Development of an Organo- and Enzyme-Catalysed Cascade Reaction

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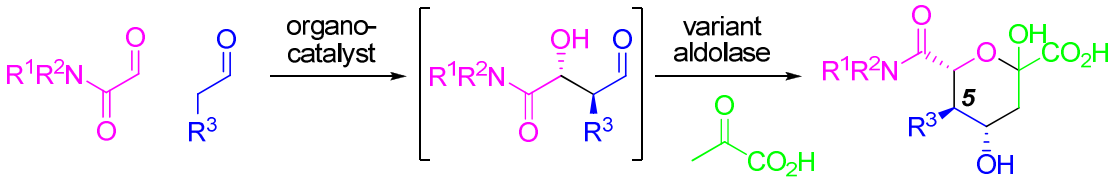
Abstract

This thesis describes the development of a novel one-pot reaction (Scheme 1). The reaction combines organo- and enzyme-catalysis in an aldol/aldol cascade for the first time. The bicatalytic, three-component assembly involves two aldol reactions: an organocatalysed reaction and an aldol reaction catalysed by an aldolase.



Scheme 1

The feasibility of the one-pot reaction was evaluated by testing whether the aldol intermediates **3** were substrates for the E192N aldolase variant of *N*-acetylneuraminic acid lyase. A range of compounds was selected as potential substrates for the enzyme. The synthesis of the compounds, and their characterisation as substrates for the aldolase variant, is described.

The development of common conditions under which both organo- and enzyme-catalysis could occur was then investigated. To do so, a number of potential organocatalysts suitable for the one-pot reaction were prepared. The catalysts were evaluated in a screening reaction which yielded optimal conditions for the organocatalysed reaction. The organocatalytic conditions were tested for compatibility with the E192N aldolase variant using an appropriate enzyme assay. The optimised process was a sequential one-pot reaction in which the organocatalysed reaction was conducted before the addition of the variant enzyme. The scope of the optimal process was determined and a range of novel acids of general structure **4**, were prepared.

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Abbreviations

ADH	Alcohol dehydrogenase
BINOL	1,1'-Bi-naphthol
br	Broad
CMP-NeuAc	Cytosine-5'-monophosphate-acetylneuraminate
COSY	Correlation spectroscopy
d	Doublet
DCM	Dichloromethane
DERA	2-Deoxyribose-5-phosphate aldolase
DEPT	Distortionless enhancement by polarisation transfer
DHAP	Dihydroxyacetone phosphate
DKR	Dynamic kinetic resolution
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMS	Dimethyl sulphide
DMSO	Dimethyl sulfoxide
d.r.	Diastereomeric ratio
e.e.	Enantiomeric excess
e.r.	Enantiomeric ratio
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact ionisation
Ent	Enantiomer
ep-PCR	error prone polymerase chain reaction
ES	Electrospray ionisation
EtOAc	Ethyl acetate
FDP	Fructose 1,6-diphosphate
FT-IR	Fourier transform-infra red
fur	Furanose
Gc	Glucose
H. influenzae	*Haemophilus influenzae*
HMBC	Heteronuclear Multiple Bond Correlation experiment
HMQC	Heteronuclear Multiple-Quantum Coherence Experiment
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IPA	*Iso*-propyl alcohol
IPTG	*Iso*-propyl β-D-1-thiogalactopyranoside
k_{cat}	Catalytic constant

KDGP	D-2-Keto-3-deoxy-6-phosphogluconate
K_M	Michaelis constant
LCMS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
lit	Literature
m	Multiplet
maj	Major
ManNAc	*N*-acetyl-D-mannosamine
MeCN	Acetonitrile
min	Minor
ms	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NAL	*N*-acetylneuraminic acid lyase
NMO	*N*-Methylmorpholine-*N*-oxide
NOESY	Nuclear Overhauser effect spectroscopy
Nu	Nucleophile
PPTS	Pyridinium *para*-toluenesulfonate
psi	Pounds per square inch
pyr	Pyranose
q	Quartet
rot	Rotomer
rpm	Revolutions per minute
s	Singlet
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
t	Triplet
TAE	Tris-acetate-EDTA
TBA	Thiobarbituric acid
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TOCSY	Total correlation spectroscopy
Tris	Tris(hydroxymethyl)aminomethane
wt	Wild type

Chapter 1

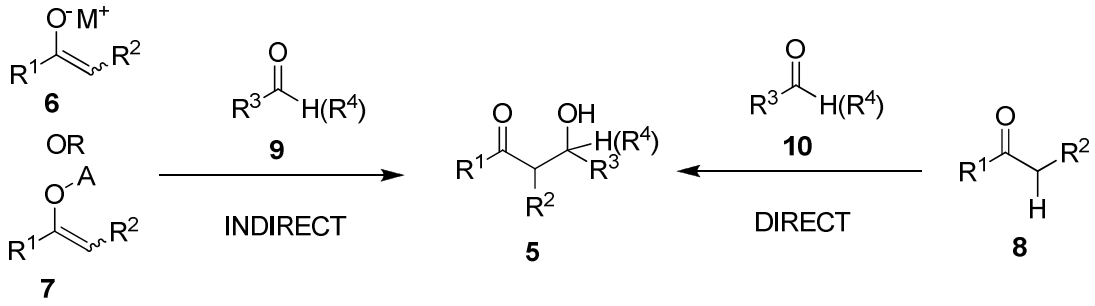
Introduction

The development of cascade reactions is an emerging field within synthetic organic chemistry. Cascade reactions can generate sophisticated chemical skeleta from simple building blocks and create molecular complexity rapidly. Cascade reactions provide the organic chemist with the synthetic weaponry to prepare complex products efficiently. Such reactions have the potential to minimise the waste of economic and chemical resources, and their synthetic utility has provided the motivation for substantial research in the area. This subject is summarised in numerous comprehensive reviews.[1-4]

This thesis describes the development of a novel bicatalytic cascade reaction which exploits both organo- and enzymatic-catalysis in two consecutive aldol reactions. To place this approach into context, the introduction will describe the value of the aldol reaction and catalytic cascade reactions to synthetic chemistry, with particular emphasis on examples from the recent literature.

1.1 The Aldol Reaction

The aldol reaction is one of the most useful and versatile transformations in organic synthesis.[5] Discovered in the 19th century, the aldol reaction is a powerful tool for the organic chemist and has been used extensively due to the occurrence of 1,3-oxygenation pattern in a wide range of natural products.[6, 7] The reaction involves the addition of an enolisable carbonyl donor to an aldehyde or ketone acceptor (Scheme 2).[8] The addition thus forms a new carbon-carbon bond and up to two stereogenic centres in the aldol product **5**.



Scheme 2: The Aldol Reaction. The two general classes are the indirect and direct aldol reactions.

There are two general classes of aldol reactions: indirect and direct aldol reactions (Scheme 2). In the indirect version, an enolate (**6**), or enolate equivalent (**7**) is pre-formed and subsequently attacks the carbonyl acceptor (**9**). Examples of enolates (**6**) or enolate equivalents (**7**) used in the indirect aldol reaction include metal enolates,[5] boron enolates[5, 9] or silyl enol ethers.[10-18]

In contrast, direct aldol reactions involve the formation of an enolate equivalent in *situ*, which then reacts with the carbonyl acceptor **10** under the same reaction conditions. This introduction will only focus on catalytic direct aldol reactions; however, there are many reviews that describe indirect aldol reactions.[13, 14, 19]

1.1.1 Catalytic Direct Aldol Reactions

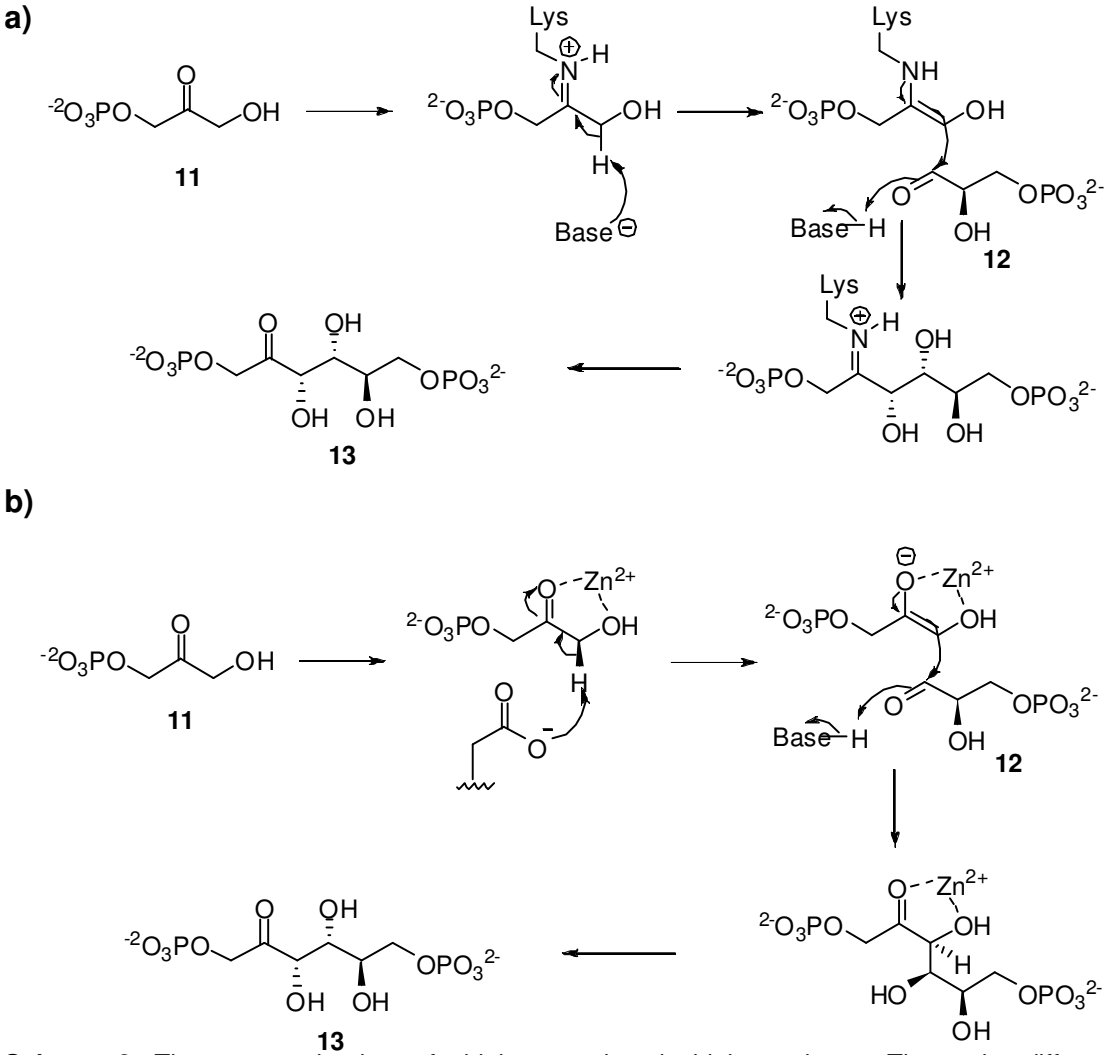
There are three major classes of catalytic direct aldol reactions: aldolase-catalysed (section 1.1.1.1), metal-catalysed (section 1.1.1.2) and organocatalysed (section 1.1.1.3) aldol reactions. The catalysis of direct aldol reactions by catalytic antibodies[20-22] is beyond the scope of this introduction.

1.1.1.1 Aldolase-Catalysed Direct Aldol Reactions

Enzymes are remarkably efficient catalysts as they can increase the rate of a reaction dramatically.[23] Enzymes operate under mild, aqueous reaction conditions and are bio-degradable, rendering them both economically and

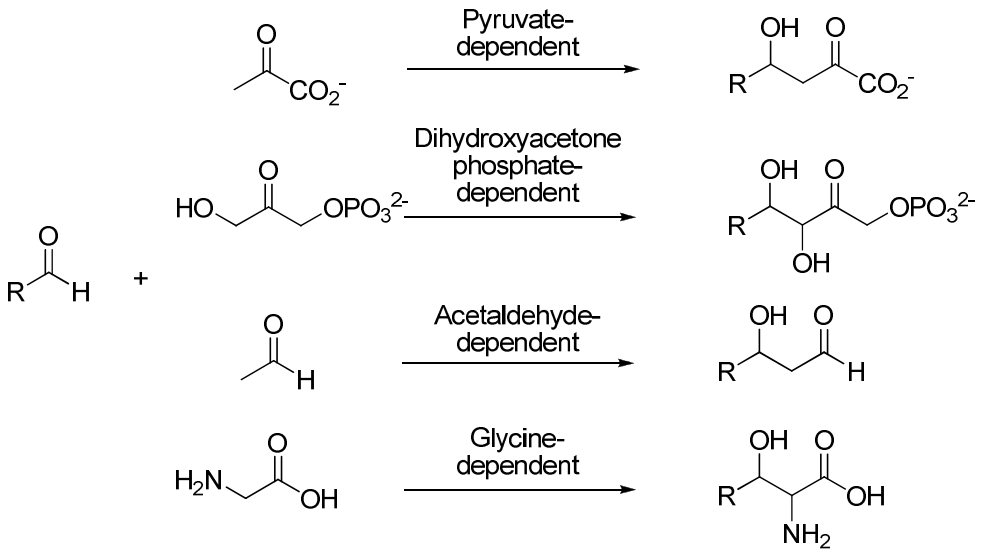
environmentally attractive. Most importantly, the regio-, diastereo- and enantio-selectivities observed in aldolase-catalysed reactions are often extremely high.[19, 24]

Over thirty aldolases have been discovered to date, many of which have been used successfully in synthesis.[25] These aldolase enzymes may be classified by their mechanism as either Type I or II (Scheme 3). The Type I aldolases form a Schiff base and thereby activate the aldehyde or ketone donor for attack on the acceptor carbonyl. The Type II aldolases utilise Zn^{2+} as a co–factor.[19]



Scheme 3: The two mechanism of aldolase-catalysed aldol reactions. The major differences between the mechanisms are illustrated for the two classes of fructose-1,6-diphosphate (FDP) aldolase which catalyse the condensation between dihydroxyacetone phosphate (DHAP, **11**) and glyceraldehyde 3-phosphate (**12**) **a)** In the rabbit muscle Type I aldolase (RAMA), the lysine residue forms a Schiff base with DHAP which attacks the carbonyl group of glyceraldehyde 3-phosphate **b)** In the Type II, FDP aldolase a zinc enoliolate is formed which attacks the acceptor aldehyde, glyceraldehyde 3-phosphate **12**.[19]

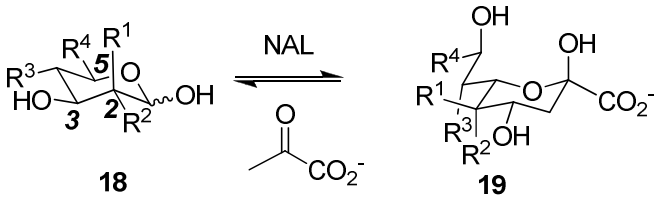
The aldolases may also be classified by the structure of their donor of which there are four major classes: pyruvate-, dihydroxyacetone phosphate-, acetaldehyde- and glycine-dependent aldolases (Scheme 4).



Scheme 4: The four major classes of aldolases classified by donor structure

1.1.1.1.1 Direct Aldol Reactions Catalysed by Pyruvate-Dependent Aldolases

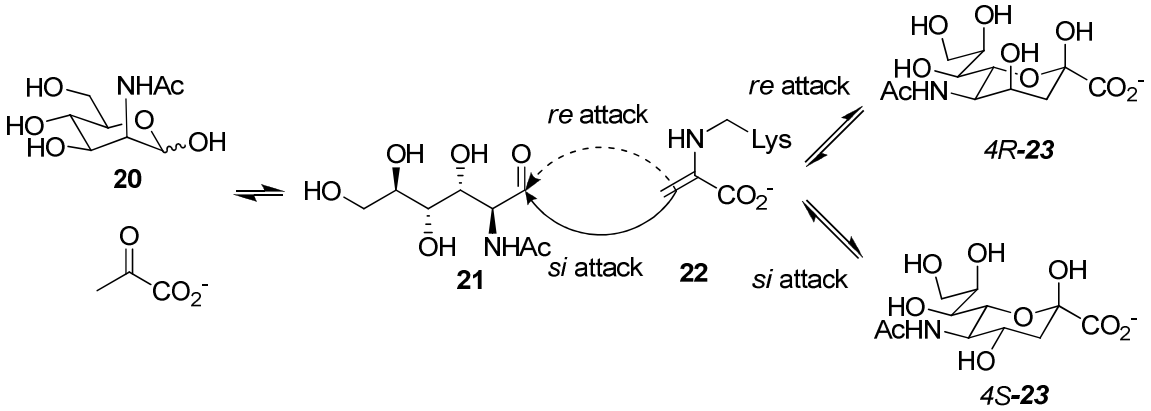
N–Acetylneuraminic acid lyase (NAL or sialic acid aldolase) is a pyruvate-dependent aldolase and has been the focus of this project. NAL is a commercially available enzyme that catalyses the reversible aldol reaction between a range of acceptor aldehydes (**18**) and pyruvate to give aldol products (**19**) (Scheme 5). The aldolase has a relatively wide substrate scope, accepting a range of substrates with differing groups in the C-2 and C-5 position (carbons are numbered based on **18**). However, aldehyde substrates normally require a hydroxyl group at C-3 and aldehydes with fewer than five carbon atoms are poor substrates. NAL is usually used synthetically with excess pyruvate, allowing the efficient synthesis of many sialic acid derivatives.[26]



R^1	R^2	R^3	R^4
AcNH	H	OH	CH_2OH
AcNH	H	OH	CH_2OAc
AcNH	H	OH	CH_2N_3
AcNH	H	OH	CH_2F
OH	H	OH	CH_2OH
OH	H	H	CH_2OH
OH	H	OH	H
H	H	OH	CH_2OH
H	OH	OH	CH_2OH
Ph	H	OH	CH_2OH
AcNH	H	OH	CH_2OCH_3
N_3	H	OH	CH_2OH
AcNH	H	OH	$CH_2OP(O)Me_2$

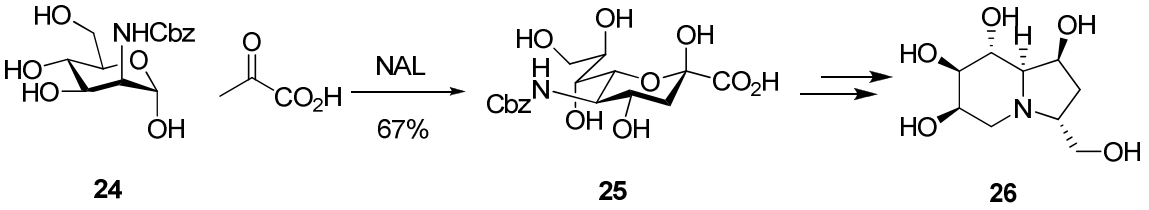
Scheme 5: Wild-type NAL substrate scope: NAL accepts a range of substrates [25]

NAL is a Type I aldolase where the formation of a pyruvate enamine intermediate (**22**) is followed by its addition to the acceptor aldehyde (*e.g.* *N*-Acetyl-D-mannosamine, ManNAc **20**) forming the carbon-carbon bond and resulting in a new stereocentre (Scheme 6). The enamine intermediate **22** can attack either the *re*- or *si*-face of the acceptor aldehyde **21** to form either diastereomeric product (4*R*- or 4*S*-**23**) Although NAL is a synthetically useful enzyme, it exhibits poor facial stereoselectivity and the stereochemical outcome is often thermodynamically controlled by the relative stabilities of the two products.[19, 26-29] In theory, the preparation of the less thermodynamically favoured product is feasible under kinetic control, by isolating the product before equilibrium is reached.



Scheme 6 Mechanism of NAL where the *re*-attack and *si*-attack of the aldehyde **21** leads to the 4*R*- and 4*S*-**23** configured aldol products respectively

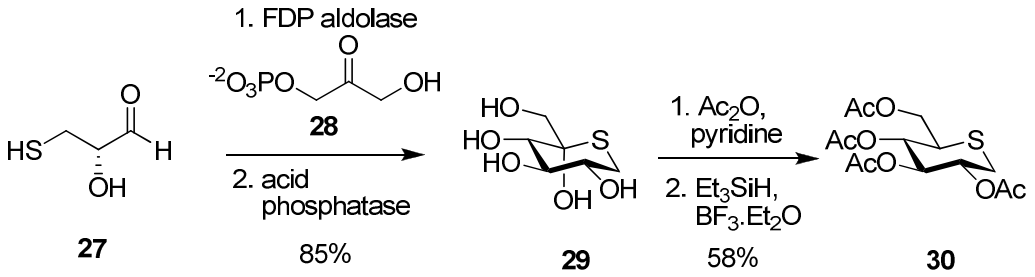
NAL has often been used synthetically for example in the preparation of aza-sugars. The condensation of *N*-Cbz-D-mannosamine **24** with pyruvate yielded the sialic acid analogue **25,** which was subsequently transformed into the glycosylase inhibitor 3-(hydroxymethyl)-6-epicastanospermine **26** (Scheme 7)[30] This chemoenzymatic approach is shorter than any previous synthesis allowing the 3-(hydroxymethyl)-6-epicastanospermine **26** to be prepared in eight steps.



Scheme 7: The synthesis of aza-sugars using NAL

1.1.1.1.2 Direct Aldol Reactions Catalysed by Dihydroxyacetone Phosphate-Dependent Aldolases

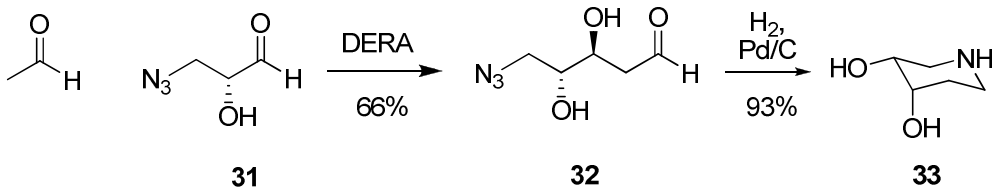
The dihydroxyacetone phosphate-dependent (DHAP) aldolases catalyse a variety of stereoselective aldol reactions.[25] The DHAP-dependent aldolase, fructose 1,6-diphosphate aldolase (FDP), has been used in a short and efficient synthesis of the thiosugar **30** which avoids the more traditional approaches typically involving protecting group chemistry and naturally occurring sugars.[31] Thus, the sugar **29** was prepared using a FDP-mediated aldol condensation with aldehyde **27**, followed by a dephosphorylation reaction and reduction to give the thio-sugar **30** (Scheme 8).[32]



Scheme 8: Chemoenzymatic Synthesis of the thio-sugar **30** using the DHAP-dependent aldolase FDP aldolase

1.1.1.1.3 Direct Aldol Reactions Catalysed by Acetaldehyde-Dependent Aldolases

2-Deoxyribose 5-phosphate aldolase (DERA) is an important acetaldehyde-dependent aldolase and is an unusual aldolase as *both* the donor and the acceptor are aldehydes.[25, 33, 34] The wild-type enzyme catalyses the reversible aldol condensation between acetaldehyde and a range of aliphatic aldehydes and has been used successfully in synthesis. For example, DERA has been exploited in the synthesis of the carbohydrate analogue **33** (Scheme 9).[34] The DERA-catalysed aldol reaction between acetaldehyde and the aldehyde **31** was stereoselective and gave the aldol product **32**; hydrogenation gave the aza-sugar **33**. DERA has also been used effectively in a one-pot enzymatic cascade reaction in the preparation of simple sugars, (section 1.2.3).



