mL). The combined organic layers were washed with brine (50 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The yellow residue was purified by silica column chromatography (heptane:EtOAc 3:1) to afford **22** as a yellow liquid (2.74 g, 84%). R_f: 0.81 (heptane:EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (dd, *J* = 8.1, 1.4 Hz, 1H), 7.54-7.47 (m, 1H), 7.39 (t, *J* = 13.8 Hz, 2H), 3.89 (t, *J* = 6.4 Hz, 2H), 3.12 (t, *J* = 6.3 Hz, 2H), 0.83 (s, 9H), –0.05 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.7, 134.4, 133.5, 132.6, 127.5, 124.7, 63.2, 36.5, 26.0, –5.4; υ_{max}(neat) cm^{-1}: 2928, 2856, 1610, 1577, 1525, 1471, 1254, 776. The analytical data were in accordance with previously reported results.7

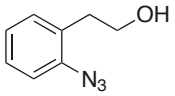
6.5.10 — 2- *O-t*-Butyldimethylsilane-2-(2-aminophenyl)ethanol (23)



Nitro compound **22** (2.74 g, 9.71 mmol) in methanol (15 mL) was sirred in the presence of Pd/C (0.1 g (10% Pd/C), 94 mmol) under a H_2 atmosphere and stirred overnight. The crude product was filtered over a Celite plug and concentrated *in vacuo* to afford **23** as a red oil (0.29 g, 80%). R_f:

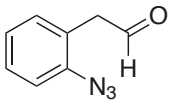
0.58 (heptane:EtOAc 3:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.02 (m, 2H), 6.69 (m, 2H), 3.88 (t, *J* = 6.0 Hz, 2H), 2.76 (t, *J* = 6.0 Hz, 2H), 0.90-0.83 (m, 9H), –0.02 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 146.0, 130.9, 127.7, 119.0, 116,2, 64.8, 35.6, 30.0, 26.2, 18.1, –5.2; υ_{max}(neat) cm^{-1}: 3442, 3352, 3021, 2927, 2883, 1622, 1587, 1254, 776. LRMS (EI^{+}) *m/z* calcd for $C_{14}H_{25}NOSi$: 251.17 found 252.20 [M+H]$^{+}$. The analytical data were in accordance with previously reported results.7

6.5.11 — 2-(2-Azidophenyl)ethanol (24)



Amine **23** (1.67 g, 6.61 mmol) was dissolved in ethanol (50 mL). Concentrated HCl (1.3 mL) was added, and the mixture was cooled on an ice bath. Isopentyl nitrite (1.0 mL, 7.4 mmol) was added dropwise at T <5 °C. After diazotization the mixture was stirred at 0 °C for 45 min. A 0.6 M aqueous solution of sodium azide (0.59 g, 9.13 mmol) was slowly added and evolution of N_2 was observed. The solution was slowly heated to ambient temperature, and MTBE and water (1:1, 50 mL) were added. To facilitate proper phase separation, NaCl was added. The aqueous phase was extracted with MTBE (2 × 50 mL) and the combined organic layers were washed with aqueous saturated $NaHCO_3$ (50 mL), water (50 mL) and dried over anhydrous Na_2SO_4. After filtration the mixture was concentrated *in vacuo*. The residue was purified by column chromatography (heptane:EtOAc 3:1 → 1:1) to obtain **24** as a yellow liquid (0.92 g, 85%). R_f: 0.6 (heptane:EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33-7.08 (m, 4H), 3.81 (dd, *J* = 6.5, 5.2 Hz, 2H), 2.87 (t, *J* = 6.7 Hz, 2H), 2.03 (t, *J* = 5.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.6, 131.6, 130.3, 128.2, 125.0, 118.4, 62.8, 34.9; υ_{max}(neat) cm^{-1} 3322, 2928, 2124, 1581, 1489, 1283, 749; LRMS (EI^{+}) *m/z* calcd for $C_8H_9N_3O$: 163.07 found 164.07 [M+H]$^{+}$. The analytical data were in accordance with previously reported results.7

6.5.12 — 2-(2-Azidophenyl)acetaldehyde (25)



2-(2-Azidophenyl)ethanol (**24**, 0.84 g, 5.1 mmol) and DMP (1.5 g, 7.7 mmol) were dissolved in DCM (50 mL) and stirred at RT for 48 h. The reaction mixture was washed with a 1:1 mixture of 0.9 M sodium thiosulfate and saturated aqueous sodium bicarbonate (2 × 50 mL) and the resulting mixture was stirred for ca. 10-15 min. The organic phase was separated, washed thoroughly with water (3 × 50 mL), brine (50 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified using silica gel column chromatography (heptane:EtOAc 3:1) to afford **25** (0.72 g, 87%) as a brownish oil. R_f: 0.55 (heptane:EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.70 (t, *J* = 1.9 Hz, 1H), 7.40-7.33 (m, 1H), 7.20-7.05 (m, 3H), 3.67 (d, *J* = 1.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.5, 138.1, 131.2, 128.4, 124.4,

123.3, 117.9, 45.5; υ_{max}(neat) cm^{-1} 2930, 2121, 1722, 1582, 1489, 1285, 751; LRMS (EI^+) *m/z* calcd for $C_8H_7N_3O$: 161.06 found 162.06 $[M+H]^+$.

6.5.13 — General procedure A for the one-pot two-enzyme cascade

To a solution of 500 mM dihydroxyacetone, 250 mM PP_i (3:2 Na_2PP_i/Na_4PP_i),[13] and 100 mM aldehyde in water, 6 U/mL RAMA was added. The reactions were initiated by adding 3 U/mL PhoNSf and incubated at 20 °C under mild shaking (350 RPM) until completion. Dephosphorylation to the end product was completed after 24 h.

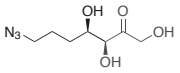
6.5.14 — General procedure B for the one-pot four-enzyme cascade

To a solution of 500 mM glycerol, 250 mM PP_i (3:2 Na_2PP_i/Na_4PP_i),[13] and 100 mM aldehyde in water 2.4 U/mL catalase from bovine liver, 6 U/mL RAMA, and 100 U/mL GPO were added. The reactions were initiated by adding 3 U/mL PhoN-Sf and incubated at 20 °C under mild shaking until completion. Dephosphorylation to the end product was completed after 24 h.

6.5.15 — General procedure C for the preparative scale one-pot four-enzyme cascade

To a solution of aldehyde (1.00 mmol) in water (total volume 10 mL) were added glycerol (2.00 mL, 2.5 M, 5.0 mmol) and PP_i (591 mg, 2.5 mmol),[13] followed by GPO (1.00 mL, 50 U/mL), catalase from bovine liver (20 µL, 10 U/mL) and RAMA (161 µL, 6 U/mL). The reactions were initiated by adding PhoN-Sf (600 µL, 3 U/mL) and incubated until completion at 20 °C under mild shaking. Silica gel (2 g) was added to the reaction mixture and it was concentrated under reduced pressure. The product was obtained by flash chromatography (EtOAc:MeOH 19:1).

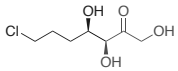
6.5.16 — (3*S*,4*R*)-7-Azido-1,3,4-trihydroxyheptan-2-one (26)



This compound was synthesized via method 6.5.15 from ***16*** *(130 mg, 1.0 mmol) and resulted in* ***26*** *(26 mg, 0.13 mmol) in 13% isolated yield.*

R_f 0.2 (DCM:MeOH 9:1); ^{1}H NMR (300 MHz, CD_3OD) δ 4.48 (q, *J* = 2.4 Hz, 2H), 4.12 (d, *J* = 2.4 Hz, 1H), 3.90 (td, *J* = 6.9, 2.4 Hz, 1H), 3.34(t, *J* = 5.9 Hz, 2H), 1.82-1.55 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 213.6, 79.4, 73.0, 67.9, 52.4, 31.4, 26.5; υ_{max}(film) cm^{-1}: 3369, 2924, 2359, 2097, 1722, 1407, 1353, 1253, 1072, 959, 907, 668, 605, 555; $[\alpha]_D^{20}$: −3.2 (*c* = 0.05, H_2O); LRMS (FAB^+) *m/z* calcd for $C_7H_{13}N_3O_4$: 203.09 found 204.20 $[M+H]^+$, 226.09 $[M+Na]^+$, 242.09 $[M+K]^+$.

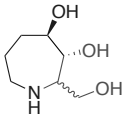
6.5.17 — (3*S*,4*R*)-7-Chloro-1,3,4-trihydroxyheptan-2-one (27)



*This compound was synthesized via method 6.5.15 from **19** (106 mg, 1.0 mmol) and resulted in **26** (69 mg, 0.35 mmol) in 35% isolated yield.*

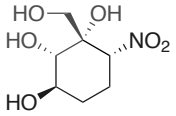
R_f: 0.45 (DCM:MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 4.53 (d, *J* = 2.6 Hz, 1H), 4.43 (d, *J* = 2.6 Hz, 1H), 4.03 (d, *J* = 2.4 Hz, 1H), 3.82 (td, 1H, *J* = 6.9, 2.4 Hz), 3.51 (t, *J* = 6.6 Hz, 2H),1.90-1.68 (m, 4H); ^{13}C NMR (75 MHz, MeOD) δ 212.2, 78.0, 71.5, 66.5, 44.3, 30.3, 28.9; υ_{max}(film)cm^{-1}: 3331, 2924, 2853, 1722, 1661, 1611, 1509, 1395, 1257,1068, 876, 644, 619, 575, 456; $[\alpha]_D^{20}$: −3.4 (*c* = 0.011, MeOH).