31 **32** **33**

Scheme 9 Synthesis of aza-sugar **33** using DERA

As the previous examples have demonstrated, enzymes offer great potential as catalysts for a variety of organic transformations, often exhibiting high efficiency and control. However, to date, the application of aldolases in synthetic chemistry has been restricted by their limited substrate repertoire.

1.1.1.1.4 Directed Evolution of Aldolases with Tailored Properties for Organic Synthesis

Directed evolution is an approach which aims to generate proteins with novel functions. Initially, diversity is introduced into the gene of interest creating a library of variant genes and, hence, a library of variant proteins. The protein library is then subjected to a screening or selection pressure, and the variant proteins with the desired function may be identified. The cycle may be repeated until the desired function is optimised.

The directed evolution approach has been used to broaden the range of enzymes with synthetically valuable properties. The approach can also offer many additional benefits to the synthetic organic chemist, such as increased tolerance to organic solvents[35, 36] or altered stereoselectivity[37-39] and has been reviewed extensively.[40-45]

The evolution of a number of important aldolases in order to yield a variety of useful mutant aldolase has been performed. The wild-type NAL enzyme has a wide substrate scope in comparison to other aldolases, but shorter-chain aldehydes are poor substrates (section 1.1.1.1.1).[25] The challenge of broadening NAL substrate scope motivated Gavin Williams to engineer an aldolase which accepted aldehyde, **35a**, to give the resultant sialic acid derivative **36a** (Scheme 10).[46, 47] A semi-rational approach was used, which involved initial analysis of the X–ray crystal structure of NAL from *H. influenzae* in complex with 4-oxo-sialic acid (Figure 1)[48] in order to identify the potential residues which were likely to be important in governing substrate specificity. Saturation mutagenesis was performed upon the identified residues, and the mutant enzyme E192N was found to be an effective catalyst for the cleavage of **36a**. The variant enzyme E192N was, in terms of specificity constants (k_{cat}/K_M) for their respective substrates (Table 1), six times more efficient at substrate cleavage than the wild-type enzyme.

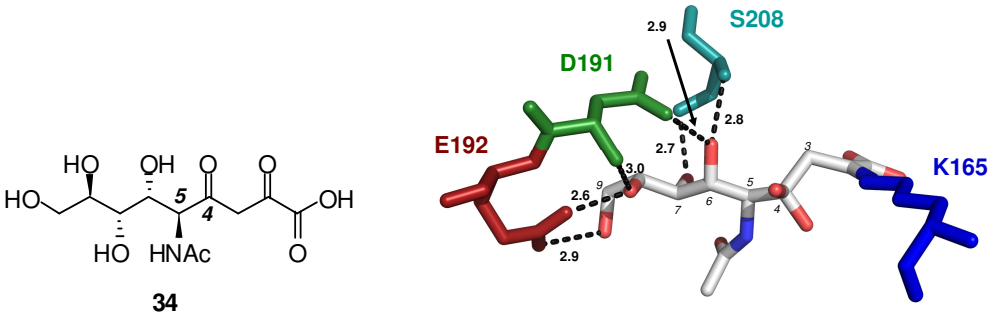
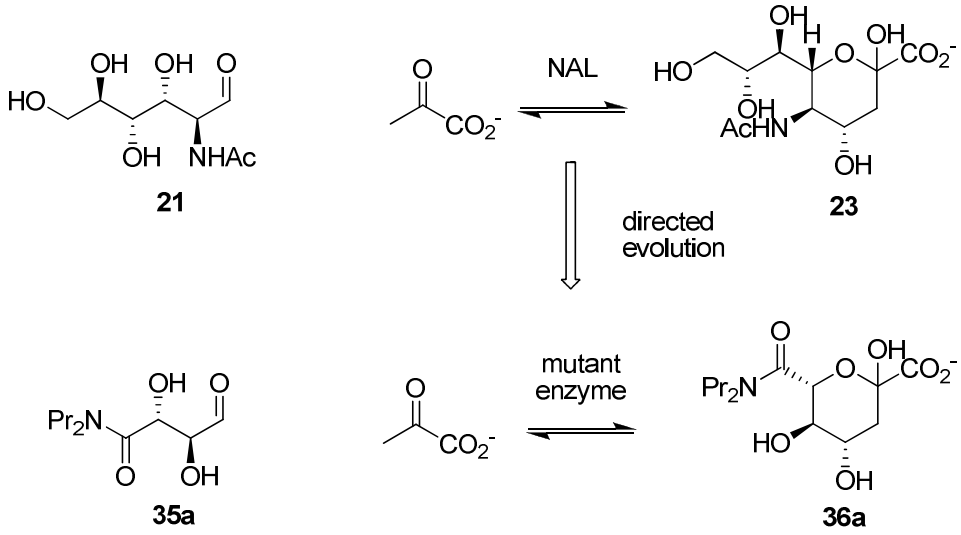


Figure 1 X-ray crystal structure of NAL from *Haemophilus influenze* in complex with 4-oxo-sialic acid (**34**).[48] The structure shows distances between the heteroatoms of the acid **34** and the residues D191, E192 and S208. The residues are numbered according to the *E.* coli and distances are in Ångströms. [49]



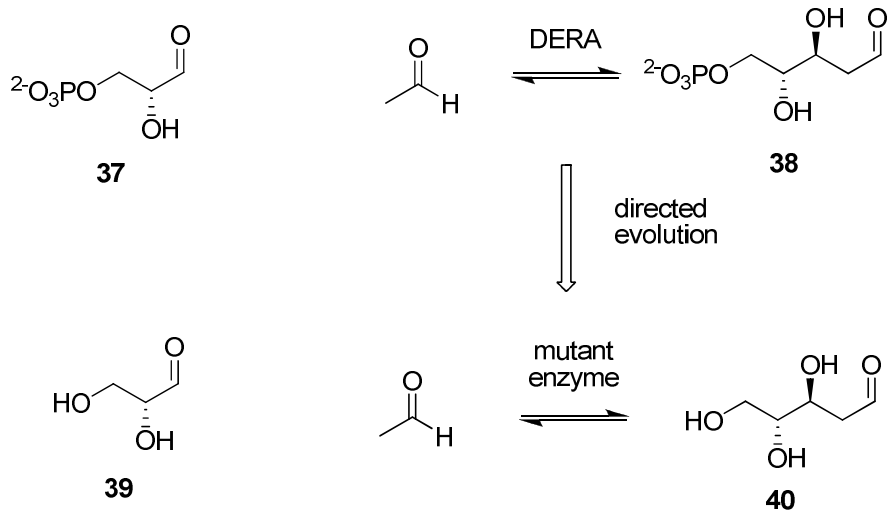
Scheme 10: Direct evolution of *N*–acetylneuraminic acid lyase

Variant	***Substrate***	k_{cat}	K_M	k_{cat}/K_M
		(min^{-1})	(mM)	($min^{-1}mM^{-1}$)
WT	**sialic acid (23)**	260 ± 6	4.4 ± 0.3	59
E192N	**36a**	130 ± 3	0.39 ± 0.04	340

Table 1: Steady-state kinetics data for wild type and E192N variant of NAL. The E192N mutant is approximately six times more efficient at substrate cleavage (**23** and **36a**) when comparing k_{cat}/K_M for their respective substrates.[46, 47]

The directed evolution of other aldolases has also been achieved. DERA catalyses the aldol reaction of D-glyceraldehyde-3-phosphate **37** and acetaldehyde to give D-2-deoxyribose-5-phosphate **38** (Scheme 11 and section 1.1.1.1.3). By analysing the active site *via* X-ray crystallography, and performing site-directed

mutagenesis, the variant, S238D, was discovered, which catalyses the aldol reaction between the unphosphorylated substrate glyceraldehyde **39** and acetaldehyde.[50] The variant was demonstrated to accept a range of substrates not accepted by the wild type enzyme.



37 **38** **39** **40**

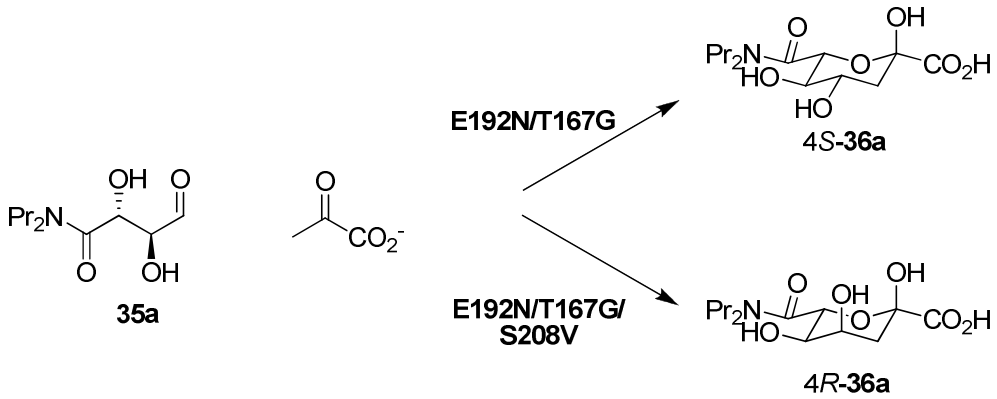
Scheme 11: The creation of a new enzyme mutant by directed evolution of DERA

The demand for enantiomerically pure molecules renders the development of highly stereoselective reactions essential in modern synthetic chemistry. NAL distinguishes poorly between the faces of aldehyde **21** (section 1.1.1.1.1) and, thus, displays poor facial stereoselectivity. In many NAL-catalysed syntheses, the stereochemical outcome of the reaction is thermodynamically controlled.[27] Therefore, an alternative approach would be to engineer a stereoselective enzyme which would control kinetically the configuration of the products.

A pair of stereochemically complementary aldolases to allow the conversion of **35a** into either possible diastereomeric of the aldol product (4*R*- and 4*S*-**36a**) was created by Gavin Williams and Tom Woodhall (Scheme 12).[51] The variant aldolase E192N was used as the starting point. Variant aldolases were screened for the ability to cleave the *4S*-configured acid (4*S*-**36a**) and not the 4*R*-configured acid (4*R*-**36a**). By applying the principle of microscopic reversibility, variants that cleaved the 4*S*- configured acid (4*S*-**36a**) and not 4*R*- configured acid (4*R*-**36a**) would also allow the synthesis of the 4*S*-product (4*S*-**36a**) in preference to the 4*R*-

product (4*R*-**36a**). The variant aldolases, which were able to synthesise the 4*R*- and 4*S*-configured compounds 4*R*-**36a** and 4*S*-**36a** respectively, were identified by screening and counter-screening variants able to cleave compounds 4*R*-**36a** and 4*S*-**36a**. Error-prone PCR (ep-PCR) was used initially to introduce diversity into the E192N scaffold, and hence identified residues important for stereoselectivity. Effective variant aldolases identified were then improved upon by saturation mutagenesis, to yield the highly stereoselective NAL variants. The *R*–selective variant is the triple mutant E192N/T167G/S208V and the *S*–selective variant is the double mutant E192N/T167G.

The stereoselectivity of NAL variants may be quantified by the selectivity ratio defined as the ratio, S, of the k_{cat}/K_M values for the cleavage of for the two diastereomeric screening substrates 4*R*-**36a** and 4*S*-**36a** {*i.e.* $[k_{cat}/K_M$(4*R*-**36a**$)]/[k_{cat}/K_M$(4*S*-**36a**$)]$}, where *R*-selective enzymes have ratios greater than 1 and *S*-selective enzymes less than 1 (Table 2).[51] The E192N variant is moderately *S*-selective, S = 0.58. The *R*–selective enzyme variant exhibited S = 48 and was 80 times more *R*–selective than the parent enzyme E192N. The *S*–selective enzyme had S < 0.02 and was at least 30 times more *S*–selective than the E192N variant.



Scheme 12: Pair of stereoselective complementary aldolases evolved from sialic acid aldolase.

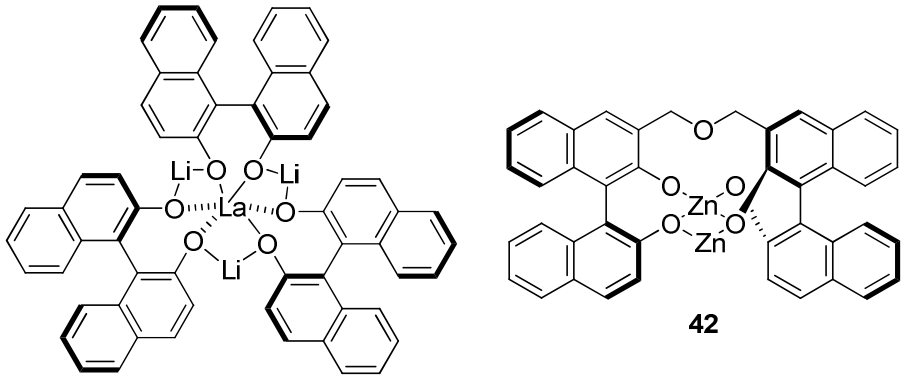
Variant	***Dipropylamide (4S-36a)***			***Dipropylamide (4R-36a)***			***S***
	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}mM^{-1}$)	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}mM^{-1}$)	$[k_{cat}/K_M$(4*R*-**36a**)]/ $[k_{cat}/K_M$(4*S*-**36a**)]
WT	230 ± 6	12 ± 1	19	73 ± 4	11 ± 2	6.6	0.35
E192N	450 ± 14	0.8 ± 0.07	563	130 ± 3	0.4 ± 0.04	325	0.58
E192N/T197G	5 ± 0.1	0.5 ± 0.03	10	NM[a]	NM[a]	<0.2[a]	<0.02[a]
E192N/T197G/S208V	0.1± 0.003	8.5 ± 0.4	0.012	1.1 ± 0.04	1.9 ± 0.2	0.58	48

Table 2 Steady-state parameters of the wild-type and variant enzymes [a] No activity greater than the background rate.

As the previous examples demonstrate, directed evolution has been an effective mechanism in broadening the substrate scope of many enzymes, and hence has provided enzyme catalysts which are useful tools in synthesis. Further examples of directed evolution of aldolases are described in recent reviews.[52, 53]

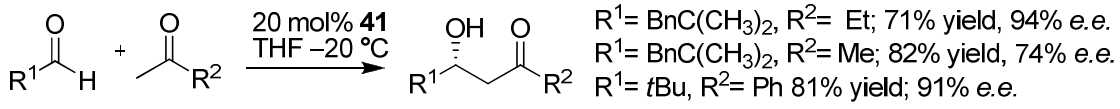
1.1.1.2 Metal-Catalysed Direct Aldol Reactions

Despite the precedent of enzyme-catalysed direct aldol reactions, the first instance of an asymmetric metal-catalysed direct aldol reaction has only been reported relatively recently.[54] The heterobimetallic BINOL complex **41**, $LaLi_3$tris(binaphthoxide) (*R*-LLB) (Figure 2) was shown to catalyse a wide range of direct aldol reactions with good diastereo- and enantio-selectivity (Scheme 13).[54-56] The LLB complex **41** is bifunctional with both Lewis acidic (Lanthanum atom) and Brønsted basic (binaphthoxide moiety) sites which activate both donors and acceptors simultaneously. The mechanism of aldol condensation is broadly analogous to the Type II aldolases.



41 **42**

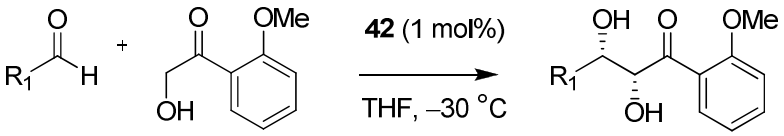
Figure 2: Metal-based catalysts for direct aldol reactions



43 **44** **45**

Scheme 13: *R*- LLB-catalysed direct cross aldol reaction

Various alternative direct aldol catalysts based on BINOL have also been developed, including the dinuclear zinc catalyst, Zn-Zn-linked BINOL, **42** (Figure 2).[57] The dinuclear zinc catalyst **42** catalyses a range of selective reactions including the synthetically useful reaction of α-hydroxy ketones **47** with aldehydes **46** (Scheme 14).



46 **47** **48**

R_1= ^{i}Pr; 92 % yield, 92% *d.e.*, 99% *e.e.*
R_1= cyclohexyl; 95 % yield, 94% *d.e.*, 98% *e.e.*
R_1= $Ph(CH_2)_2$; 94 % yield, 79% *d.e.*, 92% *e.e.*

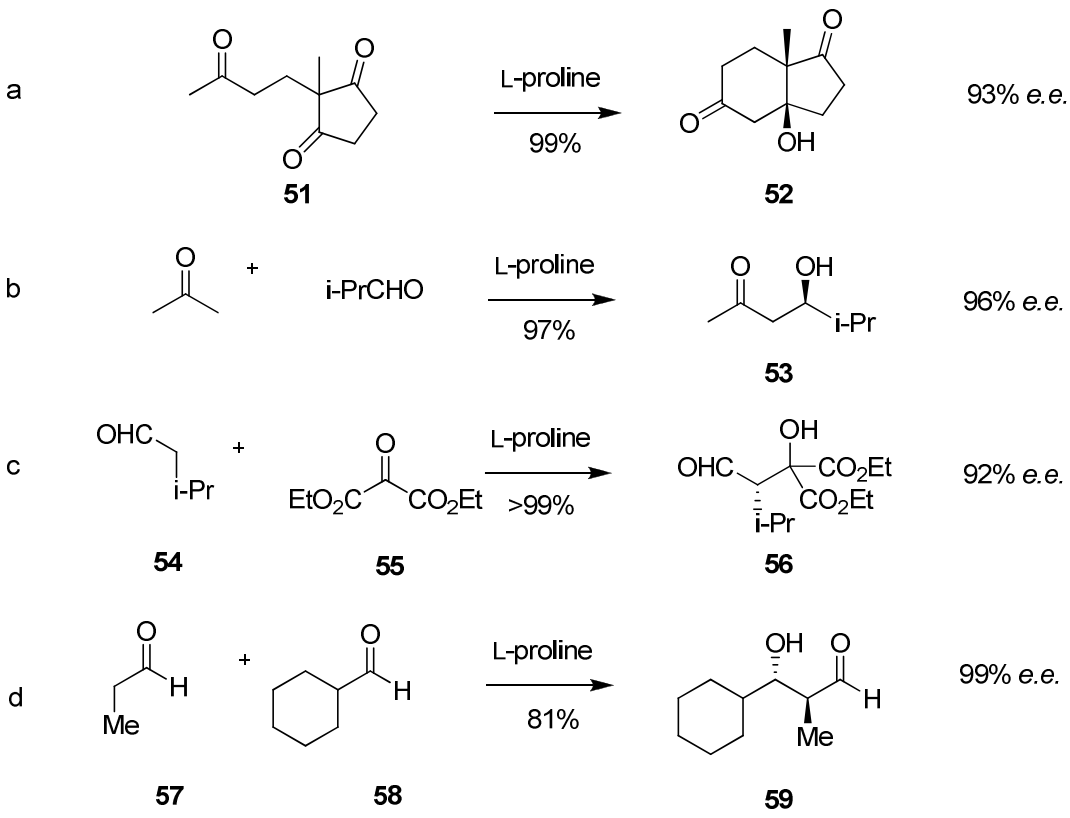
Scheme 14: Zn-Zn-linked BINOL catalysed direct aldol reaction of α-hydroxyl ketones

1.1.1.3 Organocatalysed Direct Aldol Reactions

The last decade has witnessed dramatic growth in the area of organocatalysis.[58-61] Organocatalysis is defined as the acceleration of reactions by using a substoichiometric quantity of an organic compound,[58] and the approach has been used to catalyse a wide range of organic reactions.[58-63] The use of small organic molecule catalysts has several advantages over more traditional options such as organometallic- or enzyme-catalysed systems. The benefits include: the reactions can be performed aerobically with wet solvents; the catalysts are inexpensive and normally bench-stable;[58] more efficient recycling of the catalysts by attachment to solid supports can be possible; and the lack of metal contamination in the products.[64]

1.1.1.3.1 Proline Catalysed Direct Aldol Reactions

Despite initial discovery over thirty years ago,[65-67] enantioselective proline-catalysed aldol reactions were, until recently, poorly understood and their potential scope not evident. The first reported example of an enantioselective proline-catalysed aldol reactions was the intramolecular cyclisation of triketones, such as **51** (known as the Hajos–Parrish–Eder–Sauer–Weichert reaction, Scheme 15 a).[65-67] The remarkable enantioselectivity and yield observed (93% *e.e.* and 99% yield) were not matched by intermolecular examples until 2000 (Scheme 15, b).[68]



Scheme 15: Proline-catalysed direct asymmetric aldol reaction

The proline-catalysed intermolecular aldol reaction has wide substrate scope (Scheme 15 c and d)[57, 61] and there are many examples displaying near quantitative yields and high enantioselectivities. Proline's activity has been exploited in other important and powerful reactions such as the Mannich reaction, pericyclic reactions, oxidations and reductions where good selectivity has also been observed.[69-71]

Although originally hypothesised over thirty years ago, the mechanism and stereoselectivity of proline-catalysed aldol reactions has only recently been supported by computational and experimental evidence.[72, 73] Enamine catalysis is now widely accepted to be responsible for the observed reaction.[73] The general mechanism for the proline-catalysed aldol reaction is depicted below (Figure 3) and is analogous to that of Type I aldolases. The mechanism involves attack of the key enamine intermediate **61** on the acceptor aldehyde **62**. [61] The stereoselectivity of the process has been proposed to stem from constraint of the transition state by hydrogen-bonding.

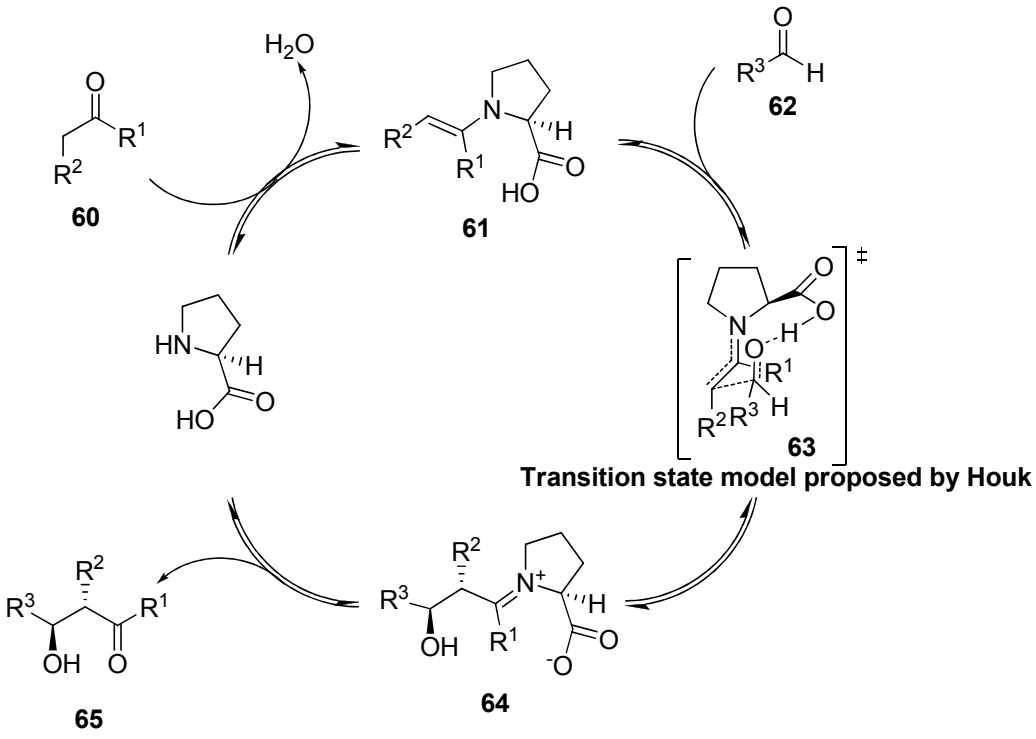


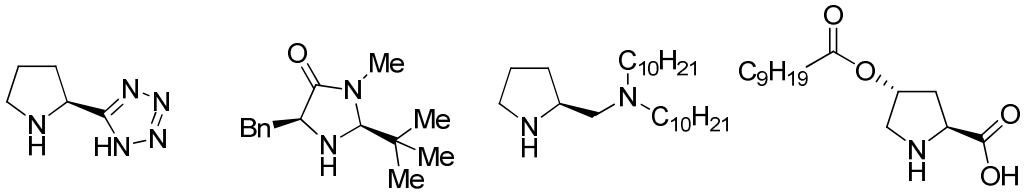
Figure 3: Proposed mechanism of proline-catalysed aldolisations.[61] The catalytic cycle is initiated by the ketone/aldehyde **60** condensation with L-proline to give the enamine **61**. The aldehyde **62** undergoes attack from enamine **61** to give the iminium ion **64**. Hydrolysis of **64** gives the aldol product **65** along with L-proline, which completes the catalytic cycle. Houk has proposed a transition state to explain the stereoselectivity observed. [61]

The enamine mechanism was first proposed in the 1970s[74] and it is also believed that only one proline is involved in the catalytic cyclic.[73, 75] The stereoselectivity observed can be explained by Houk's model (**63**, Figure 3) based

on the computations of a number of systems.[72] In general, the acceptor aldehyde is attacked on the *re*-face and thus places the acceptor aldehyde's substituents in a pseudo equatorial arrangement. Houk's model has been tested successfully by predicting correctly the enantio- and stereo-selectivities observed in a number of inter- and intra-molecular aldol reactions.[72, 76, 77]

1.1.1.3.2 Alternatives to Proline in Organocatalysed Direct Aldol Reactions

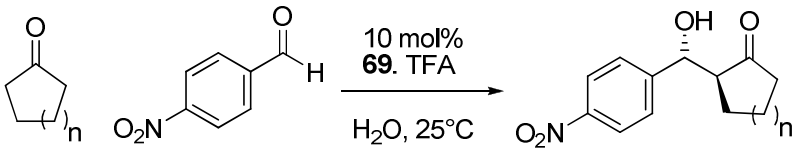
The interest in organocatalysis has led to the development of many novel organocatalysts for asymmetric aldol reactions. A plethora of asymmetric aldol catalysts have been reported which are mainly based around the proline scaffold. These catalysts can offer greater selectivities, higher yield, or greater or alternative scope (such as **68**).[78] The catalysts can also have more favourable properties, such as increased solubility in organic solvents (such as **67**)[79] or compatibility with water, (such as **69** and **70**).[80, 81]



67 **68** **69** **70**

Figure 4: Organocatalysts for asymmetric direct aldol reactions

Despite the many organocatalysts developed for asymmetric aldol reactions, very few operate in water.[82-85] The appendage of long alkyl groups onto proline and proline derivatives has led to some successful asymmetric aldol organocatalysts, which do operate in water. Barbas reported the use of the diamine **69** as a good catalyst for asymmetric aldol reactions between aliphatic ketones **71** and benzylic aldehydes **72** (Scheme 16).[86]



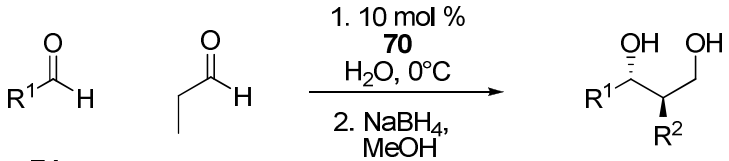
71 **72** **73**

n = 2; 98% yield, *d.r.* 6:4, 87% *e.e.*
n = 1; 40% yield, *d.r.* 1:1, 99% *e.e.*

Scheme 16: Organocatalysed asymmetric aldol reactions in water

The discovery that by increasing the alkyl chain length of the diamine **69** the enantioselectivity of the reactions is also improved, led the authors to hypothesise that the diamine **69** diminishes contact between the substrates and bulk water. A water-free transition state may help to explain the observed enantioselectivity of the reaction.

Independently Hiyashi *et al.* reported a very similar proline–based catalyst.[80] The ester **70** was shown to catalyse asymmetric aldol reactions between pairs of aldehydes **74** and **75** in water (Scheme 17). Again, the length of the alkyl group affected the enantioselectivity with a 10-carbon chain being optimal. Although the reaction mechanism is unclear, the formation of an emulsion is critical and enhances both stereoselectivity and activity.[87]



74 **75** **76**

R^1= *p*-Cl-Ph; 90% yield, *d.r.* >20:1, 99% *e.e.*
R^1= Ph; 90% yield, *d.r.* >20:1, 99% *e.e.*
R^1= cyclohexyl; 61% yield, *d.r.* 10:1, 92% *e.e.*

Scheme 17: Asymmetric aldol reaction between aldehydes in water catalysed by ester **70**

Despite the aldol reaction being one of the oldest named reactions, it still holds the attention of synthetic chemists and remains at the forefront of current research. The continued interest in the reaction is surely owed to the fact that it is

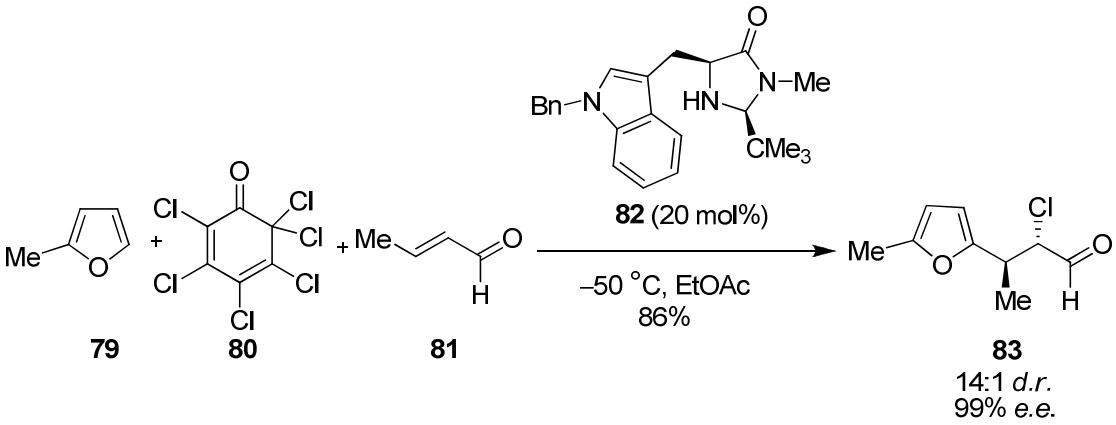
one of the most useful reactions in organic chemistry for preparing complex molecules in a diastereo- and enantio-selective manner.

1.2 Catalytic Cascade Reactions

The ability to prepare complex molecular skeletons with ease and speed has long been a goal of synthetic chemistry. Cascade reactions achieve this goal effectively, offering many advantages over traditional stepwise construction of target molecules. Cascade reactions show greater efficiency with fewer reagents, solvents, work-ups, energy, waste and labour than stepwise constructions, and often exhibit excellent selectivities.[1, 2] Although nature has long performed cascade reactions using highly efficient, and tailored catalysts (enzymes), synthetic catalytic cascade reactions have only recently been developed. Despite this fact, cascade reactions have already been used successfully with good yields and selectivities observed with many elegant examples in the recent literature.[3, 4] Catalytic cascade reactions have exploited many different types of catalysts; however, this section focuses on cascade reactions involving at least one enzyme- or organo-catalysed step.

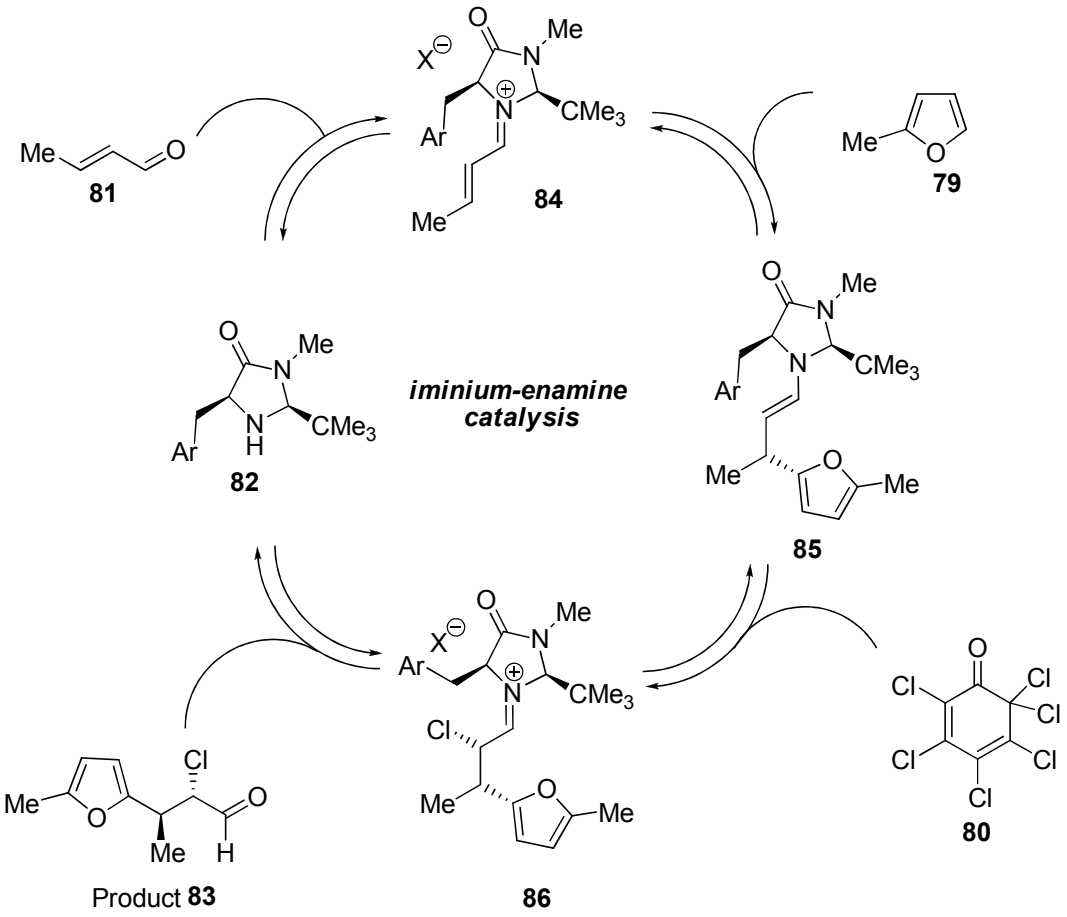
1.2.1 Organocatalysed Cascade Reactions

Cascade reactions involving organocatalysis are relatively rare. Most examples rely upon the dual reaction modes of amino catalysts: enamine and iminium catalysis, which enhance the nucleophilicity and electrophilicity of substrates respectively. Organocatalysed cascade reactions have has been pioneered by MacMillan who has used the imidazolidinone catalyst **82** in the enantioselective synthesis of a variety of products, such as the aldehyde **83**, in high yields and with exquisite enantioselectivity (Scheme 18).[88]



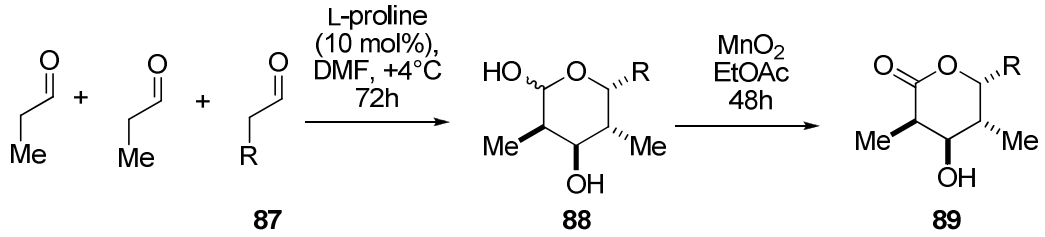
Scheme 18: An example of an organocatalysed cascade reaction

The cascade leading to the aldehyde **83** exploits both iminium and enamine activation (Scheme 19). Mechanistically, it is thought that the *α,β*-unsaturated aldehyde **81**, condenses with the catalyst **82** to give an iminium species **84**. This iminium species is attacked by a nucleophile (**79**) to form an enamine **85** which is intercepted by electrophile (**80**); hydrolysis releases the product **83** and regenerates the organocatalyst **82**, with the chiral catalyst controlling the stereochemical outcome of both steps.



Scheme 19: Proposed iminium-enamine mechanism of imidazolindinone **82** catalysed cascade reactions[88]

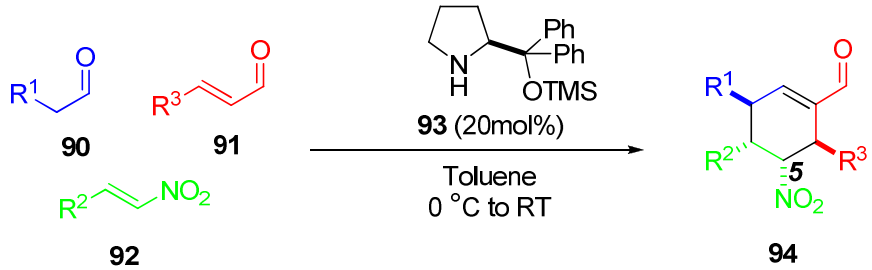
Consecutive organocatalysed aldol reactions have also been previously reported. The proline catalysed, one-pot, three-component assembly of lactols **88** is an excellent example of a tandem aldol reaction (Scheme 20).[89, 90] Unlike the previous example, this tandem process involves enamine-enamine catalysis and involves the self-condensation of propionaldehyde followed by the cross-aldolisation with a second aldehyde **87**. This cascade constructs four stereogenic centres in one pot and is the first example of an organocatalysed one-pot preparation of carbohydrate derivatives.



R= Et, 53% yield, 33% *e.e.*
R= ^{i}Pr, 32% yield, 12% *e.e.*
R= ^{i}Bu, 24% yield, 11% *e.e.*

Scheme 20: Proline catalysed assembly of carbohydrate derivates. [89, 90]

Higher enantioselectivity has been observed in the triple-cascade reaction which forms cyclohexene carbaldehydes **94** (Scheme 21).[91] This impressive three-component assembly involves a Michael/Michael/aldol reaction sequence and exhibited high levels of enantio- and diastereo-control, as well as chemo- and regio-selectivity. The reaction exploited the proline-derived catalyst **93** and involved enamine-iminium-enamine activation to generate complex small molecules **94**. Further interesting examples of organocatalysed cascade reaction have been reviewed recently.[92]



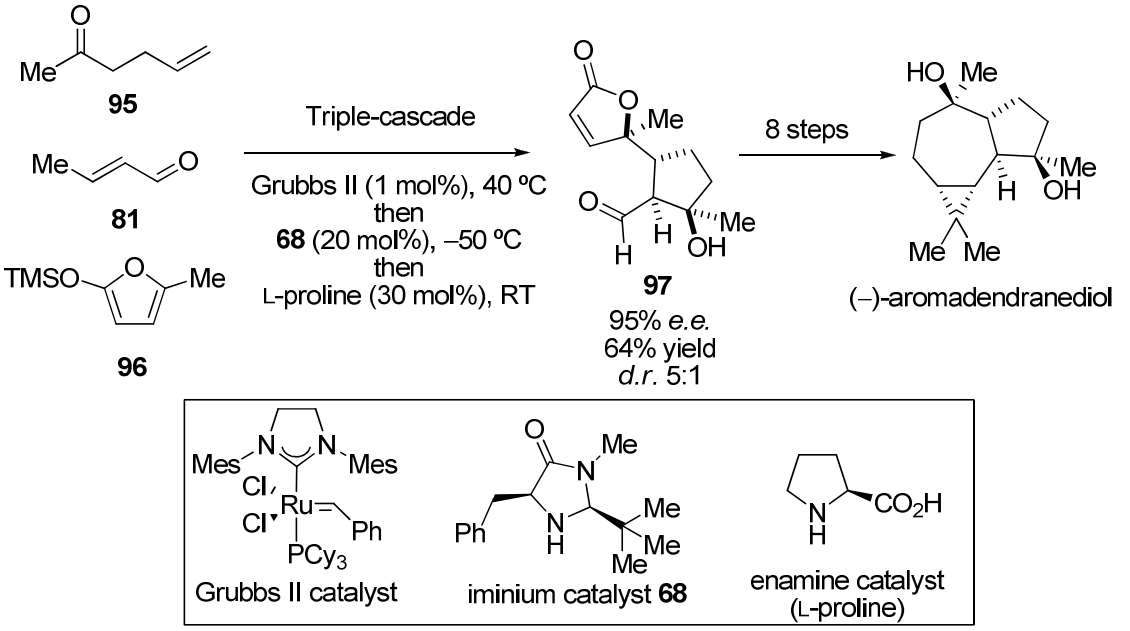
R_1= Me, R_2= Ph, R_3= Ph; 40% yield, *d.r.* 4:1, >99% *e.e.*
R_1= Me, R_2= Piperonyl, R_3= Ph; 39% yield, *d.r.* 9:1, >99% *e.e.*
R_1= Me, R_2= Ph R_3= H; 50% yield, *d.r.* 9:1, >99% *e.e.*

Scheme 21: Three-component Michael/Michael/aldol organocatalysed cascade. The cascade only produces two diastereoisomers where the minor diastereomer is the C5-epimer of the product shown.

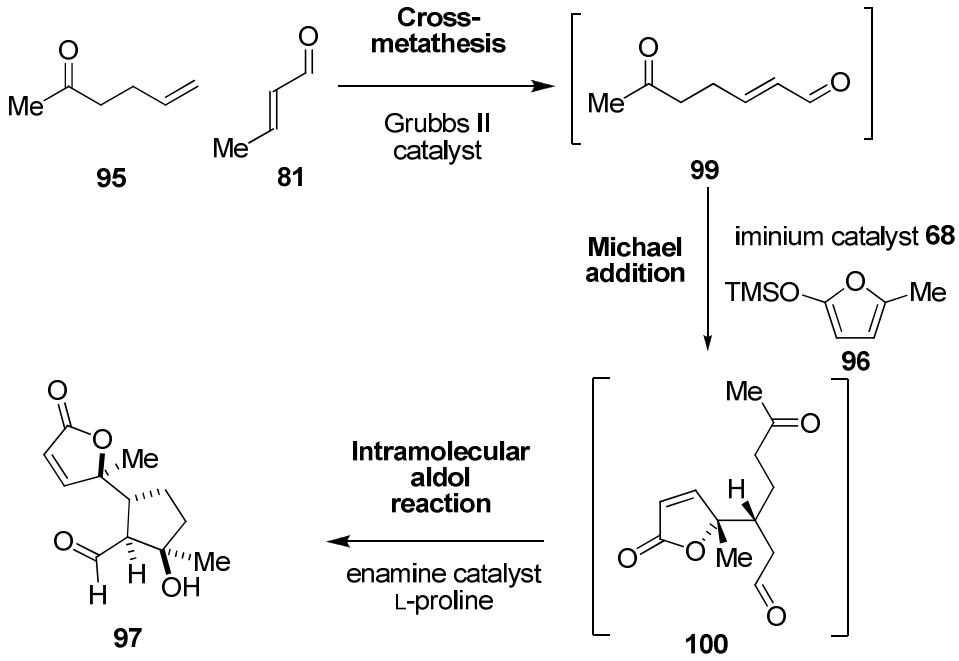
1.2.2 Combined Metal- and Organo-catalysed Cascade Reactions

Despite the array of reaction cascades involving transition metal catalysts [93-96] the combination of organocatalysis with transition metal catalysis is an area of

chemistry which has, so far, received little attention.[97-100] One of the finest reported examples is the tricatalytic triple cascade reported by MacMillan. (Scheme 22).[101] The three-step sequential cascade involves first a cross-metathesis, followed by a Michael addition and finally an intramolecular aldol reaction to generate a highly functionalised bicyclic product **97**. The three-component triple cascade operates as follows (Scheme 23): the initial cross-metathesis of the alkene **95** with the alkene **81** generates the enone intermediate **99**, which then undergoes a subsequent Michael addition by the furan nucleophile **96**, catalysed by the iminium catalyst **68**. The ketone **100** condenses with the enamine catalyst, L-proline, to produce an enamine, which undergoes an intramolecular aldol reaction. Unlike previous work, where only one catalyst is used and, hence, only one stereo-outcome can be achieved (section 1.2.1), MacMillan used two orthogonal chiral catalysts (**68** and L-proline) which control the stereochemical outcome of the iminium- and enamine-catalysed steps independently and hence control the configuration of the product. By using this system to prepare the bicycle butenolide **97**, the authors generated four contiguous stereocentres in one-pot. Butenolide **97** was then elaborated over a in eight further steps to give the marine natural product (–)-aromadendranediol.