6.5.18 — (3*R*,4*R*)-2-(Hydroxymethyl)azepane-3,4-diol (28)



(3*S*,4*R*)-7-Azido-1,3,4-trihydroxyheptan-2-one (**26**, 11.7 mg, 0.058 mmol) was dissolved in MeOH (15 ml). Pd/C (10 mg, 0.058 μmol) was added and the mixture was placed under a H_2 atmosphere and stirred at RT overnight. The reaction mixture was concentrated, the crude product was purified over a sulfonic acid column and concentrated to afford **28** (12.4 mg, 0.077 mmol, 100%) as a 1:1 diastereomeric mixture. R_f: 0.20 (DCM:MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 3.95-3.60 (m, 4.5H), 3.4-3.1 (m, 2.5H), 2.1-1.7 (m, 4H); ^{13}C NMR (125 MHz, CD_3OD) δ 74.21, 72.59, 70.95, 70.75, 64.56, 62.32, 61.27, 57.01, 47.53, 47.53, 30.49, 29.22, 20.81, 18.68; υ_{max}(film) cm^{-1}: 3129, 3042, 2815, 1753, 1628, 1402, 778, 742; HRMS (ESI$^+$) *m/z*: Calcd for $C_7H_{15}NO_3$: 161.1052, found 162.1142 [M+H]$^+$.

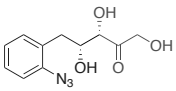
6.5.19 — (1*S*,2*S*,3*R*,6*R*)-1-(Hydroxymethyl)-6-nitrocyclohexane-1,2,3-triol (30)



*This compound was synthesized via method 6.5.15 from **14** (117 mg, 1.0 mmol) and resulted in **30** (93 mg, 0.45 mmol) in 45% isolated yield.*

R_f: 0.15 (DCM:MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 4.80 (dd, *J* = 12.9, 4.0 Hz, 1H), 3.82 (d, *J* = 11.0 Hz, 1H), 3.73 (ddd, *J* = 11.6, 9.4, 4.7 Hz, 1H), 3.37 (d, *J* = 9.4 Hz, 1H), 3.35 (d, *J* = 11.0 Hz, 1H), 2.47-2.37 (m, 1H), 2.00 (m, 1H), 1.98 (m, 1H), 1.33 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 86.33, 77.03, 75.05, 70.90, 61.78, 29.49, 24.63; υ_{max}(neat) cm^{-1}: 3448, 3290, 2931, 1550, 1468, 1364, 788; $[\alpha]_D^{20}$: 3.7 (*c* = 0.042, H_2O); LRMS (FAB$^+$) *m/z* calcd for $C_7H_{13}NO_6$: 207.07 found 208.08 [M+H]$^+$, 230.07 [M+Na]$^+$.

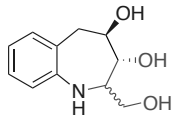
6.5.20 — 5-(2-Azidophenyl)-1,3,4-trihydroxypentan-2-one (31)



*This compound was synthesized via method 6.5.15 from **21** (161 mg, 1.0 mmol) and resulted in **31** (38 mg, 0.15 mmol) in 15% isolated yield.*

R_f: 0.5 (EtOAc:MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 7.24-7.16 (m, 2H), 7.13-7.08 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.02-6.97 (td, *J* = 7.5, 1.1 Hz, 1H), 4.42 (d, *J* = 19.5 Hz, 1H), 4.34 (d, *J* = 19.5 Hz, 1H), 4.10 (td, *J* = 7.3, 2.0 Hz, 1H), 3.91 (d, *J* = 2.0 Hz, 1H), 2.85-2.71 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 214.0, 138.7, 133.0, 130.8, 129.2, 125.8, 119.1, 78.5, 73.2, 67.9, 36.3; υ_{max}(film)cm^{-1}: 3392, 2923, 2123, 1722, 1581, 1490, 1451, 1285, 753; $[\alpha]_D^{20}$:+2.4 (*c* = 0.010, MeOH).

6.5.21 — (3*R*,4*R*)-2-(Hydroxymethyl)-2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine-3,4-diol (33)



(3*S*,4*R*)-5-(2-Azidophenyl)-1,3,4-trihydroxy-2-pentanone (**31**, 2.67 mL, 0.040 mmol) was dissolved in methanol (20 mL), followed by the addition of Pd/C (13 mg, 0.125 μmol). TES (0.20 mL, 1.25 mmol) was added dropwise to the mixture and it was stirred overnight. After completion (TLC) the reaction mixture was filtered through a Celite plug, concentrated *in vacuo* and purified with silica gel column chromatography (EtOAc:MeOH 16:1→10:1) to afford azepane **33** (10.6 mg, 0.051 mmol, 41%) as a 1:1 mixture of diastereoisomers. R_f: 0.65 (DCM:MeOH 9:1); ^{1}H NMR (500 MHz, CD_3CN) δ 7.06-6.95 (m, 2H), 6.79-6.73 (m, 2H), 3.97 (dd, *J* = 10, 5 Hz, 1H, C6-1), 3.72 (t, *J* = 5 Hz, 1H, C6-2), 3.57-3.37 (m, 3H), 3.21-3.17 (m, 0.5H), 3.11-3.07 (m, 1H), 2.98 (d, 1H, *J* = 20 Hz), 2.73-2.68 (m, 1.5H), 2.52 (dt, 0.5H); ^{13}C NMR (125 MHz, CD_3CN) δ 148.98, 132.09, 130.76, 129.33, 128.49, 127.37, 127.01, 121.55, 121.13, 119.90, 119.37, 109.97, 77.83, 73.89, 72.73, 69.39, 62.98, 62.45, 62.36, 58.09, 38.85, 36.17; υ_{max}(film) cm^{-1}: 3120, 3050, 2850, 1605, 1407, 1181, 760; HRMS (ESI$^+$) m/z Calcd. for $C_{11}H_{15}NO_3$: 209.1052, found 210.1139 $[M+H]^+$.

6.6 — References

1. (a) Moris-Varas, F.; Qian, X.-H. and Wong, C.-H. *J. Am. Chem. Soc.* **1996**, *118*, 7647–7652; (b) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683–720; (c) Albein, A. D. *Annu. Rev. Biochem.* **1987**, *56*, 497–534; (d) Ichikawa, Y.; Igarashi, Y.; Ichikawa, M. and Suhara, Y. *J. Am. Chem. Soc.* **1998**, *120*, 3007–3018.
2. (a) Mehta, G. and Lakshminath, S. *Tetrahedron Lett.* **2002**, *43*, 6525–652; (b) Painter, G. F. and Falshaw, A. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1157–1159.
3. (a) Fuetes, J.; Gasch, C.; Olano, D.; Pradera, M. A.; Repetto, G. and Sayago, F. J. *Tetrahedron: Asymmetry* **2002**, *13*, 1743–1753; (b) Merrer, Y. L.; Poitout, L.; Depazy, J.; Dosbaa, I.; Geoffroy, S. and Foglietti, M. *Bioorg. Med. Chem*. **1997**, *5*, 519–533.
4. Hongqing, L.; Blériot, Y.; Chantereau, C.; Mallet, J. M.; Sollogoub, M.; Zhang, Y.; Rodríguez-García, E.; Vogel, P.; Jiménez-Barbero, J. and Sinaÿ, P. *Org. Biomol. Chem.* **2004**, *2*, 1492–1499.
5. Kevin, K.-C., Liu R.; Pederson, L. and Wong C.-H. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2669–2673.
6. Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Leahy, E. M. and Salvino, J. *J. Am. Chem. Soc*. **1993**, *115*, 12550–12568.
7. Bucher, G. *Eur. J. Org. Chem.* **2001**, 2447–2462.
8. Blidi, E. L.; Crestia, D.; Gallienne, E.; Demuynck, C.; Bolte, J. and Lemaire, M. *Tetrahedron: Asymmetry* **2004**, *15*, 2951–2954.
9. Mandal, P. K. and McMurray, J. S. *J. Org. Chem*. **2007**, *72*, 6599–6601.
10. Shechter, H.; Ley, D. E. and Zeldin, L. *J. Am. Chem. Soc.* **1952**, *74*, 3664–3668.
11. Bates, R. W. and Dewey, M. R. *Org. Lett.* **2009**, *11*, 3706–3708.
12. Palma, A.; Cárdenas, J. and Frontana-Uribe, B. A. *Green Chem*. **2009**, *11*, 283–293.
13. PPi was added as a mixture of 3.5 equiv. (350 mg) Na_2PP_i and 2 equiv. (241 mg) Na_4PP_i resulting in a buffered solution of pH 6.

Chapter 7

Perspective Enzymatic cascade reactions:past, present and future

"Human beings make mistakes. They're supposed to. That's how they learn."
– Harry Palmer

Abstract

Herein I give my view on the past four years of research by comparison of the initial goal with the final results and encountered bottlenecks in search of novel enzymatic cascade reactions. This also results in a personal outlook on future chemoenzymatic cascade processes.

7.1 — Past: the initial concept

After million years of evolution, the diversity of functions of enzymes has reached an enormous level creating numerous applications in different scientific fields. The efficiency of sequential reactions catalyzed by enzymes in Nature is striking, especially compared to classical chemical reactions, which usually require multiple separate steps including work-up procedures and a multitude of chemical reagents. The motivation of the NWO/ACTS IBOS (Integration of Bio- and Organic Synthesis) program was to integrate both fields and use the combined knowledge of biochemists and synthetic chemists to pursue new undiscovered reaction pathways to highly functionalized molecules. Especially the idea of integrating multiple strategies in one approach appealed to me as this required a broader perspective from an organic chemist and an open mind to solutions offered by the field of biochemistry. Our project mainly focused on exploring the scope and limitations of enzymatic cascade reactions with the aim to synthesize enantiomerically pure multifunctional heterocycles via efficient and sustainable processes. Key in our research was the formation of C-C bonds using the well-known aldol reaction to prepare polyhydroxylated carbohydrate fragments. Classically, this approach requires protecting group strategies, expensive catalysts and multiple steps. Enzymes, however, are able to perform these reactions stereoselectively in water without protecting groups in a single step, thus resulting in a greener approach.

7.2 — Present: the results after four years of research

7.2.1 — The accomplishments

The research in this manuscript was focused on creating more sustainable chemistry routes using biocatalysis as a substitute for laborious multistep procedures containing multiple (de)protection steps and resulted in a one-pot four-enzyme cascade reaction allowing us to synthesize non-natural carbohydrate fragments in high enantio- and diastereoselectivities with RAMA as aldolase. With this procedure we were able to synthesize various iminosugars from natural resources.