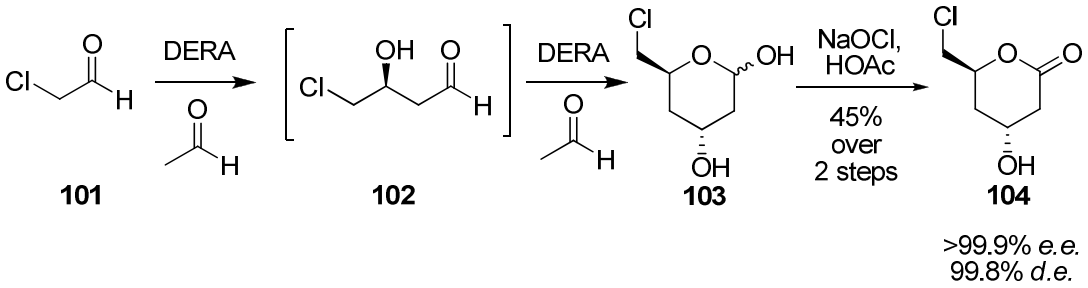
Scheme 22: Triple cascade involving combination of transition metal catalysis with organocatalysis in a sequential metathesis, Michael addition and aldol reaction



Scheme 23: Proposed mechanism for the triple cascade involving combination of transition-metal catalysis with organocatalysis in a sequential metathesis, Michael addition and aldol reaction

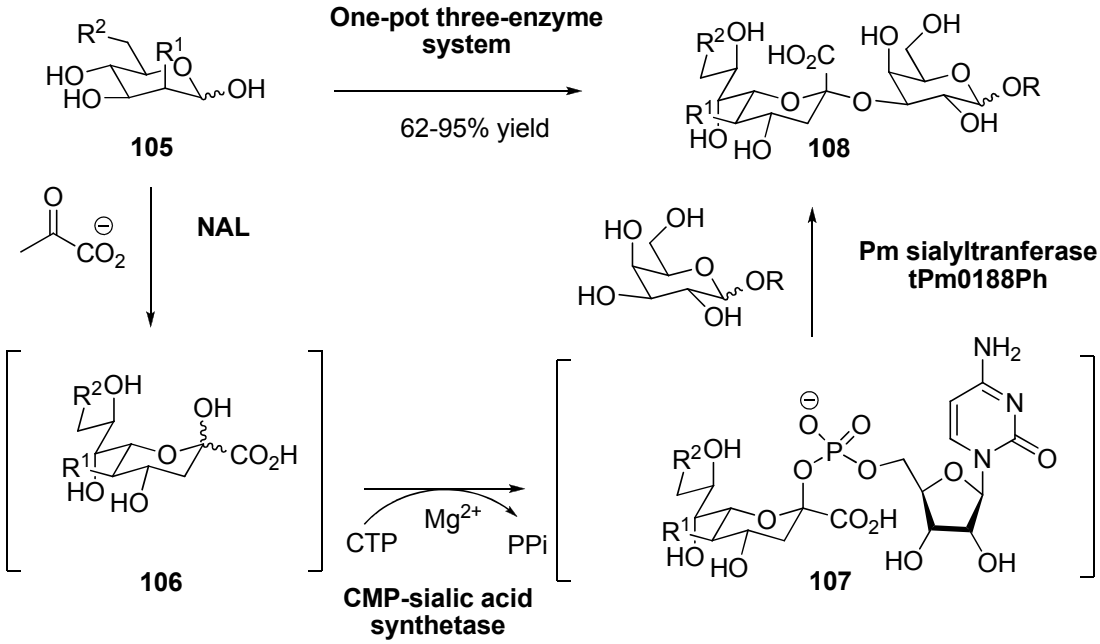
1.2.3 Enzyme-Catalysed Cascade Reactions

Although enzymatic cascade reactions have been well investigated,[1, 102, 103] tandem aldolase-catalysed reactions are relatively rare. The rarity of such processes is because in order for a cascade to occur the (aldehyde) product of the first reaction must be an acceptor for the next. In general, this means that at least one aldehyde/aldehyde coupling is needed. One of the few reported examples is the tandem aldol reaction of chloroacetaldehyde **101** with acetaldehyde (Scheme 24).[104] The sequential reaction is catalysed by DERA, which produces the lactol **103** in good yield which was converted into the lactone **104**.



Scheme 24: DERA-catalysed tandem aldol reaction

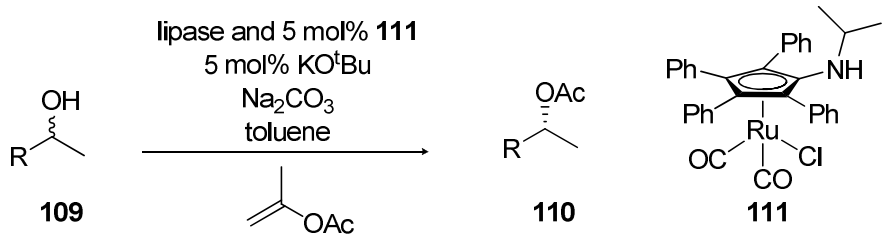
Multi-enzymatic one-pot reactions have also been developed in the synthesis of more complex products. A one-pot three-enzyme system was developed to synthesise sialosides **108** (Scheme 25)[105-107] The novel system uses NAL (section 1.1.1.1.1), Cytosine-5′-monophosphate-acetylneuraminate synthetase (CMP-NeuAc synthetase), and a silalyltransferase (*Pasteurella multocida tPm*0188Ph) and performs three sequential transformations: an aldol reaction, a phosphorylation and a glycosylation reaction. The approach allowed the preparation of a library of sialosides.



Scheme 25: One-pot three-enzyme system for the synthesis of sialosides where R^1 = OH, NHAc, $NHAcN_3$, NHGc, NHGcOMe, or NHAlkyne; R^2 = OH, N_3; R = aglycon or glycosides

1.2.4 Enzyme- and Metal-Catalysed Cascade Reactions

The combination of enzyme catalysis with metal catalysis has been relatively well investigated[108, 109] with many examples involving dynamic kinetic resolutions (DKRs). The combination of lipases with ruthenium catalysts allowed the DKR of secondary alcohols. The approach used simultaneous *in situ* racemisation of the alcohol substrate **109**, by the ruthenium catalyst **111** and kinetic resolution of the alcohol substrate **109** by the lipase. The reaction hence produced the enantiomerically pure acetate **110** (Scheme 26).[108-114]



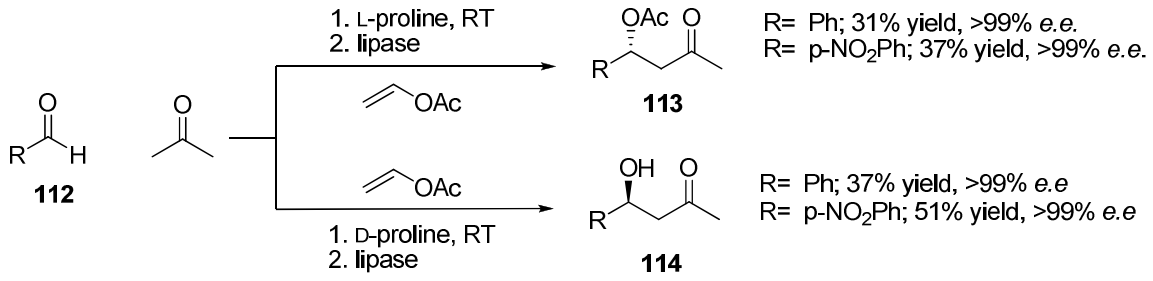
109 **110** **111**

R= *p*-Cl-Ph; 94% yield, >99% *e.e.*
R= cyclohexyl; 86% yield, >99% *e.e.*

Scheme 26: DKR of secondary alcohols using combination of enzyme and transition metal catalysis [114]

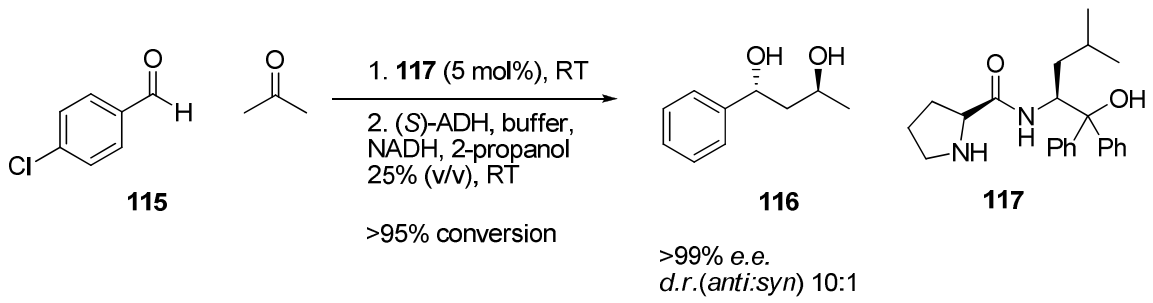
1.2.5 Enzyme- and Organo-catalysed Cascade Reactions

The combination of enzymes with organocatalysts is relatively rare with only a few reported examples.[115] Cordova *et al*. reported the first combination of organo- and enzyme-catalysis in 2004 with a tandem aldol/lipase resolution reaction (Scheme 27). The tandem reaction involved the use of either proline enantiomer to catalyse the aldol reaction, which used excess acetone as reaction solvent. The aldol reaction was followed by the addition of a lipase, which kinetically resolved the resultant aldol product. The reaction was enantioselective and yielded the either the acetate **113** or the alcohol **114**.



Scheme 27: One-pot tandem proline catalysed aldol and lipase- catalysed kinetic resolution reaction

Organo- and enzyme-catalysis have also been combined in an aldol/reduction cascade (Scheme 28).[116] Like the previous example, the cascade involved an organocatalysed aldol reaction and used catalyst **117**. The aldol reaction was followed by an enzymatic reduction of the resultant ketone with an alcohol dehydrogenase [(*S*)-ADH] and gave the diol **116**. The reaction was a sequential one-pot reaction in which the aldol reaction was performed under solvent-free conditions.[117] In the reductive step, the NADH co-factor is recycled by the *in situ* oxidation of 2-propanol to acetone and yielded the diol **116** enantioselectively. The authors have also prepared the corresponding stereoisomers of the diol **116** by varying the enantiomer of catalyst **117** and the alcohol dehydrogenase used.



Scheme 28: Enantioselective tandem aldol/reduction reaction combining organo- and enzyme-catalysis

Although the previous examples of combined organo- and enzyme catalysis are intriguing, they lead to the formation of only one carbon-carbon bond. An

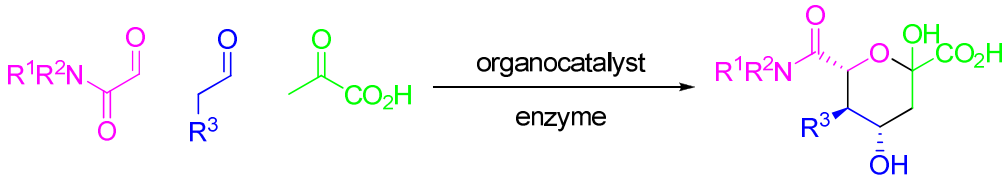
interesting area would be to develop a one-pot reaction in which both the organocatalyst and enzyme catalyse one carbon-carbon bond

1.2.6 Summary

Despite the large array of catalytic one-pot reactions reported in the synthetic literature, few reactions combine more than one form of catalysis. The challenge of developing reactions that harnesses the usefulness of a number of forms of catalysis is therefore, an interesting and challenging opportunity in organic chemistry.

1.3 Project Outline

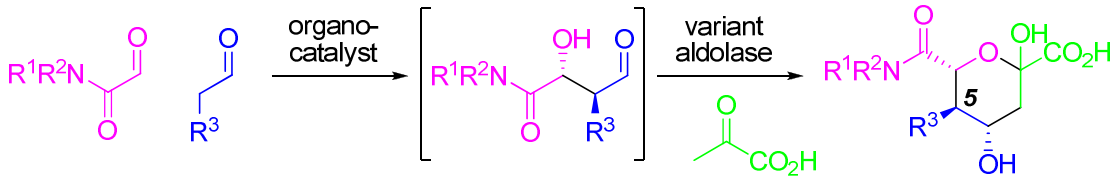
The research described aims to develop of a novel three-component reaction, which combines organo- and enzyme-catalysis in an aldol-aldol cascade (Scheme 29). The reaction aims to generate two new carbon-carbon bonds and up to three stereocentres in a one-pot process.



1 **2** **4**

Scheme 29

The envisaged aldol cascade reaction involves two catalytic aldol reactions: an organocatalysed reaction and a reaction catalysed by a variant of NAL, discovered using directed evolution (section 1.1.1.1.4.). The organocatalyst would guide a condensation reaction between glyoxylaldehyde **1** and aldehyde **2**, generating an aldol intermediate **3**. The aldol intermediate **3** would be intercepted by the E192N aldolase variant and pyruvate to yield the highly functionalised small molecule **4** (Scheme 30).



1 **2** **3** **4**

aldol intermediate

Scheme 30

The aldolase variant E192N of NAL was known to accept a range of tertiary amides; however all known substrates had a hydroxyl group in the C-5 position (R^3 = OH) of the acid **3** (Scheme 30).[46, 47, 118] We aim to vary the aldehyde **2** and, hence, generate products with alternative substituents in the C-5 position. Before investing time in the development of the one-pot reaction, we proposed to prepare

a range of potential substrates for the E192N aldolase variant. The data obtained will not only dictate whether the one-pot reaction is possible, but additionally, give further insights into the structural-activity relationship between the aldolase variant and its substrates. The selection, preparation, assay and kinetic data of the potential substrates along with an investigation into the E192N-catalysed condensations, will be described in Chapter 2.

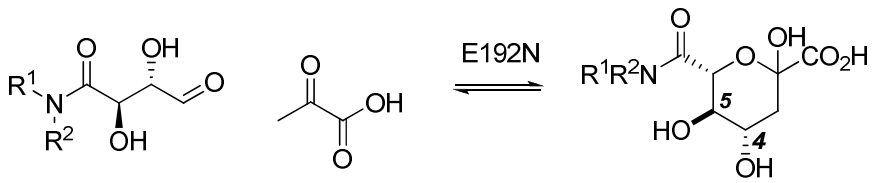
Chapter 3 describes the development of the one-pot reaction. Initially, the strategy that will be used to find common conditions under which both organo- and enzyme-catalysis could occur is discussed. This strategy involves the initial consideration of solvent systems before careful selection of organocatalysts (based on reported solubility and activity). Secondly, the organocatalysts will be prepared on multi-gram scales, the catalyst will be screened, and conditions optimised for the organocatalysed step. Organocatalytic conditions tolerated by the E192N aldolase variant, will be investigated by using an appropriate enzyme assay. The results from the enzyme assay will be used to select a number of potential one-pot reaction conditions, which will be screened and optimal conditions chosen. With conditions in place, the stereoselectivity of the one-pot reaction will be investigated. Finally, the scope of the reaction will be explored. Chapter 4 summarises the principal discoveries made in the project.

Chapter 2

Determination of the Range of Substrates Accepted by the E192N Aldolase Variant

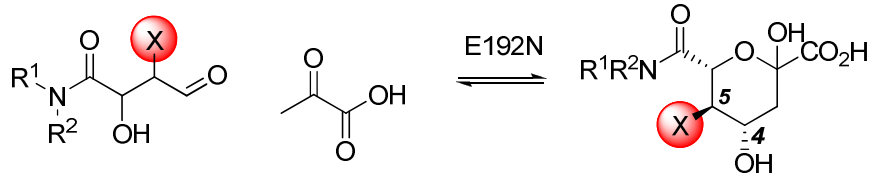
A key aim of the project was to establish if the series of compounds with the general structure **3** were substrates for the E192N aldolase variant (Figure 5, b). Achieving this goal would provide information on the feasibility of the proposed one-pot reaction and also give further insights into the structure-activity relationships of substrates for the E192N aldolase variant. Thomas Woodhall and Gavin Williams proved that compounds **35** were substrates for E192N by measuring steady-state kinetics for the substrates **36** (Figure 5, a).[46, 118, 119]

a)



35 **36**

b)



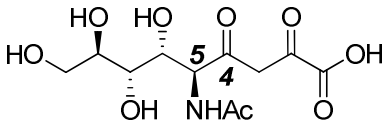
3 **4**

Figure 5: a) E192N substrates **35** and **36**; **b)** non-hydroxylated, potential substrates for the E192N variant aldolase **3** and **4**

We predicted that the compounds **3** and **4** (Figure 5, b), where the C-5 hydroxyl substituent is replaced by alternative substituents, would also be substrates for the E192N variant aldolase. The prediction was based on the following rationale. The crystal structure of wild-type NAL (Figure 6)[48] shows that the C-5 position of the enzyme substrate is solvent-exposed and therefore may not be involved in substrate recognition. It is also known that wild-type NAL accepts substrates with a wide range of substituents at the C-5 position with little or no

deterioration in catalytic activity.[25] We anticipated that the E192N variant would behave in a similar manner to its parent enzyme and would therefore tolerate a range of substituents at the C-5 position.

a)



34

b)

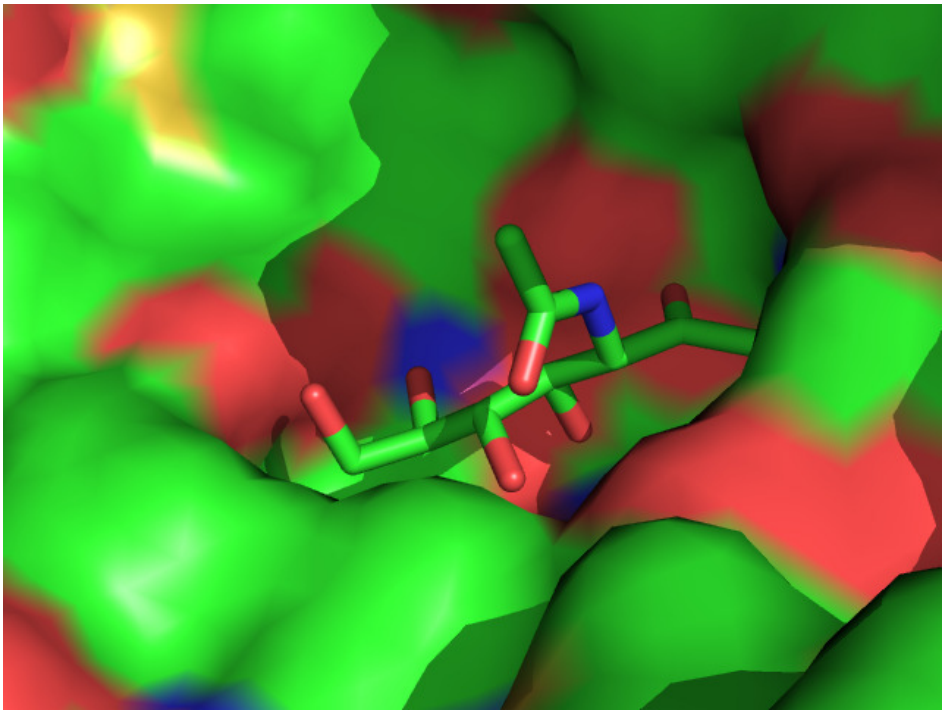
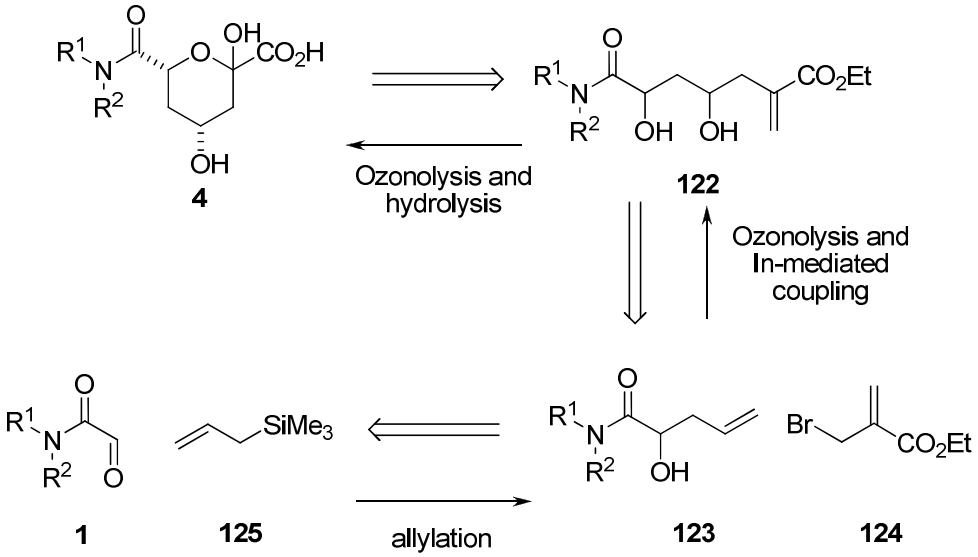


Figure 6 a) 4-oxo-sialic acid (**34**); **b)** X-ray crystal structure of NAL from *Haemophilus influenzae* in complex with 4-oxo-sialic acid (**34**); the C-5 position is exposed to bulk solvent.[48, 49]

2.1 Preparation of Proposed Enzyme Substrates

To test the range of substrates accepted by the E192N aldolase variant, a range of potential substrates were prepared using synthetic chemistry. The aim was to prepare a number of compounds in which the C-5 substituent is a hydrogen atom (X=H, Scheme 31); once prepared the compounds were screened in an appropriate enzyme assay.

The retrosynthetic analysis of the potential substrates **4** (Scheme 31) was adapted from the synthetic route used to prepare similar compounds and was developed by Thomas Woodhall.[46, 118, 119] The synthetic strategy involved three key steps. The Lewis acid-promoted allylation of glyoxylaldehydes **1** with allyltrimethylsilane **125** would yield the homoallylic alcohols **123**. Upon ozonolysis of the homoallylic alcohols **123**, the corresponding aldehydes would be exposed to a diastereoselective indium-mediated coupling that would produce the diols **122**. Ozonolysis of the diols **122**, and ester hydrolysis, should give the racemic potential substrate **4**.*



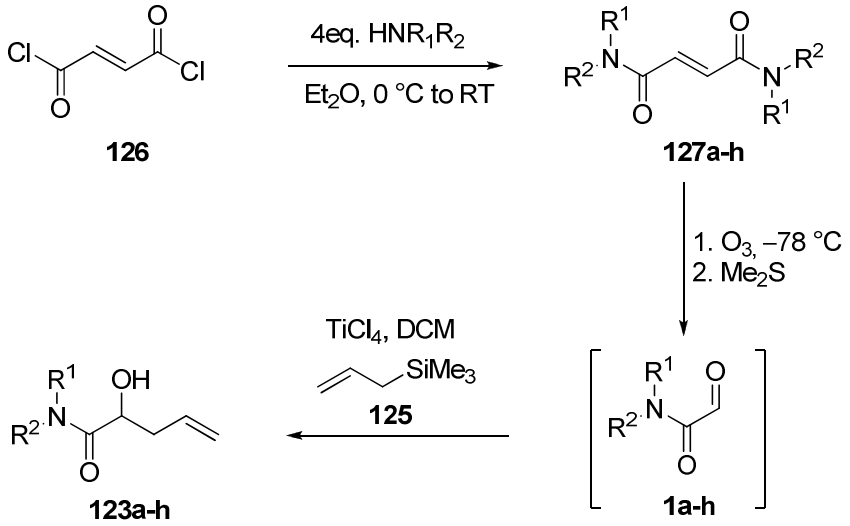
Scheme 31: The retrosynthetic analysis of potential compounds **4**. The keto-acid functionality may be unmasked by the ozonolysis and hydrolysis of diols **122**. The diols **122** might be prepared from **123** *via* an indium-mediated coupling where the bromoacrylate **124** is a pyruvate equivalent. The homoallylic alcohols **123** might be prepared by the allylation of glyoxylaldehyde **1** with allyltrimethylsilane **125**.