7.2.2 — The bottlenecks encountered

Enzymes are limited with respect to their specific mode of action and substrate specificity, which was demonstrated with the synthesis of diastereoisomeric precursors of fagomine using the stereocomplementary aldolases. The results with these aldolases were on the one hand encouraging in the sense that reactions did proceed, but on the other hand

also disappointing due to the generally moderate diastereoselectivity and yields. The diastereoselectivity could be tuned with the synthesis of more suitable acceptor aldehydes, however chemical synthesis of these aldehydes remains challenging. Previously reported results demonstrated that α-branching may lead to higher diastereoselectivities in case of RhuA and FucA.[1] Our research also demonstrated that β-branching positively influenced the diastereoselectivity of RhuA in the synthesis of a batzellaside precursor using the four-enzyme cascade strategy. These results certainly imply that in case a specific diastereoisomer needs to be produced on larger scale, the enzyme specificity could be optimized by using genetic engineering techniques such as directed evolution[2] and gene shuffling.[3] The development optimized enzymes might however require a significant investment in research.

Another drawback we encountered was the larger scale availability of enzymes in order to be able to produce sufficient amounts of carbohydrate building blocks for follow-up chemistry. This was required given the aim of the IBOS project use the enzymatic cascades for the synthesis of libraries of functionalized heterocycles of interest to the pharmaceutical and fine chemical industry. To realize this goal the enzymes had to be expressed in large quantities and preferably should be recycled. One bottleneck in the scaling up process was the availability of RAMA. This enzyme is commercially available, but extracted from rabbits. An effective recombinant system from bacteria would greatly reduce the amount of waste, but current alternative expression systems have not been efficient up to now.[4] PhoN-Sf and the other aldolases were available via recombinant systems, but even then it would require too much effort to produce the amounts of enzymes needed to synthesize grams of the required building blocks. As an example, as much as 60 U were required to synthesize 1 mmol of aldol product via the four-enzyme cascade, meaning that scaling up is laborious and expensive. This, and the bottleneck of recycling the enzymes, was elegantly tackled by the group of Wever. They initially showed that by immobilization of PhoN-Sf on beads and carrying out the phosphorylation step in a continuous flow system led to preparative amounts of the corresponding phosphate products.[5] In a later stage, they also demonstrated that by combining immobilized PhoN-Sf and inmmobilized RAMA in a flow reactor, the two-enzyme cascade strategy could be applied on larger scale to produce the carbohydrate building blocks in gram amounts.[6] Hence, the continuous flow strategy might provide an excellent opportunity to realize the scaling up process.

The last bottleneck was the timing. For a successful synthetic strategy, the optimization and modification of enzymes was preferred on forehand, but these routes were pursued simultaneously. It would have been favorable to have the enzymes optimized and in bulk available to optimize the results in the time span of the IBOS project. Nevertheless we were able to combine the two scientific fields, organic chemistry and biochemistry, into a fruitful collaboration between our university, the University of Amsterdam (Lara Babich,

MSc.), Wageningen University (Pierpaolo Falcicchio, MSc.) and in the case of synthesis of batzellaside, Catalonia Institute for Advanced Chemistry, Barcelona (Prof. Dr. Clapés). As a result the four-enzyme catalyzed cascade reaction was developed, which combines the perspectives of both research fields in an aligned fashion that resulted in a publication in Green Chemistry and three poster prizes at conferences.

7.3 — Future: outlook

From my perspective, the biggest challenge we face in the field of multiple enzyme catalysis is the availability of enzymes with a broad substrate specificity and selectivity. As mentioned in the previous paragraph, this problem could be tackled with directed evolution of the enzyme to enhance the selectivity and specificity, however optimizing these parameters might come at the expense of other parameters, such as reaction rate and stability. Clapés *et al*. have already demonstrated the effect of mutations on RhuA that allowed non-phosphorylated donor substrates such as dihydroxyacetone to give the same products with similar efficiency and selectivity.[7] It would be interesting to extend the effect of mutations towards other donor substrates such as halogen- or cyanide-containing hydroxyacetone derivatives to modify the functionality of the final heterocycles. In addition, the aldolase could also be tuned to enhance the stereoselectivity of the acceptor aldehydes to create a larger variety of functionalized heterocycles. The synthesis of modified acceptor aldehydes was challenging and we were unable to induce successful cascades with e.g. fluoride in the acceptor aldehyde. On the other hand, we were able to functionalize these heterocycles in a later stage. As demonstrated in Section 4.2.3, the introduction of fluoride on DNJ greatly enhances the activity against α-glucosidase. The group of Overkleeft demonstrated that *N*-alkylation also positively affects the activity.[8] We have made a first attempt to combine the enzymatic cascade reaction with the functionalization of the heterocycles to gain more insight in the structure activity relationship of these iminosugars for their glycosidase inhibiting properties, but further research is required.

The highly efficient acid phosphatase PhoN-Sf could be applied in cascade reactions combined with other homo- or heterogeneous catalysts that operate in acidic aqueous media. PhoN-Sf provides easy access to phosphorylated compounds that are challenging to synthesize with classic synthetic strategies that generally require multiple steps. As an example, with PhoN-Sf phosphorylated allylic alcohols can be readily produced generated that *in situ* in aqueous media can undergo palladium-catalyzed cross coupling reactions. This is a topic that is currently pursued in our research group.

With the immobilization of PhoN-Sf and RAMA and the development of a continuous flow system, the scaling up process has become successful.6 The large scale synthesis

with immobilized enzymes was first demonstrated with PhoN-Sf and 0.5 gram phosphorylated inosine was isolated.5 With the immobilization of RAMA, the two-enzyme cascade could be performed in a continuous system as well and the aldol products could be synthesized on gram scale.6 Another advantage of immobilization was suppression of the retro-aldol activity and only the aldol reaction was observed. Bottleneck of this approach is the use of DHA (Section 3.2), which makes the purification more difficult and it would be interesting to investigate the opportunities to immobilize GPO as well and perform the four-enzyme catalyzed cascade reaction in a continuous system starting from glycerol. The immobilization strategy greatly enhances the scaling up process and brings it one step closer to industrial application.

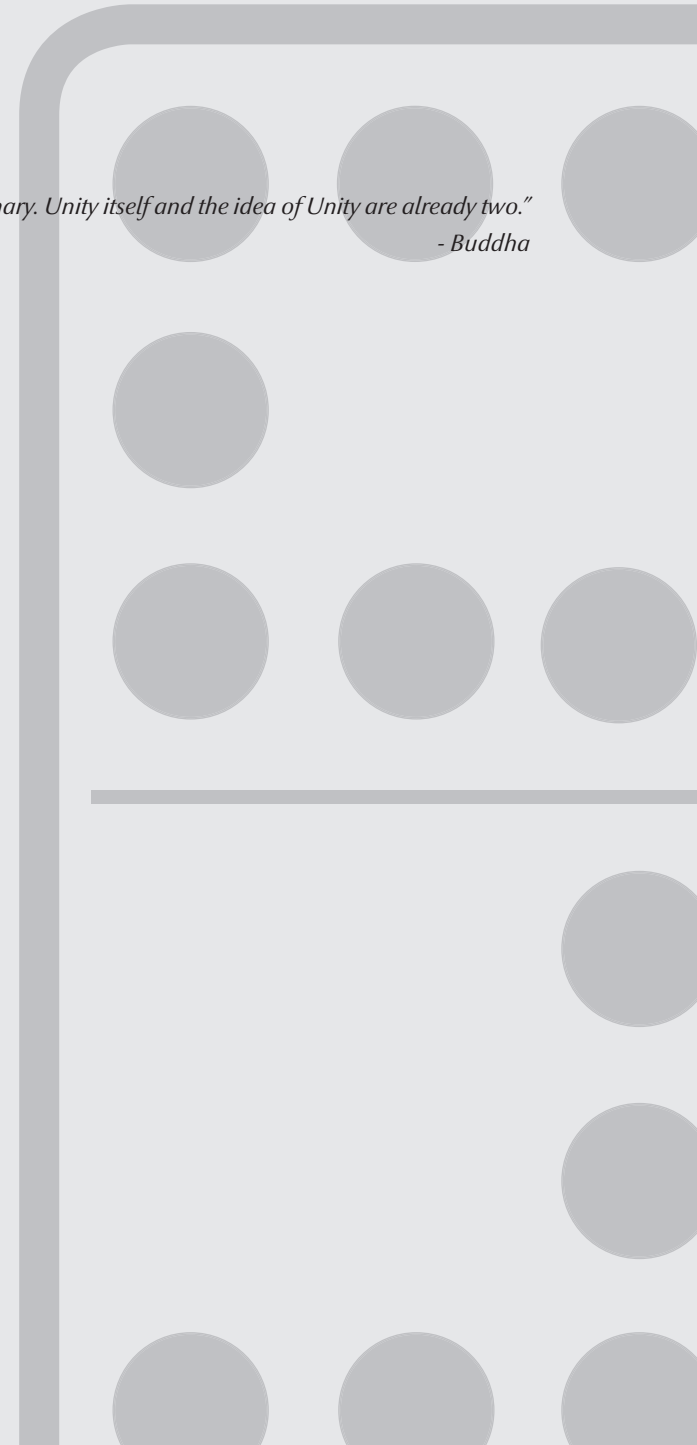
In the past four years we gained significantly more insight in enzymatic cascade reactions with the stereoselective aldol reaction as key step. In my opinion is it an elegant alternative for the classical approach, but to be industrial feasible, it requires more research in the optimization and expression of the enzymes, as well as the development of large scale immobilized enzymes to produce the aldol product on larger scale.