2.1.1 Preparation of the Homoallylic Alcohols 123

The synthesis of the homoallylic alcohols **123** was required for the preparation of the keto-acid potential substrates **4**. It was envisaged that the

* A racemic route was chosen for speed at this point in the project as all chiral routes towards single enatiomers of the alkenes **123** were unsuccessful. It is worth noting that this impacts on the results obtained as the data only shows if one of the enantiomers is a substrate for the E192N aldolase and no information will be gained if there is any difference in enzyme kinetic between enantiomers.

alkenes could be prepared using a Lewis acid-promoted allylation of glyoxylaldehydes **1**. The glyoxylaldehydes **1** were prepared from amides **127** in a two-step procedure from fumaryl chloride **126** (Scheme 32 and Table 3), and were used directly in the next step. Treatment of the glyoxylaldehydes **1** with titanium(IV) chloride and allyltrimethylsilane **125** yielded the homoallylic alcohols **123** in 22-60% yield over two steps.[†]



Scheme 32: Preparation of homoallylic alcohols **123** from amides **127** *via* a two-step ozonolysis/Lewis acid promoted allylation procedure (see Table 3)

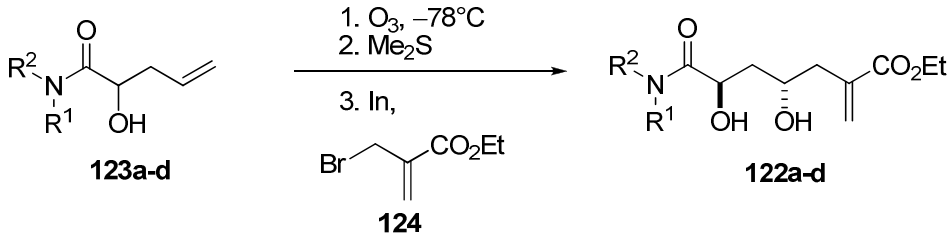
Entry	R^1	R^2	Amide	127 Yield (%)	Homoallylic Alcohol	123 Yield [c] (%)
1	Pr	Pr	**127a**	69	**123a**	91[d]
2	Bu	Bu	**127b**	69	**123b**	46
3	-$(CH_2)_5$-		**127c**	68	**123c**	30
4	$(CH_2)_2O(CH_2)_2$-		**127d**	25	**123d**	60
5	Me	Pr	**127e**	100	**123e**	22
6	Me	OMe	**127f**	81[b]	**123f**	34
7	Et	Et	**127g**	100	**123g**	37
8	Me	Me	**127h**	46[a]	**124h**	30

Table 3: [a] Reaction additive: triethylamine, [b] Reaction additive: pyridine,[c] Yield of purified product over two steps; [d] Yield from isolated glyoxylaldehyde **1a** (see Scheme 32).

[†] The compounds **123e-h** were not converted into the corresponding alcohols **122**, but were used later in the project

2.1.2 Preparation of the Diols 122

The diols **122** were prepared from the homoallylic alcohols **123** using a two-step ozonolysis and indium-mediated coupling sequence (Scheme 33 and Table 4). Ozonolysis of homoallylic alcohols **123** gave the corresponding aldehydes which were immediately exploited in the next step. Indium-mediated coupling with the bromoacrylate **124** gave the diols **122**. The allylation was moderately diastereoselective with the levels of diastereoselectivity determined for the isolated products **122**. The configuration of the products was determined by their subsequent conversion into the cyclic α-keto esters **134** (section 2.1.3). In some cases, double bond isomerisation of the diols **122** was also observed in the reaction, which may explain the low yield from the two-step procedure.



Scheme 33: Preparation of the diols **122a-d** (Table 4). The major diastereomer is shown.

Entry	R^1	R^2	Product	Yield [a] (%)	*anti:syn* [b]
1	Pr	Pr	**122a**	24	70:30
2	Bu	Bu	**122b**	12	80:20
3	$-(CH_2)_5-$		**122c**	33	68:32
4	$-(CH_2)_2O(CH_2)_2-$		**122d**	18	74:26

Table 4: Isolated yield and diastereoselectivity of the two-step ozonolysis and indium-mediated couplings of homoallylic alcohols **123a-d** (Scheme 33). [a] Isolated yield over the two step procedure; [b] Diastereomeric ratios were determined by ^{1}H NMR spectroscopic analysis of the isolated products

2.1.2.1 Rationalisation of the Diastereoselectivity of the Indium-Mediated Couplings

The diastereoselectivity of the indium-mediated couplings was determined using 500 MHz ^{1}H NMR spectroscopy and was assigned later in the synthesis of the potential substrates **4**. The indium-mediated couplings are moderately *anti* diastereoselective. This observation is consistent with an established chelation–control model (Figure 7).[120, 121] The axial attack of the nucleophile onto the chelated aldehyde from the top face of chelate **131** is favoured, [120, 121] which results in the formation of the intermediate **132** and hence, the *anti*-diol **122**.

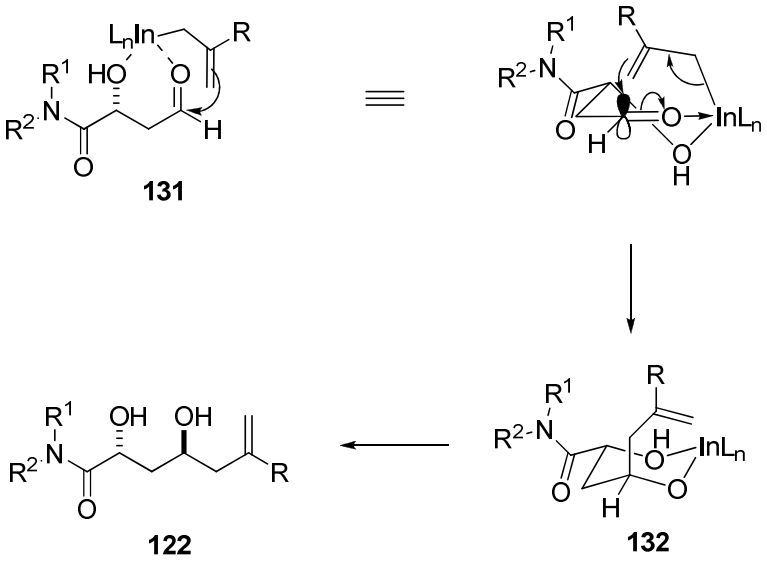
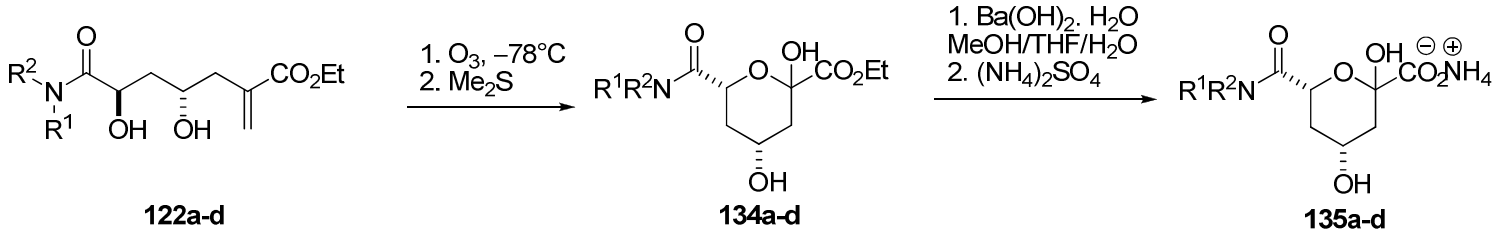


Figure 7: Chelation control model to explain the diastereoselectivity of the indium-mediated couplings

2.1.3 Deprotection of the Diols 122

The diols **122a-d** were ozonolysed to give the corresponding α-keto esters **134a-d**. Upon conversion to the α-keto esters **134a-d** the compounds became enriched with the *cis*-diastereomer due to column chromatography. Hydrolysis of diastereomeric mixtures of the α-keto esters **134a-d** using barium hydroxide gave the corresponding barium salts which were subsequently exposed to ammonium sulfate to yield the potential substrates, **135a-d**, as ammonium salts.



122a-d **134a-d** **135a-d**

Scheme 34: The preparation of the potential substrates **135a-d** from the diols **122a-d** *via* ozonolysis and hydrolysis (see Table 5). Only the major diastereomers of **122**, **134** and **135** are shown

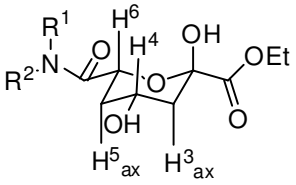
Entry	R^1	R^2	Diol	122 *Anti:syn* [a]	α-Keto ester	134 Yield (%)	134 *Cis:trans* [b,c]	Salt	135 Yield (%)	135 Cis:trans [b]
1	Pr	Pr	**122a**	70:30	**134a**	59	95:5	**135a**	>98	95:5
2	Bu	Bu	**122b**	80:20	**134b**	62	95:5	**135b**	>98	95:5
3	$-(CH_2)_5-$		**122c**	68:32	**134c**	27	88:12	**135c**	>98	88:12
4	$-(CH_2)_2O(CH_2)_2-$		**122d**	74:26	**134d**	49	78:22	**135d**	93	78:22

Table 5: Isolated yields of the ozonolysis the diols **122a-d** and the hydrolysis of the α -keto esters **134a-d** (see Scheme 34); [a] Diastereomeric ratios were determined by 500 MHz ^{1}H NMR spectroscopic analysis of isolated products **122a-d**. The relative configuration was assigned by conversion into cyclic α-keto ester **134a-d** ; [b] Determined by ^{1}H 500 MHz NMR spectroscopic analysis of isolated products; [c] Column chromatography of the α-keto esters **134a-d** yielded diastereomeric mixtures enriched with the *cis*-diastereomer

2.1.4 Determination of the Diastereoselectivity of the Indium-Mediated Couplings

The diastereoselectivity of the indium-mediated couplings was determined by conversion of the diols **122** into the α-keto esters **134**. The relative configuration of the cyclic α-keto esters **134** was determined by 500 MHz ^{1}H NMR spectroscopy (Figure 8). The major product was the *cis* diastereomer: large coupling constants (J = 12.0 and12.5 Hz) observed between H-4 and the axial protons H-3 and H-5, and between H-6 and the axial proton H-5$_{ax}$, predicted by Karplus,[122] confirmed the configuration of the product.

a)



b)

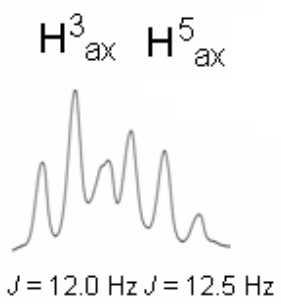


Figure 8: a) The *cis* diastereomer of the keto-ester **134**; **b)** the diagnostic 500 MHz ^{1}H NMR signals for axial H-3$_{ax}$ and H-5$_{ax}$ protons which exhibit large coupling J = 12.0 Hz and J =12.5 Hz respectively consistent with a *trans*-diaxial arrangement with H-4 and H-6.

2.2 Expression and Purification of the E192N Aldolase Variant

The His-tagged E192N aldolase was over–expressed and purified by utilising an existing method developed within the group by Gavin Williams. The aldolase was over-expressed from glycerol stocks that were kindly provided by Dr. Amanda Bolt. The cells were harvested by centrifugation and lysed using a cell dispruter. The variant aldolase was purified using nickel affinity chromatography with elution with imidazole. SDS-PAGE analysis (Figure 9) shows increased protein purity after the protein purification procedure.

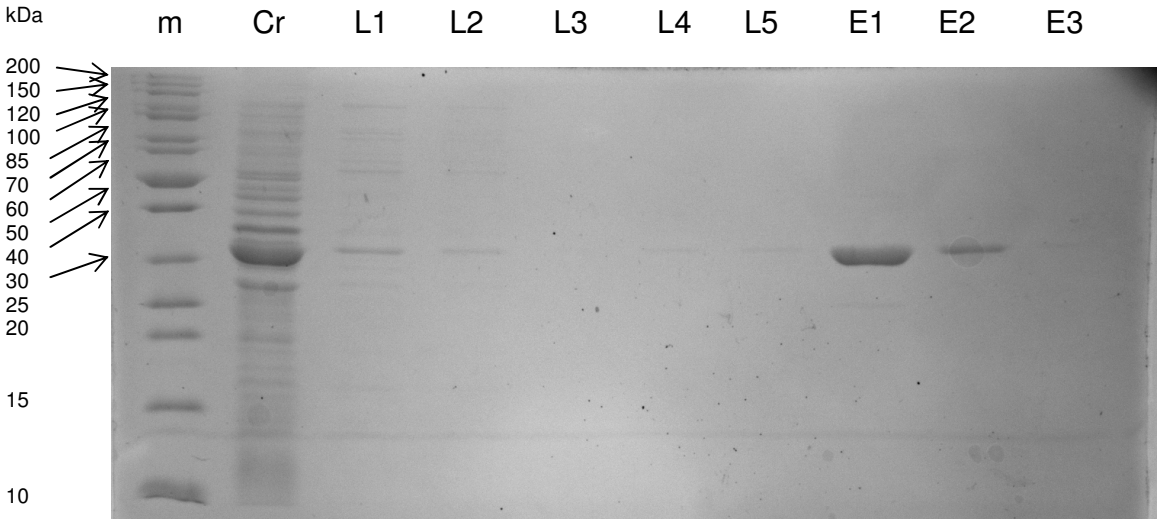


Figure 9: SDS-PAGE Gel depicting purification of the E192N aldolase variant using Nickel resin. From left to right: marker (m), the crude lysate (Cr), five sequential pre-elution washes (L1-5) and three sequential elution washes (E1-3). The aldolase was loaded with loading buffer (containing 16.2 mM potassium phosphate dibasic and 3.8 mM potassium phosphate monobasic (pH 7.4), and 300 mM NaCl and 10 mM imidazole) and eluted with elution buffer (containing 20 mM potassium phosphate buffer (pH 8.0), 200 mM NaCl and 200 mM imidazole).

2.3 Assay for Determining the Catalytic Activity of E192N Aldolase Variant

To determine whether the compounds **135** were substrates for the E192N aldolase variant, a nicotinamide adenine dinucleotide/lactate dehydrogenase (NADH/LDH) coupled assay was used (Figure 10). The assay allowed the rate of E192N reaction to be determined. As the substrate **137** is cleaved by the E192N aldolase variant pyruvic acid is produced. The consumption of NADH and hence the rate of the E192N reaction can followed by a decrease in absorbance of NADH as it converted into NAD^+.

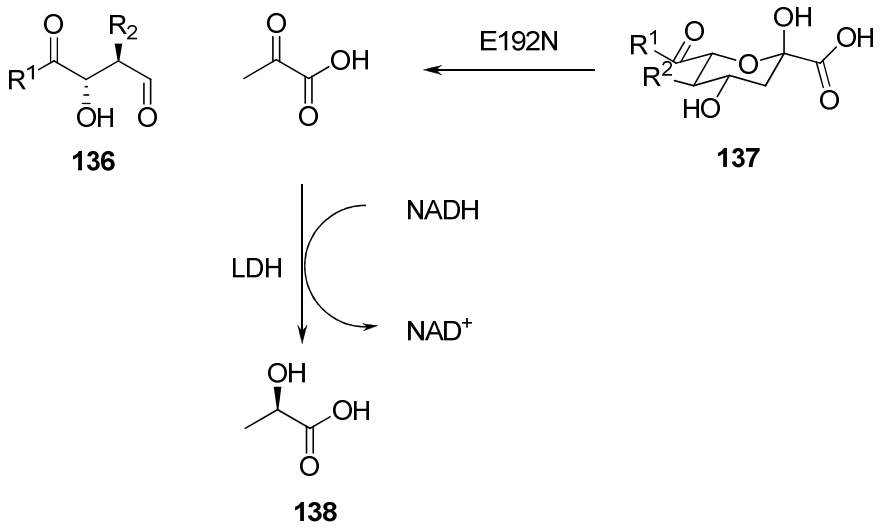


Figure 10 Coupled enzyme assay used to determine the activity of the aldolase variant. The cleavage of compounds **137** *via* a reverse aldol reaction leads to the production of pyruvate, which is reduced *in situ* by lactate dehydrogenase (LDH) to L-lactate. The decrease in the absorbance of NADH (A_{340}) can be used to characterise the aldolase activity.

2.4 Steady-State Kinetics for Potential Substrates 135 with the E192N Aldolase Variant

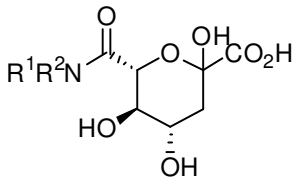
The cleavage of the potential substrates **135**, catalysed by the E192N aldolase variant was studied using the NADH/LDH assay (Figure 10). The assay was performed in a 1 mL cuvette and the initial rate of reaction was measured by following the A_{340}, using a spectrophotometer. Reaction velocities were calculated using the molar extinction coefficient of NADH (6220 $M^{-1}cm^{-1}$), plotted using Origin®

and the kinetic parameters estimated by non-linear regression analysis. The data collected for all substrates **135** is shown in Figures 11 and 13 and Table. 6. All of the compounds **135** were, as predicted, substrates for the E192N aldolase variant.

When the specificity constants (k_{cat}/K_M) are compared between substrates **135a-d** a general trend is observed. The dipropylamide **135a** shows the highest k_{cat}/K_M value and is followed by the piperidylamide **135c**, the dibutylamide **135b**, and the morpholylamide **135d**. The observation fits with the general trend shown for the hydroxylated substrates **36a-d** (Figure 11, Table 6, Figure 14) where the increased hydrophobicity of the tertiary amide tallies with increased catalytic efficiencies,[46, 118, 119] although the dibutylamide **135b** bucks this trend with much lower specificity constant than expected. Additionally, when the k_{cat}/K_M values are compared between the non-hydroxylated compounds **135a-d** and their hydroxylated counterparts **36a-d** the values are slightly elevated for the non-hydroxylated compounds **135a-d** but the values are broadly comparable. In general, the activity (k_{cat}) values are higher and the Michaelis constant (K_M) are lower observed than the values the non-hydroxylated substrates **135a-d** are slightly lower than the values for the hydroxylated substrates **36a-d**.

In conclusion, all compounds screened were indeed substrates for the E192N aldolase variant.[‡] The presence of a hydroxyl substituent in the C-5 position is not crucial for E192N catalysis and has little effect on k_{cat}/K_M. This observation fits with our initial rationale and also meant that it was possible to vary the groups in the C-5 position.

[‡] Please note, all of the compounds **135** tested were racemic and mixtures of diastereomers. This means that the steady state kinetics collected is most likely average of the data for each of the enantiomers and diastereomers. However, it is possible that only one or a number of the enantiomers and diastereomers are substrates for the E192N aldolase variant from the data presented here, although later in the project found not to be the case.

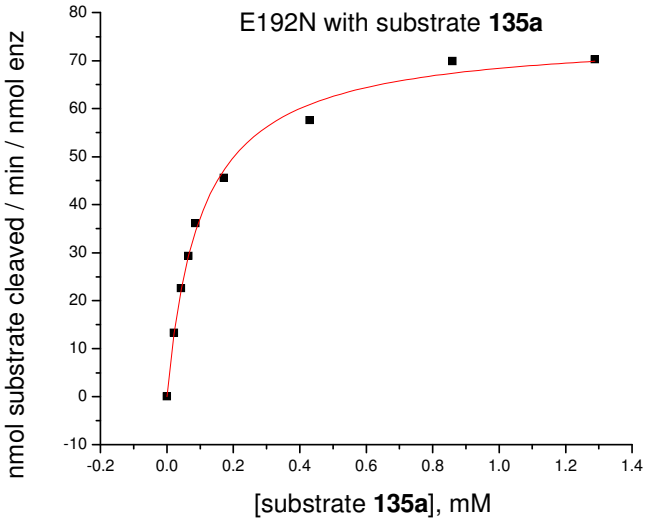


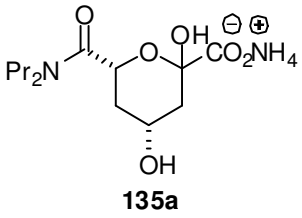
36

R^1	R^2	**Acid**
Pr	Pr	**36a**
Bu	Bu	**36b**
$-(CH_2)_5-$		**36c**
$-(CH_2)_2O(CH_2)_2-$		**36d**
Me	OMe	**36e**

Figure 11: The E192N hydroxylated substrates

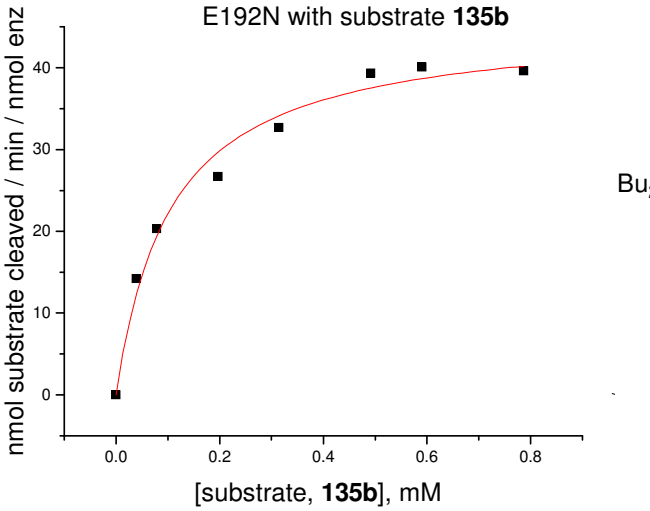
a)

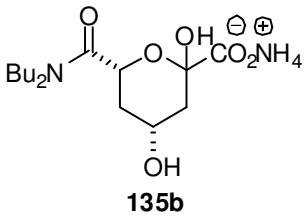




135a

b)

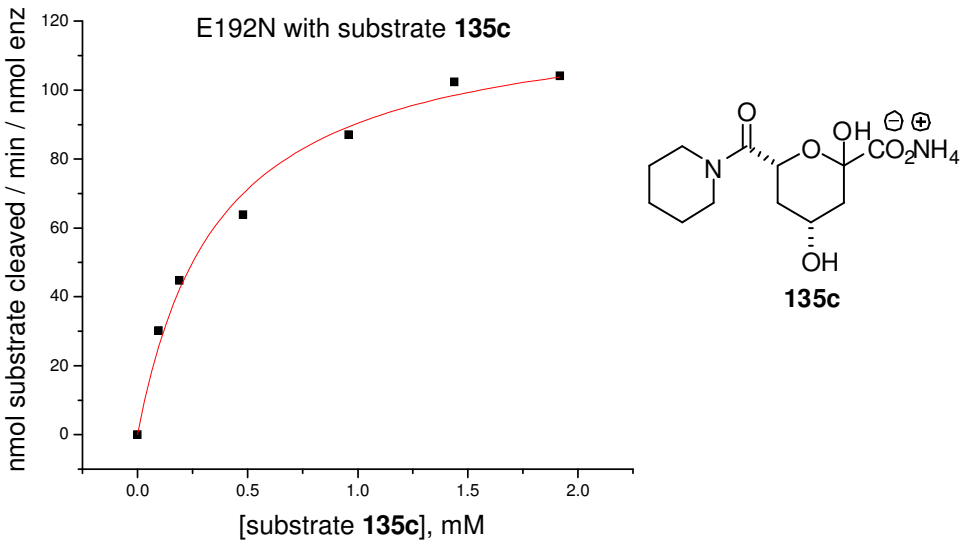




135b

Figure 12: Initial rates of E192N-catalysed cleavage of the substrates **135a-d**, the y axis scale is different on each graph. a) E192N with **135a**; b) E192N with **135bX**; c) E192N with **135c**; d) E192N with **135d**;

c)



d)