7.4 — References

1. Calveras, J.; Egido-Gabas, M.; Gomez, L.; Casas, J.; Parella, T.; Joglar, J.; Bujons, J. and Clapés, P. *Chem. Eur. J.* **2009**, *15*, 7310–7328.
2. van Herk, T.; Hartog, A. F.; Babich, L.; Schoemaker, H. E. and Wever, R. *ChemBioChem* **2009**, *10*, 2230 – 2235.
3. Stephen M. Dean, S. M.; Greenberg, W. A. and Wong, C.-H. *Adv. Synth. Catal*. **2007**, *349*, 1308 – 1320, a review on recent advancen on aldolases, including gene shuffling.
4. Labbé, G.; de Groot, S.; Rasmusson, T.; Milojevic, G.; Dmitrienko, G. I. and Guillemette, J. G. *Protein Expres. Purif*. **2011**, *80*, 224–233.
5. Babich, L.; Hartog, A. F.; van der Horst, M. A. and Wever, R. *Chem. Eur. J. accepted.*
6. Babich, L.; Hartog, A. F.; van Hemert, L. J. C.; Rutjes, F. P. J. T. and Wever, R. *Manuscript submitted*.
7. Garrabou, X.; Joglar, J.; Parella, T.; Crehuet, R.; Bujons, J. and Clapés, P. *Adv. Synth. Catal*. **2011**, *353*, 89–99.
8. Wennekes, T.; van den Berg, R. J. B. H. N.; Donker, W.; van der Marel, G. A.; Strijland, A.; Aerts, J. M. F. G. and Overkleeft, H. S. *J. Org. Chem.* **2007**, 72, 1088–1097.

Summary

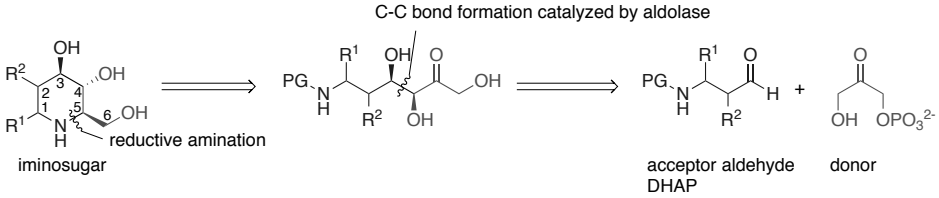
"Unity can only be manifested by the Binary. Unity itself and the idea of Unity are already two."
- Buddha



Summary

Iminosugars, cyclic sugar derivatives in which the ring oxygen atom has been replaced by nitrogen, are biologically active compounds that play an important role in the metabolic pathway. They are potent glucosidase and glycosyltransferase inhibitors and therefore may have pharmaceutical applications. In fact, two iminosugars are currently marketed as drugs. Over the years, many synthetic pathways have been developed to synthesize these types of molecules. In the search for efficient and sustainable synthetic strategies, chemists increasingly pursue approaches that are inspired by Nature. The ultimate goal is to mimic biosynthetic pathways and construct complex products via ingenious sequences of multiple enzymatic conversions in one pot, preferably with both high chemo- and stereoselectivity.

Chapter 1 provides an overview of chemoenzymatic routes towards polyhydroxylated piperidines focusing on chemoenzymatic strategies involving C–C bond formation catalyzed by aldolases (Scheme 1). Nature provides four complementary aldolases to access all four diastereoisomers at the C_3 and C_4 positions.



Scheme 1 — Retrosynthetic approach towards iminosugars.

The use of aldolases provides direct stereoselective access to carbohydrate structures without going through protecting group manipulations. The potential of existing aldolases in C–C bond formation, in particular the fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA), was extensively investigated in **Chapter 2**. With computational studies more insight was gained in the mechanistic properties of RAMA as well as its substrate specificity (Figure 1).

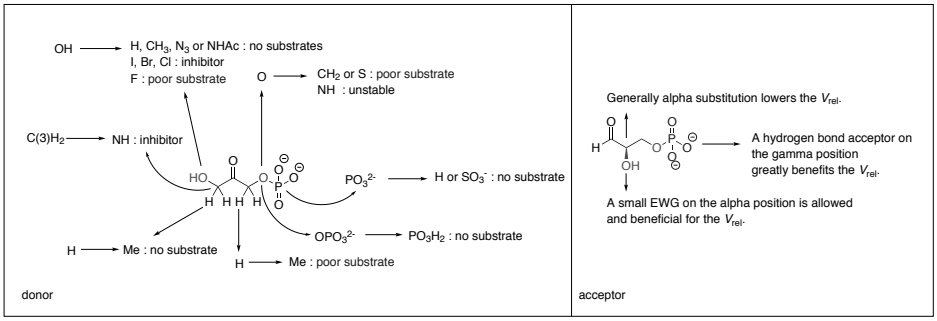
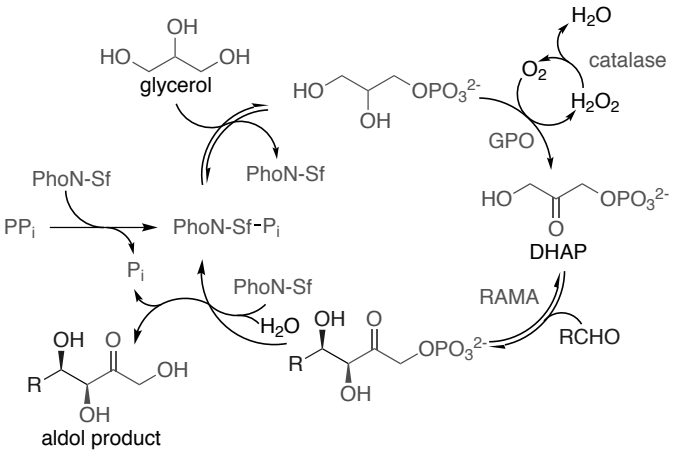


Figure 1 — Structure activity relationships of donor and acceptor substrates for RAMA.

Previously reported research shows that RAMA is highly specific towards its donor substrate, while many varieties on the acceptor substrate are allowed. Computational studies predicted which variations were allowed, based on RAMAs natural acceptor aldehyde D-glyceraldehyde-3-phosphate (D-G-3-P). These results were experimentally verified and from the resulting data we concluded that α-branching on the aldehyde resulted in a lower V_{rel}, which is in accordance with previously reported results. A small electron-withdrawing group (EWG), however, on the α-carbon resulted in a higher V_{rel} due to enhancement of the electrophilicity of the carbonyl group. Double substitution with an EWG at the α-carbon, however, lowered the V_{rel}, probably due to steric hindrance or hydration of the carbonyl group. These results led us to conclude that the high V_{rel} of D-G-3-P was caused by the stabilizing effect of the phosphate group on the γ-position of the aldehyde with the phosphate pocket of the enzyme. This effect was also demonstrated by nitro- and carboxylic acid groups. According to the computational studies, these groups could mimic the hydrogen bond interactions with the enzyme. This was also demonstrated in the experimental verification as both aldehydes showed an increased V_{rel}.

RAMA requires dihydroxyacetone phopsphate (DHAP, Scheme 1) as a donor. **Chapter 3** describes a convenient procedure that was developed for the production of carbohydrates in a highly enantio- and diastereoselective manner using an efficient one-pot four-enzyme-catalytic cascade that *in situ* generates DHAP, followed by the aldol reaction (Scheme 2).

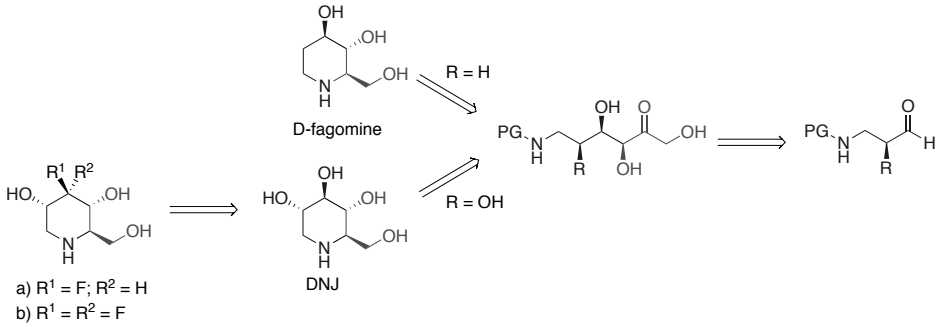


Scheme 2 — The catalytic cycle of the one pot four enzyme cascade reaction.

This cascade starts from inexpensive glycerol and the acceptor aldehyde, and proceeds without the need for protecting groups. The primary step is chemoenzymatic phosphorylation of glycerol by the phosphatase PhoN-Sf and subsequent oxidation by glycerol phosphate oxidase (GPO) to the energy rich phosphate ester DHAP at the expense of PP_i.

In the same pot, the formed DHAP is coupled to a variety of aldehydes by DHAP-dependent aldolases, such as RAMA, allowing the synthesis of all four possible stereoisomers.

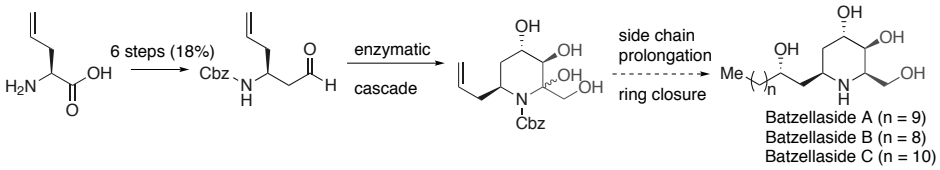
Based on the four-enzyme-catalytic cascade, a two-step strategy to yield iminosugars was successfully demonstrated in **Chapter 4**. Upon optimization, the natural product D-fagomine was obtained in an overall yield of 69% (Scheme 3).



Scheme 3 — Retrosynthesis of 6-membered ring iminosugars via the biocatalytic cascade.

A chemoenzymatic synthesis of deoxynojirimycin (DNJ) was also realized with this strategy and derivatization afterwards resulted in the corresponding C-3 (di)fluorinated iminosugars. The four-enzyme-catalytic cascade was also conducted using the complementary aldolases RhuA, TagA and FucA to synthesize epimeric precursors of D-fagomine. The results, however, demonstrated that the synthesis of the diastereoisomers is strongly dependent on the substrate specificity and stereoselectivity of the aldolases.

Chapter 5 describes an approach towards the synthesis of the so-called batzellasides, naturally occurring iminosugars (Scheme 4). The required precursor aldehyde was obtained via a straightforward synthesis starting from commercially available (*S*)-allylglycine using a cyanide substitution to introduce the aldehyde carbon atom in an overall yield of 18%.

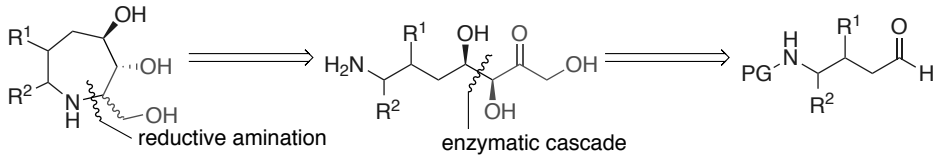


Scheme 4 — Synthetic approach towards batzellasides A–C.

The four-enzyme cascade was performed on the aldehyde at pH 6.5 using RhuA WT to provide the desired aldol product as the corresponding cyclic hemiaminal in 51% isolated yield. Unfortunately, attempts to convert the hemiaminal via reductive amination into the desired cyclic derivative were unsuccessful. To probe the feasibility of the final steps,

2-piperidylacetaldehyde was used as a model compound, on which the side chain via indium-catalyzed allylation and subsequent cross-metathesis was introduced in excellent yields.

Chapter 6 describes the application of the four-enzyme cascade in the synthesis of azepane derivatives (Scheme 5). Trihydroxy-substituted azepanes were obtained in a four- to six-step synthesis via intramolecular reductive amination of the aldol product, which in turn was obtained in the enzymatic cascade reaction. Starting materials were aminoaldehydes, of which the nitrogen atom was typically protected as an azido or nitro group.



Scheme 5 — Restrosynthesis of azepanes using the cascade reaction.

Finally **Chapter 7** places this thesis in perspective and starts with the comparison of the initial goal with the final results and encountered bottlenecks in search of novel enzymatic cascade reactions. The research in this manuscript was focused on creating more sustainable chemistry routes using biocatalysis as a substitute for laborious multistep procedures containing multiple (de)protection steps and resulted in a one-pot four-enzyme cascade reaction allowing us to synthesize non-natural carbohydrate fragments in high enantio- and diastereoselectivities with RAMA as aldolase. With this procedure we were able to synthesize various iminosugars from natural resources. But to be industrial feasible, it requires more research in the optimization and expression of the enzymes, as well as the development of large scale immobilized enzymes to produce the aldol products on industrial scale.

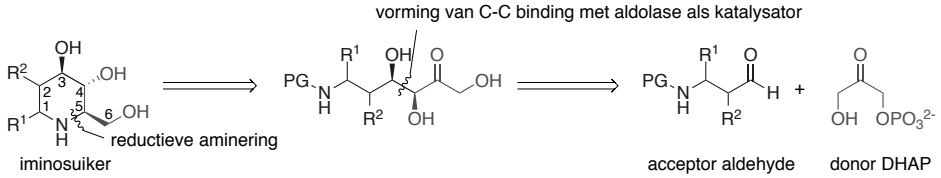
Samenvatting

"Simplicity is the ultimate sophistication."
- Leonardo da Vinci

Samenvatting

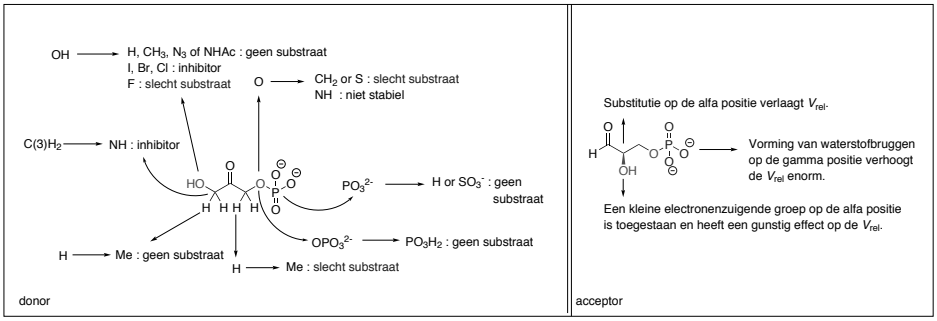
Iminosuikers zijn suikerderivaten waarbij de zuurstof in de ring vervangen is door een stikstofatoom. Deze verbindingen hebben interessante biologische activiteiten, zo worden ze bijvoorbeeld gebruikt als remmers van de enzymen glucosidase en glycosyltransferase, die weer een belangrijke rol spelen bij het metabolisme. De farmaceutische industrie heeft dit type verbindingen nader onderzocht en momenteel zijn er zelfs twee iminosuikers als medicijn verkrijgbaar tegen diabetes en de ziekte van Gaucher. In de afgelopen jaren zijn er vele syntheseroutes ontwikkeld om deze verbindingen te maken. Ons doel was om een duurzame route te ontwikkelen waarbij we gebruik wilden maken van een biosynthetische route om deze complexe verbindingen te maken met de natuur als inspiratie. Uiteindelijk zou dit moeten leiden tot een éénpotssynthese met meerdere enzymen die chemo-, enantio-en diastereoselectief deze verbindingen kunnen produceren.

In **Hoofdstuk 1** staat een overzicht van chemoenzymatische routes waarbij piperidines met een of meerdere hydroxylgroepen gesynthetiseerd worden, in het bijzonder de chemoenzymatische aanpak waarbij een van de de C–C bindingen gevormd wordt met behulp van aldolases (Schema 1). Er komen vier complementaire aldolases in de natuur voor, waarmee alle vier de diastereoisomeren op de C3- en C4-positie gevormd kunnen worden.



Schema 1 — Retrosynthese van iminosuikers.

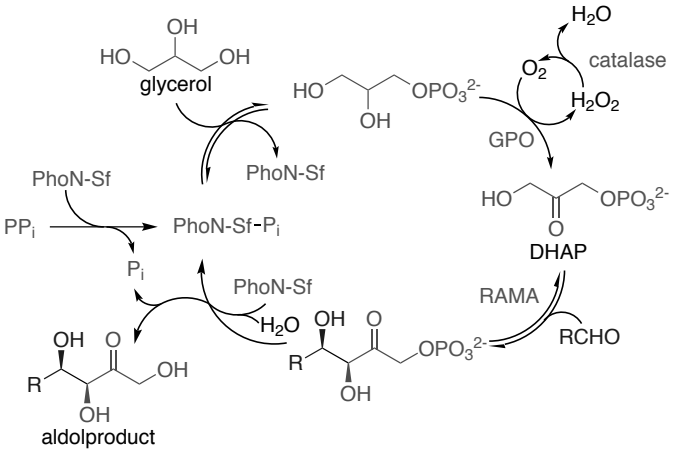
In **Hoofdstuk 2** wordt dieper ingegaan op de werking van de aldolases waarbij in het bijzonder fructose-1,6-bisfosfaat aldolase RAMA nader onderzocht is. Met behulp van computergestuurde 3D simulatie werd een model verkregen waarmee we meer inzicht kregen in de werking van de acceptor en donor (Figuur 1).



Figuur 1 — Structuur-activiteitsrelaties van donor en acceptor met RAMA.

RAMA is zeer specifiek voor het donorsubstraat, terwijl er meer mogelijkheden zijn voor het acceptorsubstraat. Deze werden met de computersimulatie voorspeld aan de hand van het natuurlijke substraat D-glyceraldehyde-3-fosfaat (D-G-3-P). Ter controle werd een aantal substraten experimenteel getest om zo de simulatie te kunnen toetsen. Dit leidde tot de conclusies vermeld in Figuur 1.

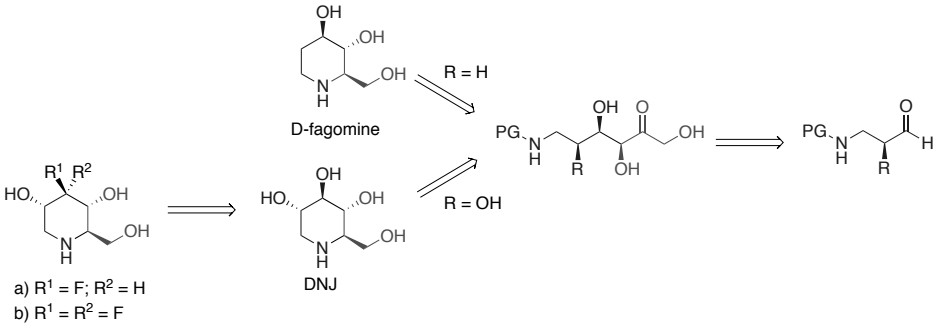
Het beste donorsubstraat is dihydroxyacetonfosfaat (DHAP, Schema 1) wat in **Hoofdstuk 3** samen met het aldolproduct in één pot gesynthetiseerd wordt. Hierdoor ontstaat er een cascade aan reacties waarbij in totaal vier enzymen betrokken zijn (Schema 2).



Schema 2 — De enzymatische cyclus van de éénpotsreactie met vier enzymen.

In de eerste stap wordt glycerol gefosforyleerd met pyrofosfaat door het fosfatase PhoN-Sf, waarna het gevormde product geoxideerd wordt tot DHAP door het enzym glycerol fosfaat oxidase (GPO). Bij deze stap ontstaat tevens waterstofperoxide dat door catalase wordt omgezet in zuurstof en water. Het gevormde DHAP wordt vervolgens met het aanwezige aldehyde gekoppeld door het aldolase (bijvoorbeeld RAMA), waarna PhoN-Sf het gefosforyleerde aldolproduct hydrolyseert en het gewenste aldolproduct ontstaat.

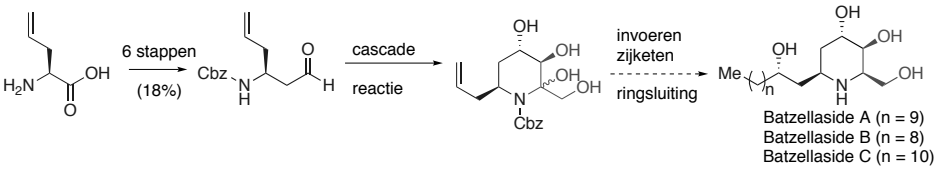
Hoofdstuk 4 laat zien dat met behulp van deze cascadereactie de gewenste iminosuikers in twee stappen gesynthetiseerd kunnen worden (Schema 3). Een van de voorbeelden is D-fagomine, dat geïsoleerd werd in een totale opbrengst van 69%. Verder werd deoxynojirimycin (DNJ) met de cascadereactie gesynthetiseerd.



Schema 3 — Retrosynthese van iminosuikers met behulp van de cascadereactie.