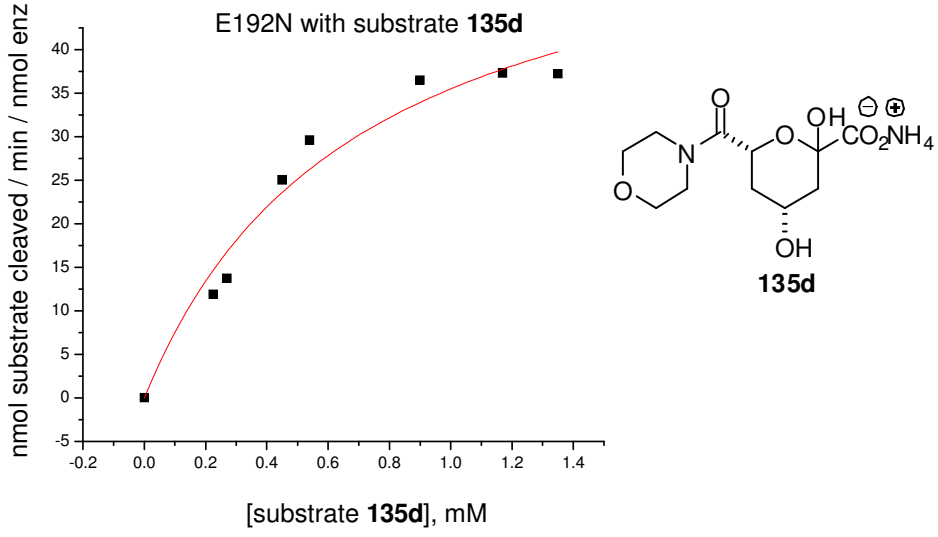


Figure 13: Initial rates of E192N-catalysed cleavage of the substrates **135a-d**, the y axis scale is different on each graph. a) E192N with **135a**; b) E192N with **135b**; c) E192N with **135c**; d) E192N with **135d**;

Substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}mM^{-1}$)	Substrate[a]	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($min^{-1}mM^{-1}$)
135a	201.8 ± 1.6	0.103 ± 0.007	1959	**36a**	130 ± 3	0.39 ± 0.04	340
135b	60.9 ± 1.9	0.105 ± 0.017	580	**36b**	90 ± 6	0.25 ± 0.04	359
135c	330.8 ± 5.7	0.338 ± 0.055	853	**36c**	95 ± 1	0.57 ± 0.02	167
135d	161.6 ± 8.0	0.703 ± 0.198	230	**36d**	72.4 ± 2.3	3.4 ± 0.2	21.3

Table 6: Steady state kinetic parameters for the cleavage of substrates **135a-d** and **36a-d** with the E192N aldolase variant. Kinetic parameters (± errors of the fit) were determined by fitting the data to the Michaelis–Menton equation; [a] Substrates prepared by Thomas Woodhall and steady-state kinetic data obtained by Gavin Williams.[46, 118, 119]

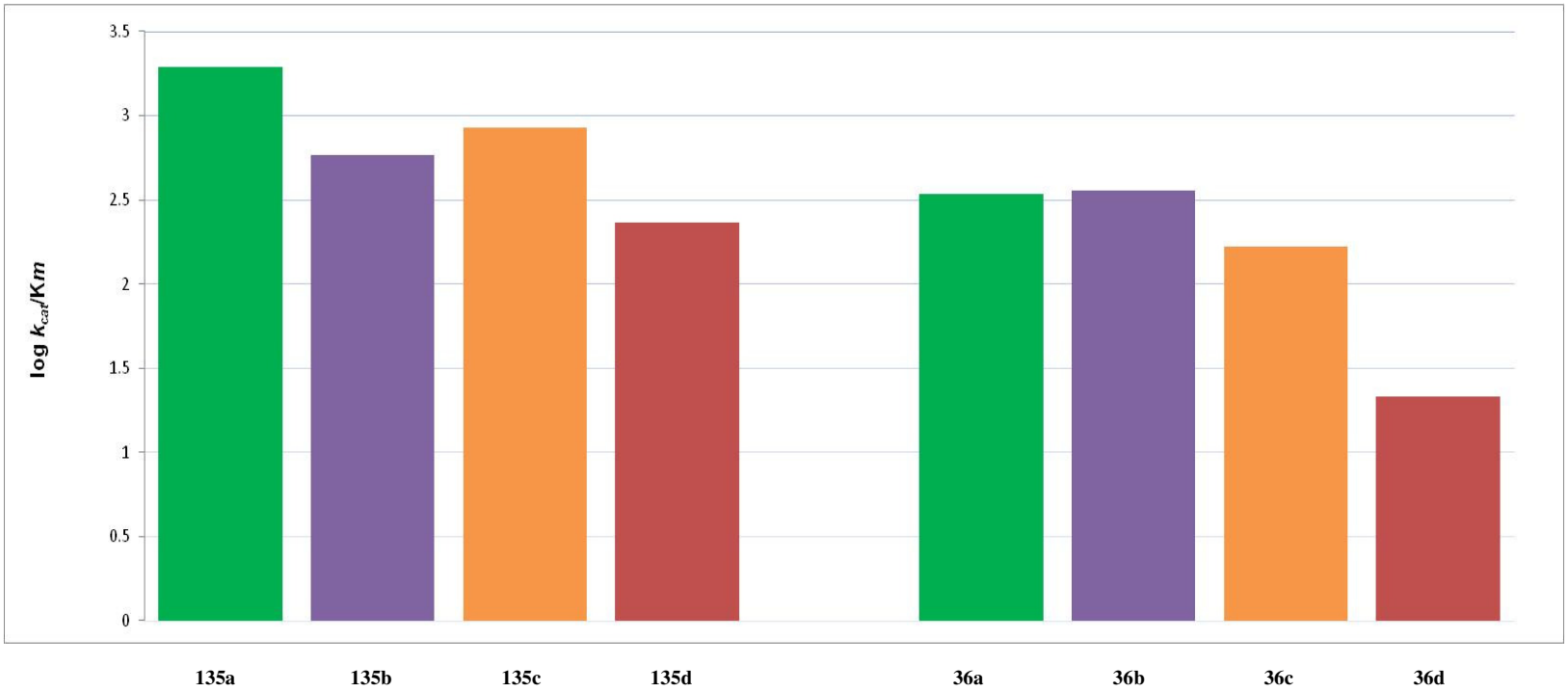
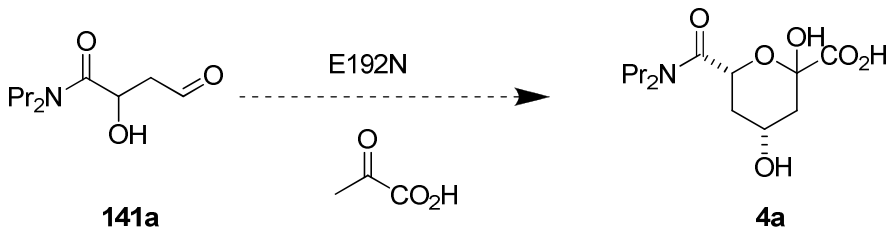


Figure 14: Comparison of specificity constants log k_{cat}/K_M for the cleavage substrates **135a-d** and **36a-d** with the E192N variant aldolase I

2.5 E192N-Catalysed Condensation Reactions with Novel Enzyme Substrates

Although the amides **135a-d** were established as substrates for the E192N aldolase variant, it was not known whether the forward direction aldol reaction was thermodynamically favoured and hence, synthetically useful. As the aldol reaction between hydroxylated substrates (**35**) and pyruvate are thermodynamically favourable processes, it was predicted that the non-hydroxylated substrates would also be thermodynamically favoured. To investigate the thermodynamics of the aldol condensation, the aldehyde **141a** and pyruvate were treated with the E192N aldolase variant (Scheme 35).



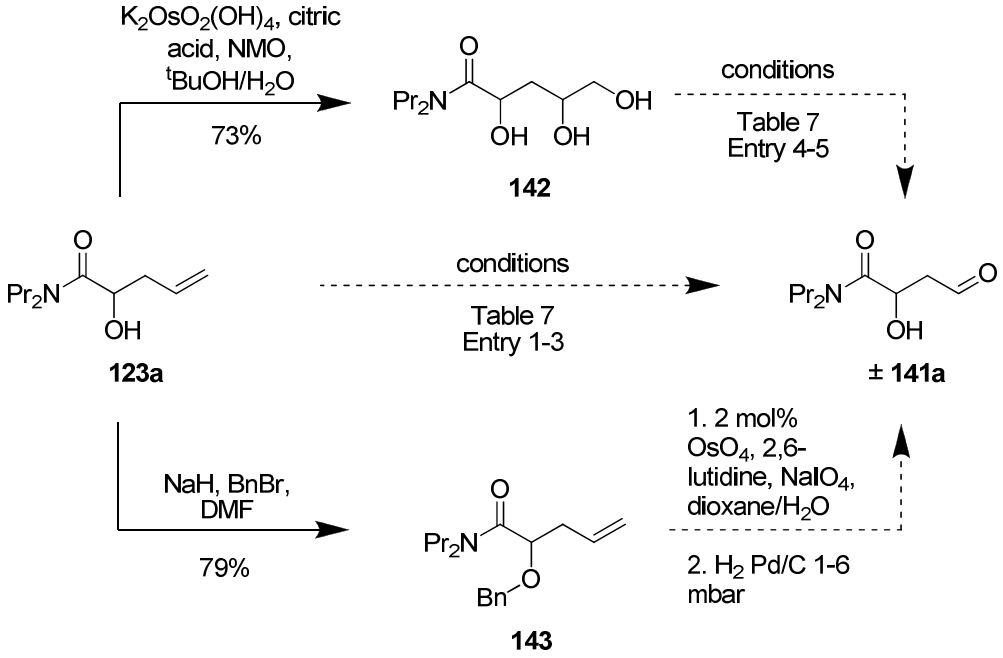
141a **4a**

Scheme 35

2.5.1 Preparation of Aldehyde 141a

The preparation and isolation of aldehyde **141a** was attempted from the racemic alkene **123a** (Scheme 36 and Table 7). Various conditions were attempted; however the aldehyde **141a** could not be isolated, despite the reactions going to completion as indicated by LCMS and TLC. Consequently, the isolation of the aldehyde **141a** was abandoned. The crude aldehyde **141a**, prepared by ozonolysis of the alkene **123a** in MeOH[§], was therefore used directly in the E192N reaction (Scheme 37).

[§] When the ozonolysis of the alkene **123a** was performed in DCM the condensation reaction did not yield any of condensation product, acid **4a**



Scheme 36: The attempted routes toward aldehyde **141a** from alkene **123a**

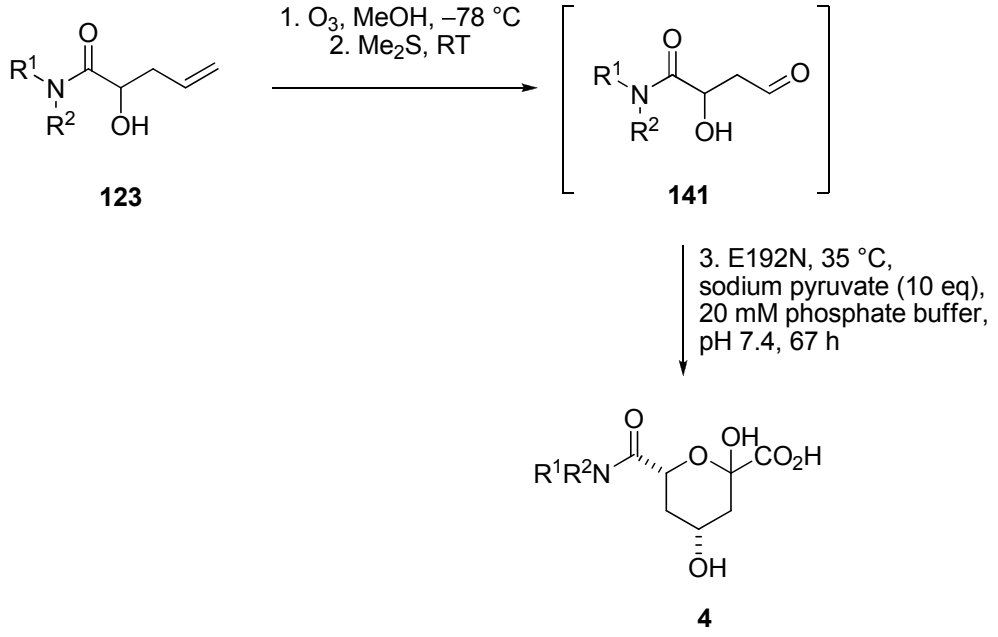
Entry	Substrate	Reaction Conditions
1	**123a**	2 mol% OsO_4, 2,6-Lutidine, $NaIO_4$, dioxane/H_2O
2	**123a**	30 mol% OsO_4, NMO, $NaIO_4$, MeCN/H_2O
3	**123a**	O_3, MeOH, –78 °C; DMS
4	**142**	Periodic acid, DCM
5	**142**	$NaIO_4$ DCM/H_2O

Table 7: Attempted reaction conditions in the attempted preparation of the aldehyde **141a** from the alkene **123a**.

2.5.2 E192N-Catalysed Condensation Reactions

As anticipated, the E192N-catalysed aldol condensation between the aldehyde **141a** and pyruvate was a thermodynamically favoured reaction (Scheme 37 and Table 8, entry 1). The alkenes **123** were also ozonolysed and the corresponding aldehyde reacted with pyruvate to give the acids **4** (Scheme 37 and Table 8). These reactions were also thermodynamically favoured as the reactions proceeded well with, on

the whole, good yields after purification by preparative HPLC. The yields observed are comparable to those observed for the hydroxylated derivatives **35** in the forward direction.[118] The corresponding aldehyde of alkene **123f** (R^1 = Me; R^2 = OMe) is not a substrate for the E192N aldolase variant; however there is no evidence that the hydroxylated version (**35f**) is a substrate for the E192N aldolase variant either.



123 **141** **4**

Scheme 37: The preparation of the acids **4** using E192N-catalysed aldol condensation (see Table 8). Only the *cis* diastereomer is depicted.

Entry	Alkene	R^1	R^2	Aldehyde 141	Product	4 Yield[b] (%)	*Cis:trans*[a]
1	**123a**	Pr	Pr	**141a**	**4a**	73	70:30
2	**123c**	$-(CH_2)_5-$		**141c**	**4c**	53	74:26
3	**123d**	$-(CH_2)_2O(CH_2)_2-$		**141d**	**4d**	52	83:17
4	**123e**	Me	Pr	**141e**	**4e**	19	76:24
5	**123f**	Me	OMe	**141f**	**4f**	[c]	-

Table 8: Isolated yields of acids **4** from two-step ozonolysis and E192N catalysed aldol reaction sequence (see Scheme 37) [a] Diastereoselectivity of isolated products determined using 500 MHz ^{1}H NMR spectroscopy; [b] All products were purified by preparative HPLC; [c] No product observed

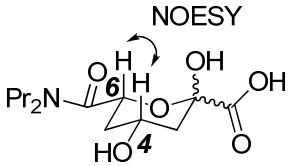
The diastereoselectivity of the reactions was determined for the isolated products (section 2.5.3). The reactions are moderately *cis*-diastereoselective and thermodynamically controlled. This was confirmed by recent work within the group by Thomas Harman, who determined the kinetic and thermodynamic stereoselectivities of the reaction with the substrate **141a**. The ratios of *cis*:*trans* observed in the above reactions are comparable to the ratios observed by Thomas Harman when the forward direction reaction of **141a** is followed, using 500 MHz ^{1}H NMR, to completion and hence under thermodynamic control. It is possible that the E192N aldolase variant is selective for one enantiomer of the aldehydes **141** and hence a resolution could be occurring in the enzyme reaction. However, recent work by Thomas Harman has also confirmed that both enantiomers of the substrate **141a** were substrates for the E192N aldolase variant and the enzyme shows no kinetic preference for either enantiomer of **141a**.[**]

2.5.3 The Sense of Diastereoselectivity of the E192N-Catalysed Condensation Reactions

The diastereoselectivity of the E192N-catalysed aldol reactions was determined using NMR analysis of the isolated products **4**. A combination of 2D NMR experiments was used to identify diastereoisomeric and anomeric species of the acid **4a**. NOESY analysis of the mixture allowed the identification of the *cis*-diastereomer with the correlations between protons H-4 and H-6 confirming the axial orientation of both hydrogens (Figure 14). TOCSY analysis confirmed ^{1}H peak corresponding to both anomers of the *trans* diastereomer. This was achieved by identifying cross peaks in the TOCSY spectrum which correspond to protons in the same spin system and hence, in this case, in the same species.

[**] personal communication

cis diastereomer

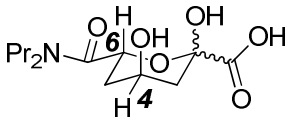


cis-**4a**

maj anomer H-6 *J* = 10.5 Hz

min anomer H-6 multiplet

trans diastereomer



trans-**4a**

maj anomer H-6 *J* = 9.5 and 3.3 Hz

min anomer H-6 *J* = 6.7and 4.9 Hz

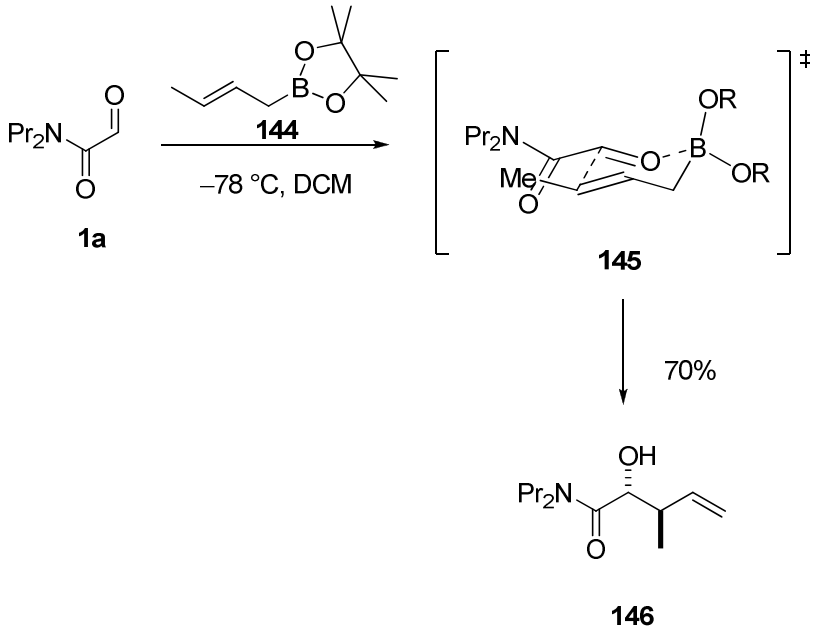
Figure 15

The vicinal coupling constants for the H-6 signal vary between anomeric species and *cis* and *trans* diastereomers of the acids **4** (for example **4a** Figure 14). These differences can be explained by the likelihood that the species exist in different conformations. It is likely that both the anomers of the *trans* diastereomer *trans*-**4a** exist in a mixture of chair conformations, and the coupling constants observed are therefore an average between the constants for each conformation. The *cis* anomers *cis*-**4a** most likely exist in mainly one conformation (as depicted in Figure 14) and hence a large *trans*-diaxial coupling is observed to the axial H-5_{ax} proton.

2.5.4 E192N-Catalysed Condensation Reactions to Yield Products with C-5 Substituents

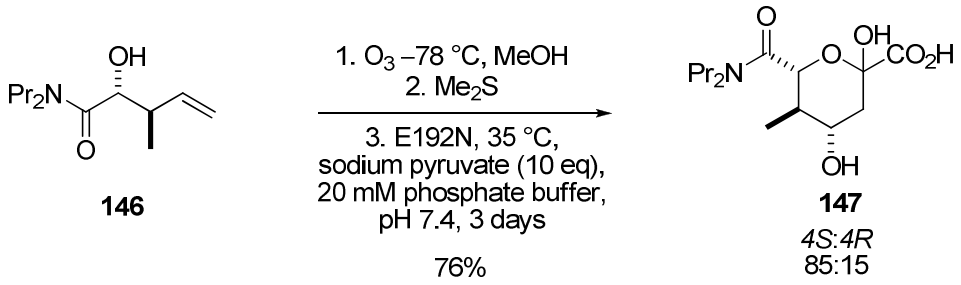
To investigate E192N substrate specificity further, the aldehyde derived from alkene **146**, where a methyl group is installed at the position corresponding to C-5, was investigated as a potential substrate for the E192N aldolase variant. The aldol condensation of the corresponding aldehyde of the alkene was performed. The alkene **146** was prepared from the glyoxylaldehyde **1a** and the *E*-crotyl boronate **144** (Scheme 38) which gave the *anti*-alkene **146**. The

diastereoselectivity of the allylation was high (>99:1) and may be explained in terms of a cyclic transition state **145**.[123]



Scheme 38: Preparation of the alkene **146.**

The alkene **146** was ozonolysed and treated with sodium pyruvate and the E192N variant aldolase (Scheme 39). The corresponding aldehyde of alkene **146** was, as predicted, a substrate for the E192N aldolase variant. The diastereoselectivity was determined by analysis by 500 MHz ^{1}H NMR spectroscopic analysis of the isolated products. The diastereoselectivity at the C-4 position (85:15) is most likely, as with the previous examples **4**, controlled by the relative thermodynamic stabilities of the two possible diastereomeric products.



Scheme 39: Preparation of the acid **147** using E192N-catalysed aldol condensation. Only one anomer of the major diastereomer is depicted.

2.6 Chapter 2 Summary

As predicted the ammonium salts **135a-d** have been shown to be substrates for the E192N aldolase variant. This observation fits with the initial hypothesis that the C-5 hydroxyl is not crucial for enzymatic activity. Full steady-state kinetic data were obtained for all the substrates which were comparable to those of the hydroxylated compounds' **36a-d**. The aldol condensations were shown to be thermodynamically favoured, with isolated yields comparable to the hydroxylated variants **36**. Additionally, it was found that substituents other than hydrogen, can be placed in the C-5 position of the E192N substrate without affecting the condensation reaction. All of these findings provided evidence that the proposed one-pot reaction may be feasible.

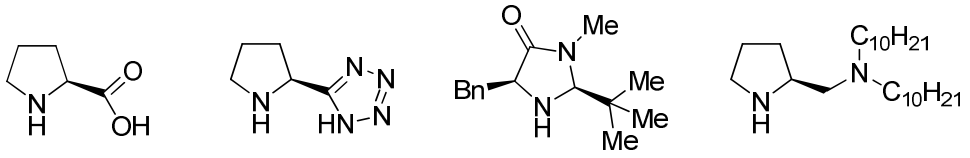
Chapter 3

The Development of a One-Pot Reaction

The next key goal of the project was to find common conditions under which both organo- and enzyme-catalysed steps could occur. To establish these conditions, three main targets were set: to develop conditions for the organocatalysed step; to ascertain whether the optimised conditions for the organocatalysed step are compatible with the E192N aldolase variant; and to perform one-pot reactions combining organo- and enzyme-catalysis.