Naast RAMA is de cascade ook uitgevoerd met de stereocomplementaire aldolases Rhamnulose (RhuA), Fuculose (FucA)en Tagatose aldolase (TagA) om epimeren van fagomine te maken, maar hierbij bleek dat de diastereoselectiviteit erg afhankelijk is van het acceptorsubstraat.

In **Hoofdstuk 5** is een start gemaakt met de synthese van batzellaside, ook een natuurlijk voorkomende iminosuiker (Schema 4). Het gewenste acceptoraldehyde werd gesynthetiseerd uit (*S*)-allylglycine in een totale opbrengst van 18%, waarna het werd gekoppeld in de cascadereactie en een cyclisch hemiacetaal gevormd werd.

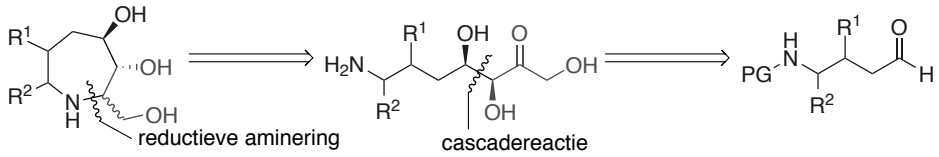


Schema 4 — Synthetisch plan voor de synthese van batzellasiden A–C.

In de cascadereactie werd gebruik gemaakt van RhuA om het gewenste diastereoisomeer te krijgen, dat geïsoleerd werd als hemiacetaal in een opbrengst van 51%. Helaas verliepen de vervolgstappen minder succesvol, zodat deze uiteindelijk gedaan zijn met de modelverbinding 2-piperidylacetaldehyde. De zijketen werd ingevoerd met behulp van een indium gekatalyseerde allylering en vervolgens werd met alkeenmetathese de gewenste keten ingevoerd.

De laatste toepassing van de cascadereactie staat beschreven in **Hoofdstuk 6**, waarbij uitvoerig wordt ingegaan op de synthese van trihydroxyazepanen (Schema 5). Deze azepanen werden verkregen door het reductief amineren van de overeenkomstige aldol-

producten uit de cascadereactie. De stikstof in de acceptoraldehyden werd beschermd als azido- of nitro-groep.



Scheme 5 — Restrosynthese van azepanen.

Ten slotte wordt in **Hoofdstuk 7** het vierjarige promotieonderzoek in perspectief geplaatst. Het doel van dit onderzoek was het ontwikkelen van een duurzame syntheseroute met behulp van enzymatische katalyse dat als vervangende methode gebruikt kan worden voor de klassieke aanpak waarbij meerdere bescherm- en ontschermstappen vereist waren. Met dit onderzoek is de cascadereactie ontwikkeld waarmee suikerderivaten gesynthetiseerd kunnen worden met hoge enantio- en diastereoselectiviteit in één pot met vier enzymen. Hoewel dit op labschaal een uitstekend alternatief is, zal voor de industriële toepasbaarheid meer onderzoek nodig zijn waarbij het grootste knelpunt de beschikbaarheid van de enzymen is. Voor industriële toepassingen zou een methode ontwikkeld moeten worden waarbij de enzymen op grote schaal tot expressie gebracht kunnen worden, evenals het immobiliseren van deze enzymen op industriële schaal.

Dankwoord

"Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We ask ourselves, Who am I to be brilliant, gorgeous, talented, fabulous? Actually, who are you not to be? You are a child of God. Your playing small does not serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others."[1]

1 *A Return To Love: Reflections on the Principles of A Course in Miracles - Marianne Williamson, quoted in Nelson Mandela's inaugural speech.*

Dankwoord

Hier staat het dan ineens allemaal op papier, mijn proefschrift. Vier jaar onderzoek, groei, ontmoediging, levenslessen, ontmoetingen en perspectieven. Dit had ik niet alleen kunnen en willen doen. Ik geloof niet dat de taal toereikend is om mijn waardering in woorden uit te drukken, maar ik zal een poging wagen ;-) Ik ben ontzettend dankbaar voor iedereen die op welke wijze ook een steentje heeft bijgedragen aan dit werk. Dat kan 'm zitten in experimenten en wetenschap, maar ook in de kopjes thee of tedere schopjes onder mijn kont. Ik bedank jullie allemaal voor elke bijdrage hieraan!

Toch is er een aantal mensen dat ik graag nog even in de spotlights wil zetten en ja dat zijn er veel ;-) Allereerst Floris en Floris! Floris, bedankt voor de plaats die je me hebt aangeboden in je groep. Eerst als masterstudent bij Jorge, daarna als promovendus zelfstandig. Ook wil ik je bedanken voor je steun en perspectieven. Het was af en toe niet gemakkelijk, maar na een gesprek met jou waren er altijd weer nieuwe ideeën en mogelijkheden en konden we weer verder. Zelfs de tijdschriften hebben ons niet weten tegen te houden en mede dankzij jouw vertrouwen in ons werk is het toch gepubliceerd!

Floris, mijn co-promotor, jou wil ik ook graag bedanken voor de sparpartijtjes en nuttige tips. Jouw kennis over de (suiker)chemie is vaak zeer nuttig gebleken als we weer een uitdaging tegenkwamen! Daarnaast wil ik jou, Alide, Maxim, Jolijn en Lauke ook ontzettend bedanken voor de leuke avondjes bij jullie thuis. Ik heb ervan genoten! Dit was vaak een welkome afleiding in de afgelopen jaren!

Dan misschien wel de belangrijkste personen die dit boekje tot stand gebracht hebben: de studenten die zich met hart en ziel gestort hebben op het onderzoek. Bram, jij mocht het spits afbijten. En meteen kreeg je een pittige klus. Je werk met nitrilhydratase is helaas niet in dit boekje verschenen, maar je inspanningen voor batzellaside wel! Het was een feest om met je te werken en ik wens je heel veel succes en vooral plezier met je promotieonderzoek!

Daarna nam Danny het stokje van je over. Danny, jouw zelfstandigheid en kunde op het lab maakten de begeleiding voor mij erg gemakkelijk. Je hebt uitstekend werk geleverd, wat zich ook uitte in je eindpunt. Ik wens je heel veel plezier bij Avantium en wie weet kruisen onze wegen elkaar nog wel eens.

Luuk, jij hebt het misschien van alle studenten wel het lastigst gehad. Wat op papier zo simpel leek, bleek in de praktijk heel wat uitdagender. Ik ben enorm trots op wat we uiteindelijk samen hebben weten te creëren. Ik denk dat onze samenwerking wel bewijst dat we beiden niet vies waren van een uitdaging ;-) Bedankt voor jouw bijdrage aan dit boekje in de vorm van hoofdstuk 7!

In deze periode werd mijn groepje ineens uitgebreid met Freek, Sybrin en later Sander en toen was het hek van de dam ;-) Oké jongens ik heb echt ontzettend veel lol met jullie gehad! De foute muziekkeuzes, rare uitspraken en grapjes op het lab hebben regelmatig voor hilarische momenten gezorgd! Freek(alicious), jij hebt het batzellaside hoofdstuk mogen afronden en ook dit bleek niet zo simpel als het eruit zag. Maar met een beetje hulp uit het buitenland is het uiteindelijk een heel mooi hoofdstuk geworden, dankjewel! Veel succes in Leiden, ik kom graag over vier jaar naar je verdediging ;-)

Sybrin, jij werd eerst opgezadeld met een chemisch vraagstuk dat helaas op geen enkele manier op te lossen was. Gelukkig hadden we nog een alternatief in de koelkast staan en lag deze chemische uitdaging je heel wat beter. Je kon weer lachen en produceerde echt de strakste NMR spectra die ik in tijden gezien had! En hoeveel mensen kunnen nu zeggen dat een ton in handen hebben gehad en ook rustig een stufie aan reacties verspilden, Bz ≠ Cbz ;-) Zonder jouw bijdrage was hoofdstuk 4 heel wat korter, thanx!

Sander G., ook bij jou wilde het maar niet van een leien dakje gaan. De computational studies gingen heerlijk vlot, maar ook hier bleek het gebruik van aldehyden in de praktijk ingewikkelder. Gelukkig heb je een mooie methode ontwikkeld om het toch allemaal in beeld te brengen en is dankzij jouw bijdrage hoofdstuk 2 ontstaan. Bedankt voor je inzet en aanwezigheid op het lab! En samen met Sander hoort er ook nog een dikke vette dank naar Sander N. uit te gaan! Voor je begeleiding van Sander en mij tijdens ons 3D RAMA avontuur, maar ook voor je input in de hoofdstukken en artikelen.

Stefan, ik wil jou heel erg bedanken voor je scriptie! Dankzij jouw kunde heb ik duidelijk inzicht gekregen in de wereld van chemoenzymatische piperidine synthese. Ik wens je waanzinnig veel succes met je promotie!

Ook heb ik nog Saskia en Frank voor een aantal weken bij me gehad. Saskia, jij kreeg de lastige taak van de fluorchemie, mede dankzij jouw werk heeft Sybrin dit later met succes af kunnen ronden, bedankt! En dan Frank, ook jij mocht worstelen met aldehyden ;-) En dankzij jouw werk hebben we nu een reproduceerbare route, thanx!

Naast de Nijmeegse inbreng was dit werk ook nooit tot stand gekomen zonder de hulp van de Wever groep! Allereerst jij Ron, bedankt voor de mogelijkheid om op de UvA kennis op te komen doen van de enzymen. Ook wil ik je bedanken voor het vele correctiewerk dat je hebt gedaan, zowel voor de artikelen als mijn manuscript. Louis, dankzij jouw kennis en jarenlange ervaring heb ik in de afgelopen jaren heel veel geleerd op biochemisch/microbiologisch vlak! Daarnaast heb je me kennis laten maken met Amsterdam tijdens de lunchwandelingen en hebben we Mexico verkend! Samen met Lara heb ik genoten van ons avontuur met de cocktails, tequila, Tulum en uiteraard ons tropische congres. Lara, we had a blast these past four years! I loved working together with you and creating our green chemistry masterpiece ;-) Thank you for your help and company in the lab! Good luck with finalizing your thesis and I am looking forward to

your defense! Teunie hoort ook bij dit rijtje uiteraard, want zonder jouw onderzoek was dat van ons niet eens mogelijk geweest! Ook wil ik je bedanken voor het plaatsnemen in de corona! En dan zijn er uiteraard nog Aleksandra, Michael en Rokus, thank you for your contributions! Han en Jan, jullie bedankt voor de moeite die jullie hebben gestoken op mijn massa en NMR spectra interpreteerbaar te krijgen. En uiteraard de Hiemstra-groep bedankt voor alle gezellige koffiepauzes!

Dat brengt me bij het IBOS team, Pierpaolo, John en Maurice van de WUR, Bernard en Martin van DSM, Prof. Kellogg van Syncom (een bijzonder dank voor uw deelname in de oppositie) en Gerjan van MSD, allen bedankt voor de input en discussie tijdens de meetings! In het bijzonder Pedro, allereerst bedankt voor het corrigeren en lezen van mijn manuscript, maar ook voor de overleguurtjes waarbij ik en mijn studenten al onze problemen op tafel konden gooien, dat gaf ons altijd weer mogelijkheden om verder te gaan!

Hans Scheeren, ook u wil ik nog even speciaal bedanken voor het lezen van mijn manuscript en deelname bij de verdediging, dankuwel!

I had a great opportunity to visit Barcelona and do some serious research, while staying in this awesome city. Prof. Clapés thank you so much for your hospitality! Without your help and that of Xavi, we would have never been able to create chapter 5. I learned a lot and really enjoyed the two weeks and I have you and your group to thank for that!

Dan is er nog een select groepje bijzondere mensen op de afdeling die ook zeker een speciale benoeming verdienen! Allereerst Jacky, wat zou ik (zouden we!) zonder je moeten! Met alles (maar dan ook echt alles!) kon ik bij je terecht, dankjewel. En dit geldt ook zeker voor Peter vD. Jouw kantoor stond altijd open voor een praatje of om leeg te roven qua glaswerk en andere benodigdheden ;-) Ook heb ik altijd erg genoten van onze filosofische gesprekken. En dan zijn er natuurlijk Peter vG. Ad, Paul en Helene, het powerteam dat ervoor gezorgd heeft dat mijn experimentelen compleet zijn. En om het lijstje volledig te maken: Marieke, Paula en Desiree, dankjulliewel voor alle hulp bij organisatorische problemen! Ook de heren van Chiralix, Richard en Peter, wil ik graag bedanken voor alle goede tips en adviezen als ik weer eens hun koelkast leeg kwam plukken!

Roseri, jou wil ik ook bijzonder bedanken voor al je steun tijdens de afgelopen jaren. Het was heel fijn om tegelijk met jou dit traject te doorlopen. Ook heb ik genoten van onze nachtelijke babbelsessies tijdens congressen en uiteraard ook voor de suikertoevoer tijdens de lezingen ;-) Je eerlijkheid en directheid vond ik heerlijk verfrissend. Bedankt

voor je taalpurisme tijdens het lezen van mijn manuscript. Egoïstisch gezien hoop ik dat je een baan als klinisch chemicus vindt in Amsterdam e.o., maar zo niet, dan weet ik zeker dat er ergens hier in Nederland een machtig mooi plekje op je wacht! Ook jou Jorge wil ik bedanken voor je kritische oog tijdens het lezen van de hoofdstukken. En daarnaast voor je humor en geweldige kookkunsten! Ik kijk er naar uit om je te feliciteren als Dr. Verkade, het zal je goed staan ;-) Uiteraard kan Marloes hierin niet ontbreken. Ook jij was er altijd voor een goed advies of om even mijn hart te luchten. Je bent samen met Jorge een heel fijn referentiepunt geweest tijdens mijn gehele RU tijd. Ik wens je samen met Sander heel veel plezier en geluk, waar jullie uiteindelijk ook terecht komen! En ook Britzen en Martijn verdienen een benoeming. Britzen voor de vunzige taal en uhm Limburgse meningen tijdens onze dinertjes met Roseri, Jorge en Marloes en Martijn voor zijn muzikale afleiding met Solid.

Dan wil ik iedereen van Lab 03.130 heel erg bedanken. En in het bijzonder Mariëlle, Nanda, Ahne, Mark, Sander vB, Berry, Moniek, Tim, Zaskia en Sophie voor alle heerlijke labpicknicks en fantaaastische muziek! En iedereen van de Rutjes-groep van 2005 tot nu.

En zijn er naast de labgenoten nog een aantal anderen die ik wil benoemen. Om te beginnen Patrick, onze avondjes NAC waren legendarisch! Er is maar één club in Brabant ;-) En de rest van mijn Breda posse: Kim, Monique, Margot, Yves, Marnix, Mike, Chris en Denise. En ook de Groene Sael cq Staalmeesters (of moet het inmiddels gewoon breed Rembrandtpark) posse: Kelly, Mauz, Sindy, Clemens, Lilian, Stephen, Ruud en Gertjan, ik ben zo blij met jullie afleiding (lees theeleutsessies, wijnproeverij, eindeloze BBQs, parkhangsessies) geweest! Heerlijk!

Poffer, Marieke jij verdient uiteraard ook een dikke vette dankjewel! Sinds 2004 ben je een humoristisch, geduldig en wijze vriendin in Nijmegen geweest! Uren kletsen met heel veel thee, kickfitten, spelletjes spelen en ooit nog een legendarische avond met chopstick Rik ;-)

En dan zijn er nog mijn lieve (Avatar)vriendinnetjes Carolien, Ellen, Marieke, Nathalie en Jos. Jullie hebben me van mijn zuiverste kant mogen zien. Bedankt dat jullie er voor me waren, altijd! En dan mijn skypemaatjes in de ochtend: Erma, Joke, Annemieke, Marieke, Natasja, Betina, Betty, Arienne en Paulien, jullie zorgen ervoor dat ik 's ochtends weer fris en gemotiveerd mijn dag kon beginnen! Ook wil ik Marieke, Tonny en Pieta bedanken voor hun steun en wijsheid. And Harry thank you for the tools, they were mighty useful ;-)

Marc, ik ben jou heel erg dankbaar voor je liefde in de afgelopen jaren. Ik heb heel veel van jou en ons geleerd en ben heel blij dat je er tijdens mijn promotietijd als een rots in

de branding was. Onze wegen scheidden zich net voor het afronden hiervan en ik wens je heel veel liefde en een waanzinnig leven in de toekomst!

Ten slotte mijn familie, uiteraard wil ik jullie ook bedanken voor de steun in de afgelopen jaren. En in het bijzonder mijn broeders Janton en Lennar, tevens ook mijn paranimfen. Op Wikipedia (ja wetenschappers ik durf deze in mijn dankwoord wel als bron te gebruiken ;-)) staat het volgende vermeld bij paranimf: "Honderden jaren geleden, toen academische disputen bij een promotie nog wel eens hoog wilden oplaaien en de gemoederen danig verhit konden raken, dienden paranimfen als lijfelijke beschermers." Ik kan me geen betere beschermers bedenken dan mijn twee (letterlijk!) grote broers. Inger en Nova, jullie verdienen ook een bijzonder plekje als de twee grote liefdes van mijn broertje, jullie zijn een hele waardevolle aanvulling op ons gezinnetje!

En dan mama, wat had ik zonder jou gemoeten. Soms was het niet eens nodig om te bellen, maar wist je al op afstand hoe het met me ging. Onze band is niet in woorden te vatten, altijd ben je eerlijk en wind je er geen doekjes om. Ik kon nog wel eens struisvogel spelen met mezelf, maar bij jou lukte dit nooit ;-) Dankjewel voor je onvoorwaardelijke liefde!

Zo het is een hele lap geworden, hoe kon het ook anders. Luitjes hiermee zet ik er echt een punt achter.

Veel liefs,

Het begin.....

Abbreviations

Abbreviations

Ac	acetyl
AcOH	acetic acid
AIBN	2,2′-azobisisobutyronitrile
All	allyl
Alloc	allyloxycarbonyl
aq.	aqueous
Ar	aryl
arom	aromatic
ATP	adenosine triphosphate
ax	axial
Bn	benzyl
Boc	*tert*-butoxycarbonyl
br	broad
Bu	butyl
Bz	benzoyl
Cbz	benzyloxycarbonyl
CSA	camphorsulfonic acid
d	days
d (NMR)	doublet
DAST	diethylaminosulfur trifluoride
dd (NMR)	doublet of doublets
d.e.	diastereomeric excess
DCM	dichloromethane
D-G-3-P	D-glycerol-3-phosphate
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DIBAL	diisobutylaluminum hydride
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMP	dess-martin periodane
DMSO	dimethyl sulfoxide
DNJ	deoxynojirimycin
DMJ	deoxymannojirimycin
dt (NMR)	doublet of triplets
e.e.	enantiomeric excess
e.g.	*exempli gratia* (for example)

EI	electron impact
eq	equatorial
equiv.	equivalents
ESI	electrospray ionization
Et	ethyl
et al.	*et alia* (and others)
EWG	electron withdrawing group
FBP	D-fructose-1,6-bisphosphate
FHAP	fluorohydroxyacetone phosphate
Fmoc	9-fluorenylmethoxycarbonyl
GC	gas chromatography
h	hours
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
i.e.	*id est* (that is)
IR	infrared
K_m	Michaelis constant
LRMS	low resolution mass spectrometry
m (NMR)	multiplet
M	molar
m-CPBA	*meta*-chloroperoxybenzoic acid
Me	methyl
MeCN	acetonitrile
MIC	minimum inhibitory concentration
min	minutes
MOE	molecular operating environment
MS	mass spectrometry
m/z	mass to charge ratio
NAD(H)	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
o	ortho
p	para
PDB	protein database
pHTX	perhydrohistrionicotoxin
PG	protecting group
Ph	phenyl
PP_i	pyrophosphate
q	quartet

quant.	quantitatively
RCM	ring-closing metathesis
R_f	retention factor
RT	room temperature
s	singlet (NMR)
SAR	structure activity relationship
sat.	saturated
sp.	species
t (NMR)	triplet
TBDMS	*tert*-butyldimethylsilyl
t-Bu	*tert*-butyl
TEA	triethylamine
TES	triethylsilyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
TsOH	*p*-toluenesulfonyl acid
U	units
UV	ultraviolet
V_{rel}	relative reaction rate