3.1 The Development of Conditions for the Organocatalysed Step

To establish suitable organocatalytic reaction conditions, a comprehensive literature search was conducted and a number of organocatalysts were selected as potential catalysts for the envisaged one-pot reaction. The organocatalysts were chosen based on their reported catalytic activity in cross-aldol reactions and their solubility.[78, 86, 124] In order to investigate the effect of different solvent systems in the proposed one-pot reaction, it was important to choose a range of organocatalysts with varying solubility in organic and aqueous solvents. Organocatalysts which performed well in aqueous conditions were attractive targets due to their potential compatibility with enzymatic catalysts, which usually operate in aqueous buffered conditions. However, organically soluble catalysts were also of interest in case biphasic systems or sequential reaction conditions needed to be explored as alternative approaches.



67 **68** **69**

Figure 16: Selected Catalysts

L-Proline is the most extensively studied organocatalyst for asymmetric cross aldol reactions including aldehyde-aldehyde aldol reactions.[124] However, most of the proline-catalysed aldol reactions reported in the literature, are performed in DMF or DMSO.[125, 126] The diamine **69** has been reported as an active catalyst for cross aldol reactions under aqueous conditions (see Introduction),[86] and is therefore suitable for our investigation. The tetrazole catalyst **67** has increased catalytic activity for cross-aldol reactions when compared to proline and has been reported as an effective catalyst in solvents such as acetonitrile.[127] The imidazolidone **68** has been reported by MacMillan and is an active organocatalyst for various transformations including cross aldol reactions in ether.[79]

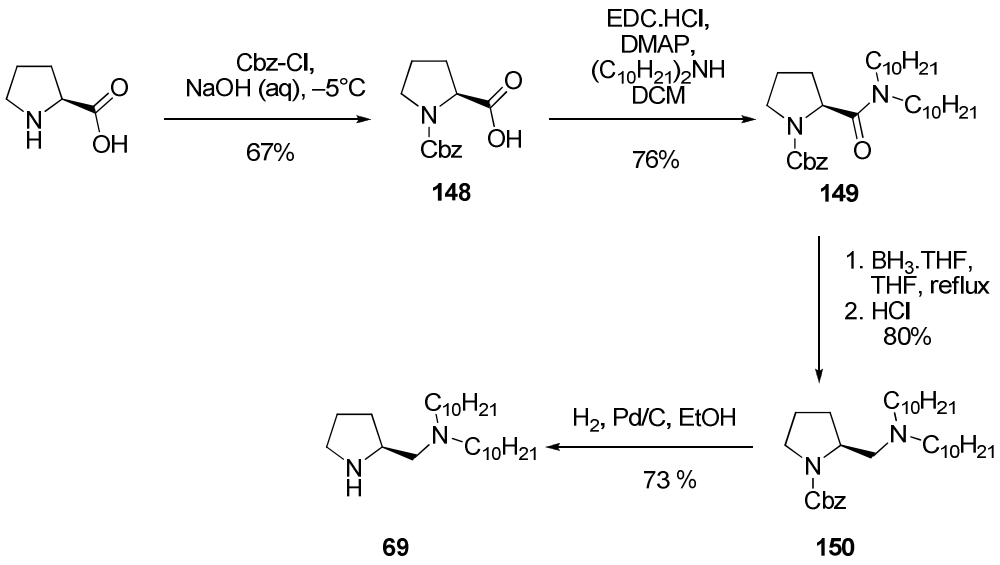
As the catalysts **67**-**69** are not commercially available, it was necessary to synthesise the compounds to investigate their catalytic activity. The catalysts were all synthesised on a multi-gram scale in order to have sufficient stocks for the planned investigations.

3.1.1 Synthesis of Organocatalysts 67-69

3.1.1.1 Synthesis of the Diamine 69

The diamine **69** was synthesised in four steps from L-proline using a known procedure reported by Mase *et al*. (Scheme 40).[86, 128] Cbz protection of L-proline with benzyl chloroformate gave the carbamate **148** which underwent an EDC-mediated coupling with didecylamine to yield the amide **149**. The chemoselective reduction of

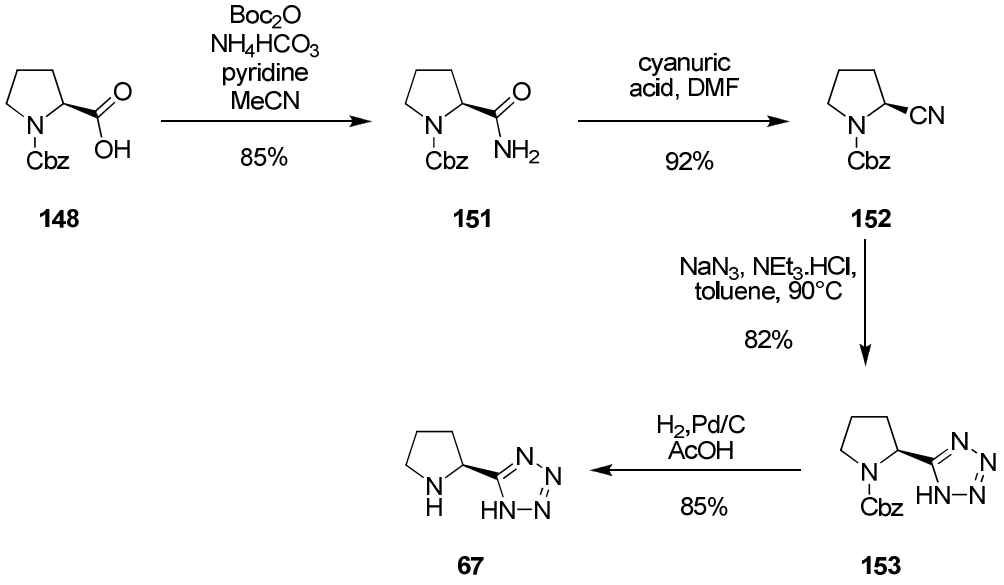
the amide **149** in ethanol with borane·THF complex afforded the amine **150**. The synthesis was completed by the deprotection of the Cbz-group by hydrogenation which gave the free diamine **69**. The diamine **69** was prepared in a 30% overall yield from L-proline.



Scheme 40: Preparation of the diamine **69**.

3.1.1.2Synthesis of the Tetrazole 67

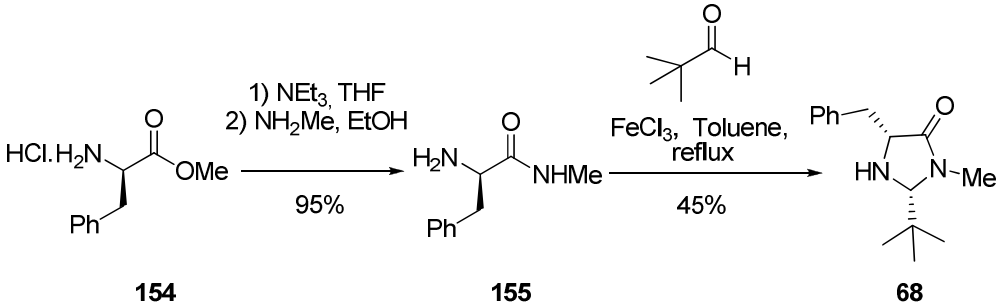
Preparation of the tetrazole catalyst **67** was achieved *via* literature procedures and involved four synthetic steps from Cbz-protected L-proline **148** (Scheme 41).[129] Cbz-protected L-proline **148** was transformed into the primary amide **151** by conversion into a mixed anhydride and treatment with ammonium bicarbonate. The primary amide **151** was dehydrated by exposure to cyanuric acid in DMF to give the nitrile **152**. The nitrile **152** underwent a 1,3-dipolar cycloaddition[130] with sodium azide to give the protected tetrazole **153**. The tetrazole **67** synthesis was completed by the hydrogenolysis of the protected tetrazole **153**, to give the deprotected tetrazole **67**. The synthesis of the tetrazole **67** was completed in an overall 37% yield from L-proline.



Scheme 41: Preparation of the tetrazole **67**.

3.1.1.3Synthesis of the Imidazolidinone 68

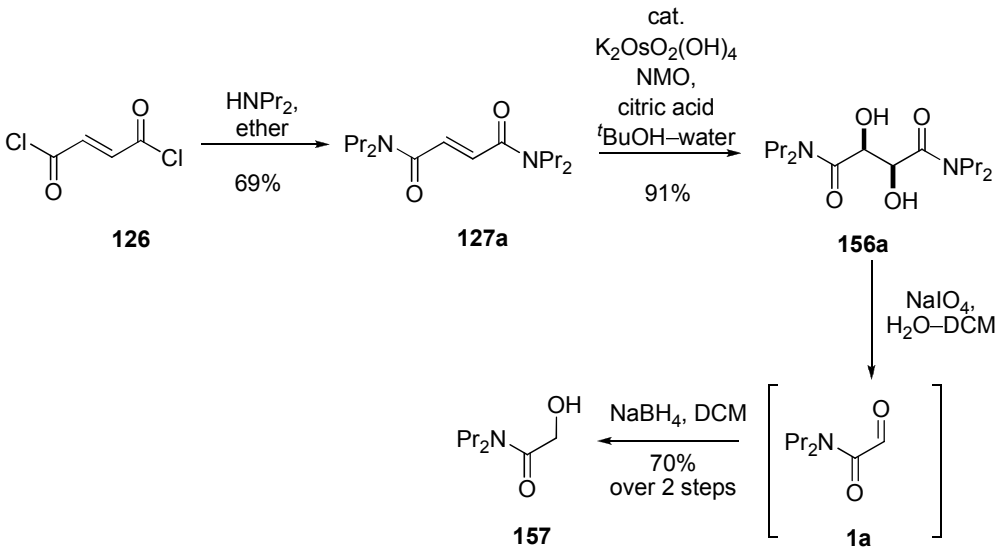
The imidazolidinone **68** was synthesised from the phenylalanine derivative **154** using existing synthetic methods (Scheme 42).[131, 132] Thus, D-phenylalanine methyl ester hydrochloride (**154**) was amidated with methylamine to give the secondary amide **155**, which was cyclised with pivaldehyde to give both *cis* and *trans* imidazolidinones **68** in a 1:1 ratio. The diastereomers were separated by column chromatography and the *cis* product **68** was obtained in a 45% yield. The overall yield from the phenylalanine derivative **154** was 43% and was comparable to literature reports.[132]



Scheme 42: Preparation of imidazolidinones **68**.

3.1.2 Preparation of the Glyoxylaldehyde 1a

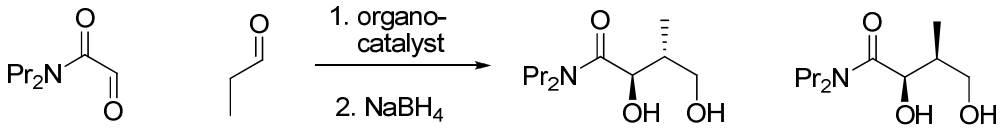
The glyoxylaldehyde **1a** was a proposed substrate for the organocatalysed-aldol reactions. The glyoxylaldehyde **1a** was prepared by the oxidative cleavage of the diol **156a** (Scheme 43). The diol **156a** was prepared in two steps from fumaryl chloride. The two step preparation involved the reaction with dipropylamine which gave the intermediate amide **127a.** The amide **127a** was dihydroxylated to give the diol **156a**; oxidative cleavage of the diol **156a** under biphasic reaction conditions yielded the glyoxylaldehyde **1a**. The glyoxylaldehyde **1a** was not isolated at this point; instead the product was used directly in subsequent aldol reactions. A lower limit for the yield for the periodic cleavage step was determined by the reduction of the glyoxylaldehyde **1a** to the alcohol **157** with sodium borohydride. In later work, an alternative procedure was used which allowed the glyoxylaldehyde **1a** to be isolated (section 3.3.2.1).



Scheme 43: Synthesis of glyoxylaldehyde **1a.**

3.1.3 Organocatalysed Aldol Reactions

The organocatalysts (L-proline, **67**, **68** and **69**) were evaluated as catalysts of the aldol reaction between the glyoxylaldehyde **1a** and propionaldehyde (Scheme 44). Initially, attempts were focussed on the conditions that have been established for each catalyst that would be potentially compatible with the enzyme-catalysed step. Consequently, organic solvents (*e.g.* DMF) and temperatures which were unlikely to be tolerated by the aldolase were, when possible, avoided.[133-135] The crude reaction products were reduced directly with sodium borohydride; the yields of the diols *anti*-**158** and sy*n*-**158** allowed the success of the crossed aldol condensation to be observed.



1a *anti*-**158** *syn*-**158**

Scheme 44: Organocatalysed aldol reactions

The study revealed that L-proline, the diamine **69** and the tetrazole **67** were the best catalysts for the crossed aldol reaction between glyoxylaldehyde **1a** and propionaldehyde (Table 9). The isolated yields of the L-proline-catalysed reactions were highest in DCM with one equivalent of propionaldehyde (Table 9, entry 1); no product was observed in DMSO (Table 9 entry 1). The tetrazole catalyst **67** did not yield the diols **158** in DCM or MeCN but, in more polar solvents such as DMF and DMSO, the product was obtained (Table 9, entry 11 and 12). The imidazolidone catalyst **68** was a poor catalyst for the aldol reaction even with high catalyst loading and excess propionaldehyde (Table 9 entries 5-7); dehydration of the aldol product was observed by LC-MS. In addition, L-alanine did not yield the diols **158**, under the conditions screened (Table 9, entry 4).

Entry	Catalyst	Catalyst loading (mol%)	Propionaldehyde (eq)	Additive	Solvent	Temp	*syn:anti* [a]	158 yield [b]
1	L-proline[g]	50	1	-	DCM	RT	62:38	25%
2	L-proline[h]	50	82	-	DCM	RT	84:16	3%
3	L-proline	50	1	-	DMSO	RT	-	[e]
4	L-alanine	30	2		DMSO	RT	-	[e]
5	**68**	20	1	TFA[c]	Ether	+ 4ºC	-	[e]
6	**68**	50	10	TFA[d]	Ether	RT	-	[e]
7	**68**	50	10	TFA[d]	DCM	RT	-	[e]
8	**67**	5	2	-	MeCN	RT	-	[e]
9	**67**	20	82	-	MeCN	RT	-	[e]
10	**67**	20	82	-	DCM	RT	-	[e]
11	**67**[h]	20	82	-	DMF	+ 4ºC	40:60	6%
12	**67**[h]	20	82	-	DMSO	RT	78:22	20%
13	**69**[i]	20	2	TFA[c]	H_2O	RT	31:69	0.2%
14	**69**[i]	20	2	TFA[c]	buffer[f]	RT	64:36	19%

Table 9: Organocatalyst screening. [a] Determined by 500 MHz ^{1}H NMR spectroscopic analysis of isolated product; [b] combined yield of both diastereomers; [c] 20 mol% additive was used; [d] 50 mol% additive was used; [e] The diols **158** were not formed in the reaction;[f] pH 7.4, 20 mM potassium phosphate buffer; [g] 0.254 mmol/mL concentration of glyoxylaldehyde **1a**; [h] 0.0254 mmol/mL concentration of glyoxylaldehyde **1a**; [i] 0.506 mmol concentration of glyoxylaldehyde **1a**

The diamine **69** catalysed the aldol reaction in aqueous conditions, and interestingly, the reaction could be performed in pH 7.4 buffer (Table 9, entry 14). Reactions involving this catalyst further were further investigated (Table 10). It was discovered that the isolated yield varied with the solvent used. In water and buffer (Table 10, entries 2,3 and 5), the reaction proceeded; in contrast, in DMSO, no diol **158** was isolated. The yield was higher with TFA as an additive than with scandium(III) triflate (Table 10). The highest yield of product was observed with 10 equivalents of propionaldehyde and 10 mol% of the catalyst in pH 7.4 buffer (Table 10, entry 5).

The pH of the reaction was varied over the range that the wild type-NAL is active (between pH 6 and 9) (Table 11, entries 1-5). The pH had little effect on the yield of the reaction within the range tested. Although the highest yield of the diols **158** was observed at pH 6 (Table 11, entry 1), this pH may be too low for the E192N variant aldolase. However, a good yield of diol **158** was obtained at pH 7.5, the optimal pH for the wild type-NAL.

The catalyst loading of the diamine-catalysed reactions was investigated. At low catalyst loadings (1-5 mol% diamine, Table 11, entries 7-8), no reaction was observed. With 10 mol% catalyst, the diamine-catalysed reaction took place (Table 11, entry 3). All organocatalysed conditions screened exhibited low to medium yield of the diols **158**. It is possible that the low yields stemmed, in part, from the instability of the intermediate aldehyde.

Entry	Catalyst loading (mol%)	Propionaldehyde (eq)	Additive	Solvent	*Syn:anti*[a]	158 yield[b]
1	20	2	TFA[c]	DMSO	-	[f, g]
2	20	2	$Sc(OTf)_3$[c]	H_2O	12:88	0.2%[f]
3	20	2	$Sc(OTf)_3$[c]	buffer[e]	24:76	8%[f]
4	20	2	$Sc(OTf)_3$[c]	DMSO	-	[f, g]
5	10	10	TFA[d]	buffer[e]	68:32	45%[f]

Table 10: Diamine-catalysed **69** aldol reaction. [a] Determined by 500 MHz ^{1}H NMR spectroscopic analysis of isolated product; [b] Combined yield of both diastereomers; [c] 20 mol%; [d]10 mol%;[e] pH 7.4, 20 mM potassium phosphate buffer;[f] All reactions were performed at room temperature; [g] The diols **158** were not formed in the reaction

Entry	pH	Cat loading (mol%)	*Syn: anti*[a]	158 yield[b]
1	6.0	10	62:38	41%
2	6.4	10	82:18	26%
3	7.0	10	68:33	33%
4	7.4	10	75:25	33%
5	8.0	10	81:19	33%
7	7.0	1	-	[c]
8	7.0	2	-	[c]
9	7.0	5	-	[c]

Table 11: Diamine-catalysed aldol reactions performed with phosphate buffer. All reactions used, 10 eq. propionaldehyde in 100mM potassium phosphate buffer for 25 hrs at room temperature; [a] Determined by 500 MHz ^{1}H NMR spectroscopic analysis of isolated product; [b] Combined yield of both diastereomers; [c] The diols **158** were not formed in the reaction

3.1.3.1 Stereoselectivity of the Organocatalysed Aldol Reactions

The diastereoselectivity of the reactions was determined by analysis of the isolated products by 500 MHz ^{1}H NMR spectroscopy. The relative configuration of the *anti*- and *syn*-diols **158** was determined by conversion into cyclic derivatives (section 3.1.3.1.1). Most of the aldol reactions were moderately *syn* diastereoselective. The *syn* diastereoselectivity is unexpected as it contrasts the *anti* sense of induction observed in many other organocatalytic crossed aldol reactions. However, aldehydes such as glyoxylaldehyde **1a** have not previously been investigated as substrates for organocatalysed aldol reactions.

It is notable that the pH of the diamine-catalysed reactions affected the diastereoselectivity.[††] At lower pH the *syn* diastereoselectivity decreases in buffer (Table 11, entries 1-5). In addition, in water with TFA as an additive, the reaction is *anti*-diastereoselective (Table 9, entry 14). It can be envisaged that the reaction may proceed *via* the open chain (**161**) or the closed chain transition state (**160**) (Figure 17).[72, 73] The pH of the reaction may control the population of these transition states and, hence, the diastereoselectivity. It has been demonstrated that cross aldol reactions, catalysed by the amide **162** (Figure 17) at low pH (pH 1-4) proceed at slower rates but with higher *anti*-diastereoselectivity and enantioselectivity than at higher pH.[136]

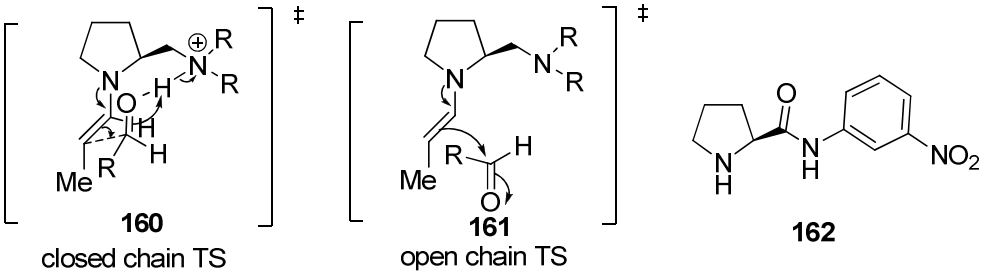


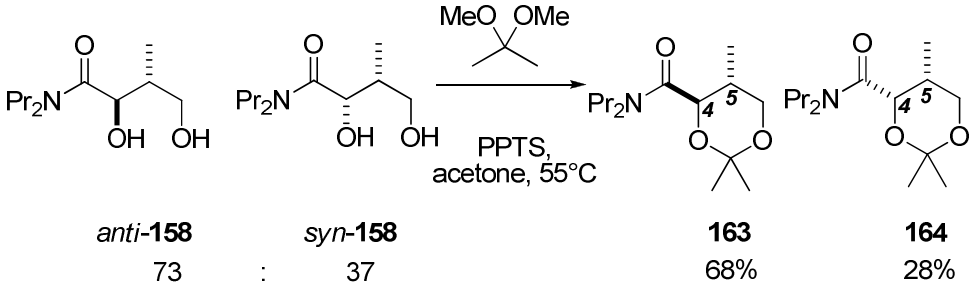
Figure 17

3.1.3.1.1 Determining the Diastereoselectivity of the Organocatalysed Aldol Reactions

In order to determine the diastereoselectivity of the organocatalysed aldol reactions, the identification of the *anti*- and *syn*-diastereomers was necessary. To identify the two respective diastereomers cyclic derivatives, acetonides **163** and **164** were prepared (Scheme 45). The separable acetonides **163** and **164** were prepared from a diastereomeric mixture of the *anti*- (*anti*-**158**) and *syn*-diols (*syn*-

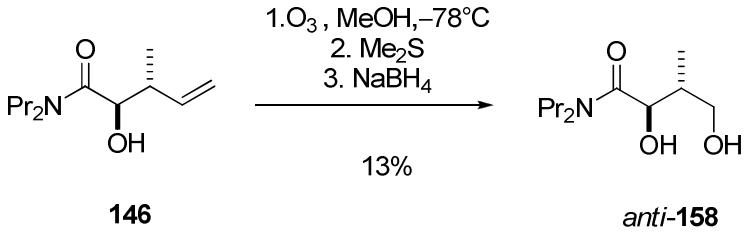
[††] The enantioselectivtiy of the organocatalysed reactions was not determined at this point in the project. This was despite attempts to establish the enantioselectivity of the organocatalysed reactions using chiral HPLC and chiral superfluid chromatography (SFC), good resolution was not achieved.

158). The configuration of the acetonides **163** and **164** was assigned on the basis of the magnitude of vicinal coupling constants. The *anti* diastereomer *anti*-**158** yielded the acetonide **163** with large coupling (*J* = 10.7 Hz, Figure 18, a) between H-4 and H-5 which is consistent with both protons being axial; the *syn* diastereomer *syn*-**158** yielded the acetonide **164** exhibited a smaller coupling between H-4 and H-5 (*J* = 3.0 Hz, Figure 18, b).