Amino acids

Arg	arginine (R)
Asn	asparagine (N)
Asp	aspartate (D)
Glu	glutamate (E)
Lys	lysine (K)
Ser	serine (S)
Tyr	tyrosine (Y)

Enzymes

AA-1	aspergillus acylase 1
Cal	*Candida antarctica* lipase
CRL	*Candida rugosa* lipase

FruA	fructose-1,6-bisphosphate aldolase
FSA	D-fructose-6-phosphate aldolase
FucA	fucose-1-phosphate aldolase
GPDH	glycerol-3-phosphate dehydrogenase
GPO	L-glycerol-3-phosphate oxidase
HNL	hydroxynitrile lyase
PCL	*Pseudomonas cepacia* lipase
PhoN-Sf	recombinant *Shigella flexneri* acid phosphatase
PLE	pig liver esterase
RAMA	rabbit muscle aldolase
RhuA	rhamnulose-1-phosphate aldolase
ROL	*Rhizopus oryzae* lipase
SPH	*Sphingomonas* sp. HXN-200
TagA	tagatose-1,6-bisphosphate aldolase
WT	wild type

List of Publications

Publications

van Hemert, L. J. C.; van Rootselaar, S. and Rutjes, F. P. J. T. **Chemoenzymatic strategies to enantiopure piperidines**, *manuscript submitted.*

Babich, L.; Hartog, A. F.; van Hemert, L. J. C.; Rutjes, F. P. J. T. and Wever, R. **The synthesis of carbohydrates in a continuous-flow reactor by immobilized phosphatase and aldolase**, *manuscript submitted*.

van Hemert, L. J. C.; Groenen, A. J. J.; Wilders, L. J. A.; Babich, L.; Wever, R.; Nabuurs, S. and Rutjes, F. P. J. T. **Expanding the scope of one-pot four-enzyme cascade aldol reactions**, *manuscript in preparation.*

van Hemert, L. J. C; Babich, L; Bury, A; Hartog, A. F; van Herk, T; Wever, R and Rutjes, F. P. J. T. **Synthesis of non-natural carbohydrates from glycerol and aldehydes in a one-pot four-enzyme cascade reaction**, *Green Chemistry*, **2011**, *13*, 2895-2900.

Verkade, J. M. M.; van Hemert, L. J. C.; Quaedflieg, P. J. L. M. and Rutjes, F. P. J. T. **Organocatalysed asymmetric Mannich reactions**, *Chemical Society Reviews*, **2008**, *37*, 29-41.

Verkade, J. M. M.; van Hemert, L. J. C.; Quaedflieg, P. J. L. M.; Schoemaker, H. E.; Schuermann, M.; van Delft, F. L. and Rutjes, F. P. J. T. **Laccase-mediated deprotection of para-methoxyphenyl (PMP)-protected amines**, *Advanced Synthesis & Catalysis*, **2007**, *349*, 1332-1336.

Verkade, J. M. M.; van Hemert, L. J. C.; Quaedflieg, P. J. L. M.; Alsters, P. L.; van Delft, F. L. and Rutjes, F. P. J. T. **Mild and efficient deprotection of the amine protecting p-methoxyphenyl (PMP) group**, *Tetrahedron Letters*, **2006**, *47*, 8109-8113.

Recent presentations

Synthesis of carbohydrate fragments through biocatalytic cascades – presentation at the NWO KNCV meeting in Wageningen, April 8th 2011.

One-Pot Four-Enzyme Cascade Catalysis: Two-Step Synthesis of D-Fagomine – presentation at the Zing Biocatalysis conference in Puerto Morelos, Mexico, December 10th – 13th 2010.

Toward a Cascade Catalysis-based Synthesis of Batzellaside – poster presentation at NWO studygroup meeting in Lunteren, October 25 th– 27th 2010.

Synthesis of industrially relevant heterocycles through biocatalytic cascades – presentation at sIMMposium in Nijmegen, May 17th 2010

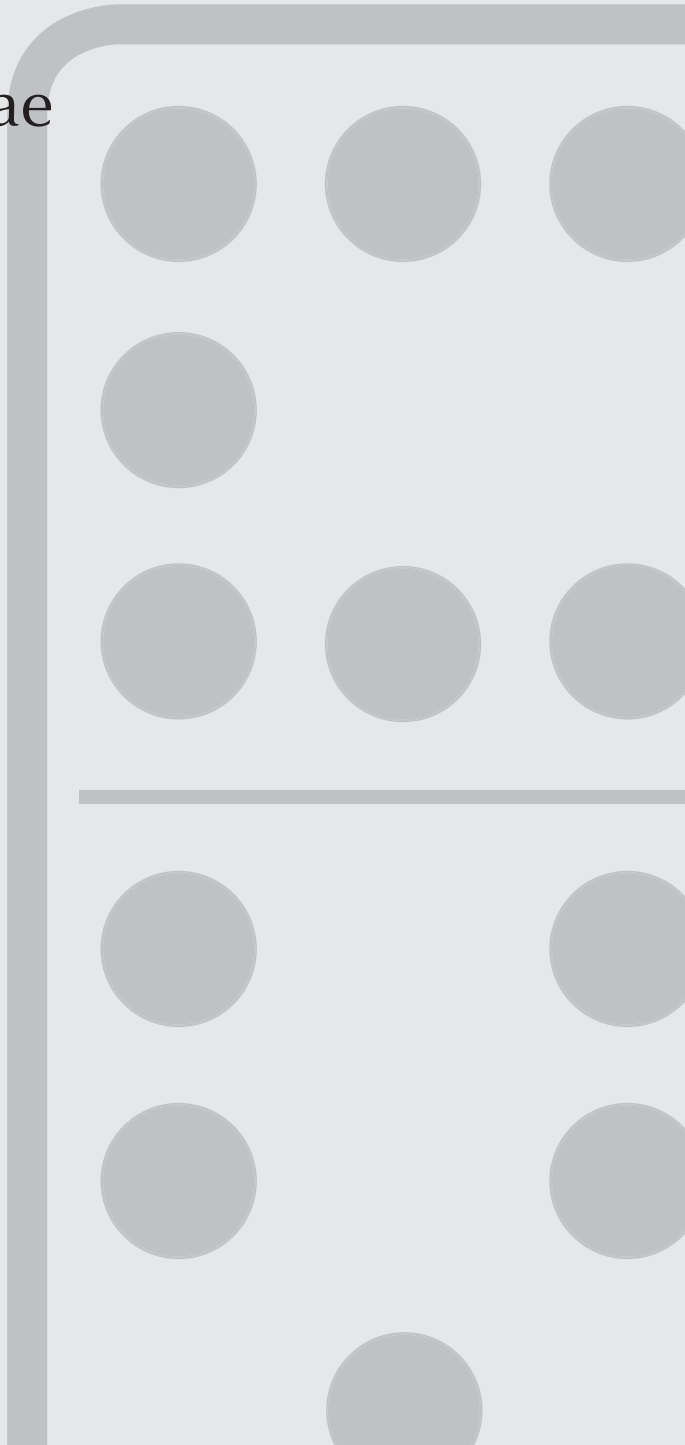
Four-enzyme-catalytic cascade to synthesize carbohydrate fragments – poster presentation at NWO study group meeting in Lunteren, October 19th – 21th 2009.
This poster was awarded the first poster prize.

Facile synthesis of D-Fagomine via a stereo-selective one pot cascade reaction – poster presentation at Tetrahedron conference in Paris, June 23th – 26th 2009.

Industrially relevant heterocycles through biocatalytic cascades – presentation at the NCCC conference in Noordwijkerhout, March 1st – 3rd 2009.

Facile synthesis of D-Fagomine via a stereo-selective one pot cascade reaction – poster presentation op NWO study group meeting in Lunteren, October 20th – 22nd 2008.
This poster was awarded the first poster prize.

Curriculum Vitae



Curriculum Vitae

Lieke is op 19 april 1980 in Middelburg geboren. Haar middelbare schooltijd op het atheneum is begonnen in 1992 op Colegio Arubano te Aruba en voltooid aan het Mencia de Mendoza Lyceum in Breda. Haar chemische carrière begon met de opleiding Chemie aan de Hogeschool West-Brabant (nu Avans) in 1998. In het laatste jaar (2001-2002) heeft ze met veel plezier een stage voltooid bij Janssen Pharmaceutica op de afdeling Johnson & Johnson Pharmaceutical Research and Development. Dit beviel haar zo goed dat ze er nog twee jaar werkzaam is geweest. De honger naar kennis was sterker en in 2004 is ze gestart met de master opleiding Scheikunde aan de Radboud Universiteit Nijmegen. Tijdens haar vrije uurtjes kluste ze als vakantiekracht bij MercaChem. In 2005 is ze begonnen met haar hoofdvakstage onder begeleiding van prof. dr. Floris Rutjes en Jorge Verkade, waarbij ze onderzoek heeft gedaan naar de enzymatische ontscherming van de paramethoxyfenyl beschermgroep. Nadat ze *cum laude* haar master diploma behaalde in 2007 wachtte het promotieavontuur. Dit werd even op pauze gezet door een korte stop bij Schering Plough (Organon/MSD), maar in september 2007 kon het van start binnen het IBOS programma. Het onderwerp "Chemoenzymatische aldolreacties: het toepassen van de vier-enzym-cascadereactie" staat in dit proefschrift nader toegelicht. In augustus 2012 is Lieke aan de slag gegaan als docent chemie op de HLO van InHolland.

I see trees of green, red roses too.
I see them bloom, for me and you.
And I think to myself, what a wonderful world.

I see skies of blue, and clouds of white.
The bright blessed day, the dark sacred night.
And I think to myself, what a wonderful world.

The colors of the rainbow, so pretty in the sky.
Are also on the faces of people going by.
I see friends shaking hands saying, "How do you do?"
They're really saying "I love you".

I hear babies cry, I watch them grow.
They'll learn much more than I'll ever know.
And I think to myself what a wonderful world.

Oh yeah.

What a wonderful world – Bob Thiele and George David Weiss