Scheme 45: The preparation of the acetonides **163** and **164** from aldol products *anti*- and *syn*-diols **158** in order to determine diastereoselectivity of the organocatalysed aldol reaction

The *anti*-diol, *anti*-**158**, was also independently prepared from the alkene **146** (prepared previously section 2.5.4) by ozonolysis and reduction using sodium borohydride. This experiment enabled an independent verification of the relative configuration of the *anti* diol **158** (Scheme 46).



Scheme 46

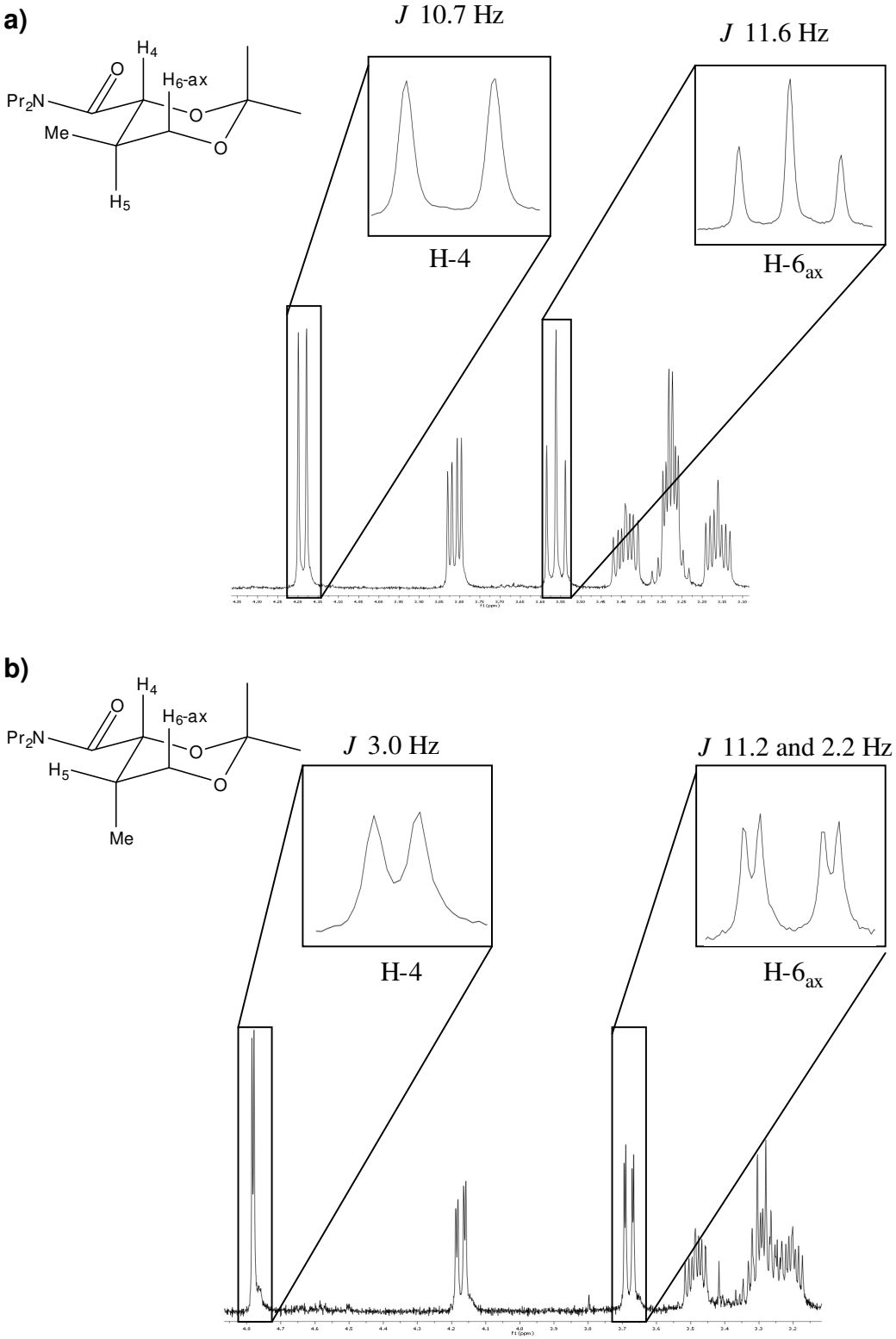


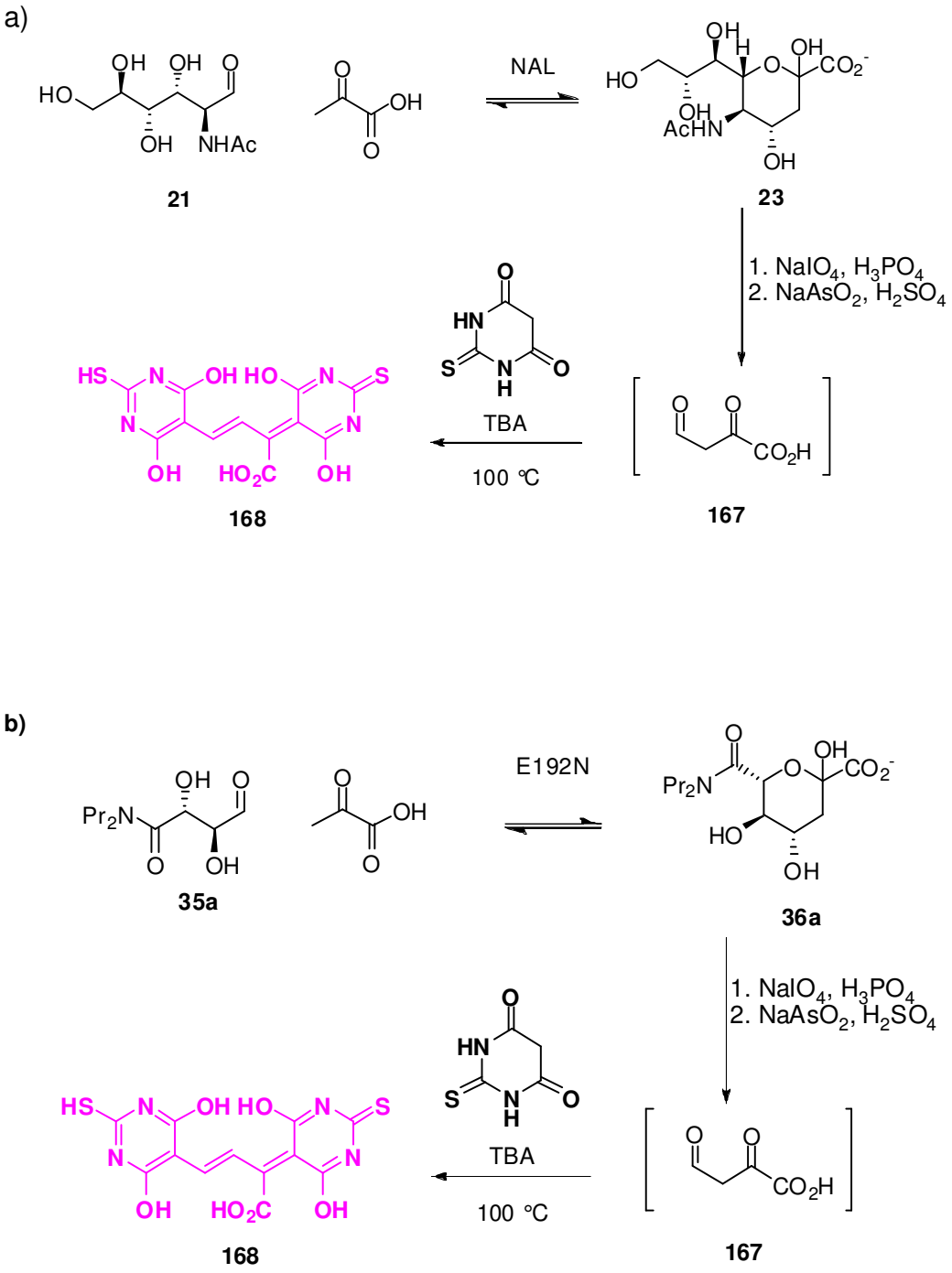
Figure 18 500 MHz ^{1}H NMR spectrum of the acetonides **163** and **164**. The signals due to the H-4 and H-6$_{ax}$ are expanded. a) 500 MHz ^{1}H NMR of the acetonide **163** , where both the H-4 and the H-6$_{ax}$ shows large couplings to H-5 , J = 10.7 Hz and J = 11.6 Hz respectively,; b) 500 MHz ^{1}H NMR of the acetonide **164**, where the H-4 shows coupling constant of J = 3.0 Hz, and H-6$_{ax}$ shows coupling constant of J = 2.2 Hz to the H-5.

3.2 Identification of Mutually Compatible Conditions for Organo- and Enzyme Catalysed Steps

With organocatalytic reaction conditions established, the next goal of the project was determining whether the conditions were compatible with the E192N aldolase variant. To do so, an enzyme assay was used to test whether various conditions affected the activity of the E192N aldolase variant.

3.2.1 Development of an Assay to Identify Mutually Compatible Conditions for Organo- and Enzyme-Catalysed Steps

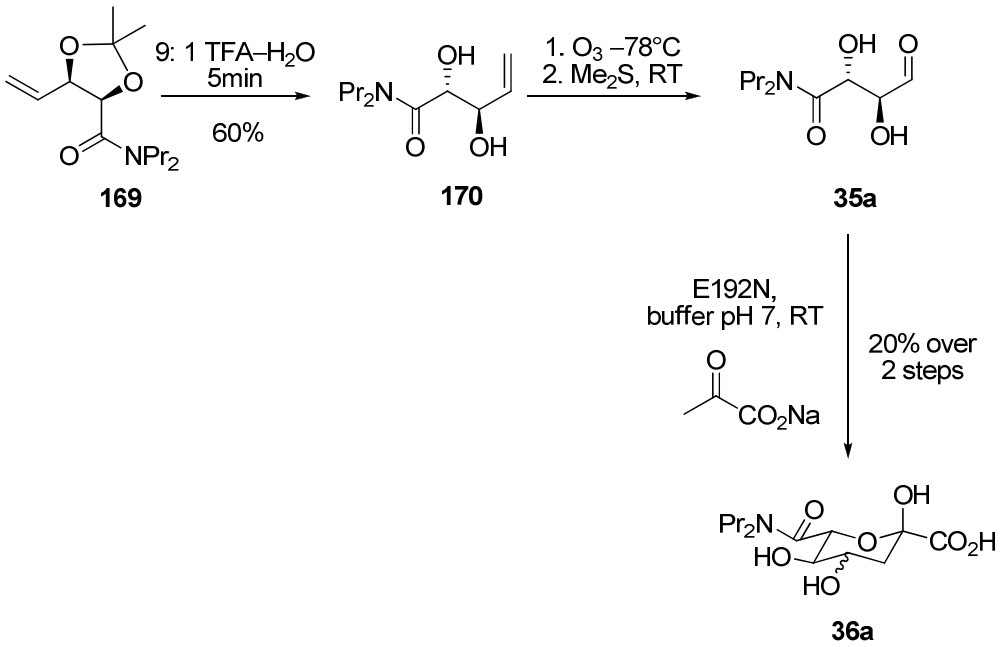
To investigate mutually compatible conditions for organo- and enzyme-catalysis, an enzyme assay was developed.[137, 138] First developed for *N*-acetyl neuraminic acid, the literature thiobarbituric acid (TBA) enzyme assay is a colourimetric assay which follows the enzyme-catalysed condensation of aldehyde **21** with pyruvate (Scheme 47). The assay tests for the production of the acid **23** by its conversion into the highly conjugated product **168**. We envisaged that this assay would be useful in our system in following the E192N-cataysed reaction which would instead involve the aldehyde **35a** being converted to the acid **36a**. The acid **36a** would also be converted to the acid **168** when exposed to TBA assay conditions.



Scheme 47: a) TBA Assay enzyme assay used to determine NAL activity. The assay converts the NAL product **23** into the pink coloured compound **168**. The compound **168** is a highly coloured compound and hence reactions which produce the enzyme product **23** can be easily detected by eye.[137-140] **b)** TBA Assay enzyme assay used to determine E192N activity.

3.2.1.1 Preparation of the Aldehyde 35a and the Acid 36a

To make use of the TBA assay, a known substrate for the E192N variant aldolase was prepared. The acid **36a** and corresponding aldehyde **35a** have proved to be good substrates for the E192N variant (Scheme 48). Both the aldehyde **35a** and the acid **36a** were prepared *via* an established synthetic route developed by Thomas Woodhall (Scheme 48). Thus deprotection of the acetonide [118, 119] **169**, kindly donated by Dr. Ieaun Davies, was performed under strongly acidic conditions and gave the alkene **170**. The alkene **170** was ozonolysed to give the aldehyde **35a**, which was used in the TBA assay without further purification. The concentrations of the aldehyde **35a**, used in the TBA assay, were based on the assumption that the ozonolysis was a quantative reaction. The acid **36a** was prepared by the E192N-catalysed aldol reaction between the aldehyde **35a** and sodium pyruvate, which, after ion-exchange, gave the acid **36a**.[‡‡] The acid **36a** was used as a standard in the TBA assay.

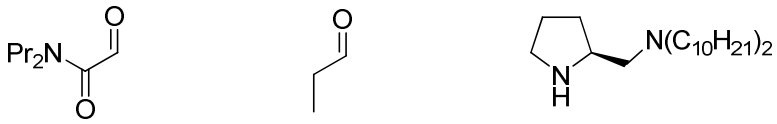


Scheme 48: Synthesis of the aldehyde **35a** and the acid **36a** from the acetonide **169** *via* acid-mediated acetonide deprotection, ozonolysis and E192N-catalysed aldol reaction.

[‡‡] Acid 36a exists as a mixture of pyraonose and furanose forms, the major product is the *S* diastereomer in one of its furanose forms.

3.2.1.2 Thiobarbituric Acid Assay Development

To test whether the organocatalytic conditions were compatible with the E192N aldolase variant, the E192N-catalysed aldol reaction of aldehyde **35a** was conducted in the presence of the components of the organocatalytic reaction. Specifically, we determined the effect of the glyoxylaldehyde **1a**, propionaldehyde and the diamine **69** at concentrations similar to those used in the organocatalysed reaction.



1a **69**

Figure 19: Compounds screened in E192N TBA assay

As expected, the literature protocol[137] converted the acid **35a** into the acid **36a** and hence generated a pink colour (Figure 20, a). However, the diamine **69** (37 mM, corresponding to 10 mol% in the organocatalysed reaction) and propionaldehyde (3.77 M corresponding to 10 equivalents in the organocatalysed reaction) interfered with the TBA assay leading to the formation of a precipitate upon exposure to the TBA assay conditions (Figure 20 b and c). The observation led us to introduce a chloroform extraction step to remove the diamine **69** and propionaldehyde before standard assay conditions (treatment with sodium periodate, sodium arsenite and thiobarbituric acid). The extraction approach worked well and no interference was observed in positive controls with propionaldehyde, the diamine **69**, the glyoxylaldehyde **1a** and sodium pyruvate.

a) b) c)

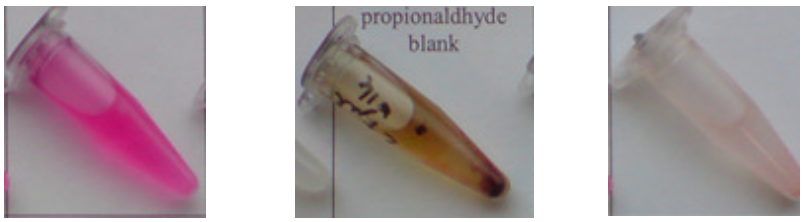


Figure 20: TBA assay controls. All of the photos show the result of standard assay conditions. All eppendorfs contained stated concentration of acid **36a**, propionaldehyde or the diamine catalyst **69** in 100 µL 100 mM, pH 7 potassium phosphate buffer; **a)** the photo of the result of the acid **36a** , and showing a positive result in the TBA assay containing 0.45 M acid **36a**; **b)** the photo of the result of a propionaldehyde control, brown precipitate formed when assay contained 3.77 M propionaldehyde; **c**) the photo of the result of the diamine **69** control, a pink precipitate appeared in the sample when assayed with 37 mM diamine **69**

To minimise pipetting errors, it was necessary to use robotic liquid handling techniques to pipette the E192N-catalysed reactions into 96-well plates. The liquid handling robot was used to pipette the diamine **69** and the appropriate volumes of the acid **36a** (2.27-0.45 mM), aldehyde **35a** (1.18 mM), buffer, the glyoxylaldehyde **1a** and sodium pyruvate into a glass-based 96 deep-well plate. Propionaldehyde and the E192N aldolase variant were pipetted manually. All reactions were performed in triplicate and the reactions were then left overnight at room temperature. The reactions were first extracted with chloroform and the aqueous layer treated sequentially with sodium periodate, an aqueous acidic sodium arsenite solution and a solution of TBA.

A range of concentrations of all the reaction components were tested, all of which were comparable to the conditions used in the original diamine-catalysed aldol reaction. Propionaldehyde was added at concentrations which corresponded to 1-10 equivalents; the concentrations of the diamine **69** screened were equivalent to 1-33 mol%; and the glyoxylaldehyde **1a** was assayed at concentrations which corresponded to 1-40 equivalents. The components were also assayed in combination to test whether there were any additional effects when components were combined (Figure 21 shows a typical assay plate).

The activity of the variant aldolase could be assessed by eye. Initially we aimed to measure the effect of various conditions on the E192N aldolase by using absorbance spectroscopy. This would allow quantification of the level of E192N activity under different conditions. However, upon assaying the enzyme reactions, turbidity occurred in a number of the samples and hence obtaining accurate absorbances was not achievable. Instead, the amount of E192N activity was assessed by eye, by comparison to a control reaction and a scale of enzyme activity established (see Appendix iv).

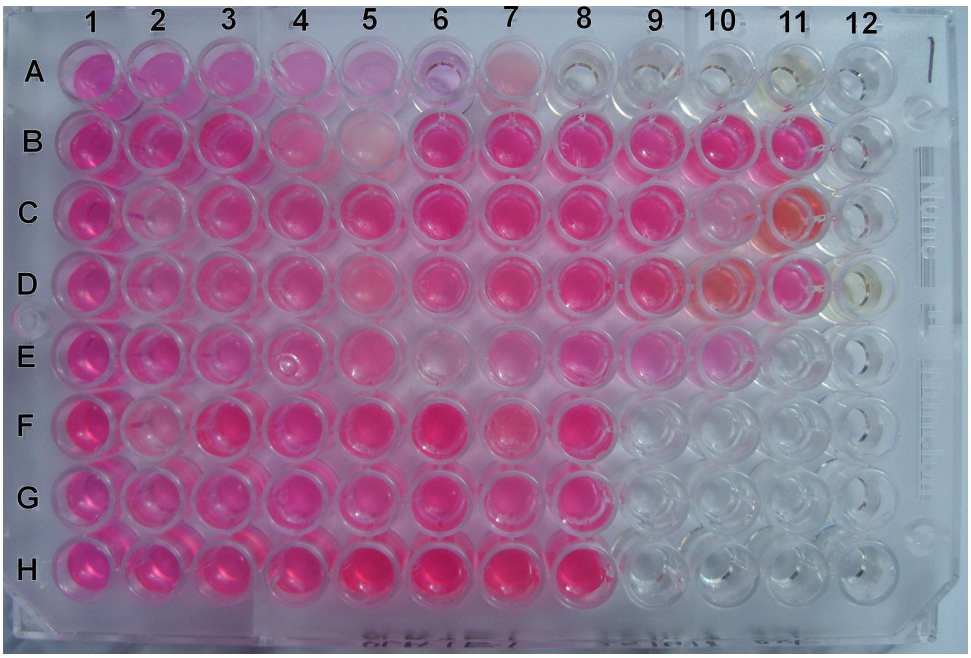


Figure 21 An example of thiobarbituric acid (TBA) assay plate. The pink colouring indicates enzyme activity under specified screening condition.

It was observed that at the concentrations screened, propionaldehyde had no effect on the catalytic activity of the E192N aldolase variant. This was in terms of the production of the acid **36a** (Figure 22a), as no decrease in colour intensity was seen across the various concentrations. The result was surprising as relatively high concentrations (4.35 mM- 4359 mM) appeared to be tolerated by the E192N aldolase.

The glyoxylaldehyde **1a** did not have any noticeable effect on the E129N aldolase variant either (Figure 22, b). The glyoxylaldehyde **1a** was the limiting reagent in the diamine-catalyst reaction, however high concentrations were tolerated by the variant aldolase.

The diamine **69** was the additive that had most effect on the E192N variant aldolase (Figure 22, c). The diamine **69** was tolerated by the E192N aldolase at low concentrations (corresponding to 1-6 mol% catalyst loading) but at medium to high concentrations (corresponding to 6-33 mol%[§§] catalyst loading), the E192N aldolase was affected and little or no product was formed.

It is interesting to note that some additional affects were observed when the enzyme reaction was exposed to the diamine **69**, in the presence of either propionaldehyde or the glyoxylaldehyde **1a** (Figure 22 c)[***]. When the components were combined, the E192N activity was higher than that observed at the same concentration of the diamine **69** alone. In all of the combined conditions assayed, the concentration of the aldehydes (propionaldehyde or glyoxylaldehyde **1a**) was always much higher than that of the diamine **69** present. The increase in E192N activity when there were two additives present can be perhaps be explained by the aldehyde's ability to sequester the diamine **69**, possibly forming an enamine species, and hence somewhat reduce the diamine's **69** effect on the E192N aldolase variant.

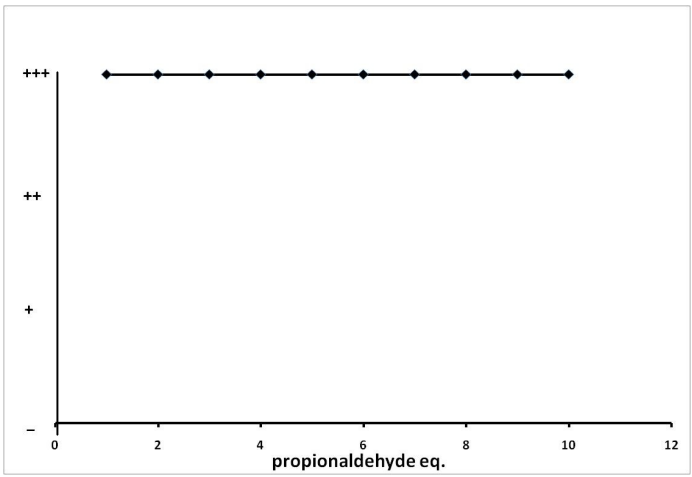
Although the E192N aldolase variant tolerated the appropriate concentrations of propionaldehyde and the glyoxylaldehyde **1a**, the required catalytic loading of the diamine **69** had a severe effect on the

[§§] Figure 22 c) shows 1-10 mol% only, no E192N activity was seen above 10 mol% at the catalyst loading studied (up to 33 mol%)

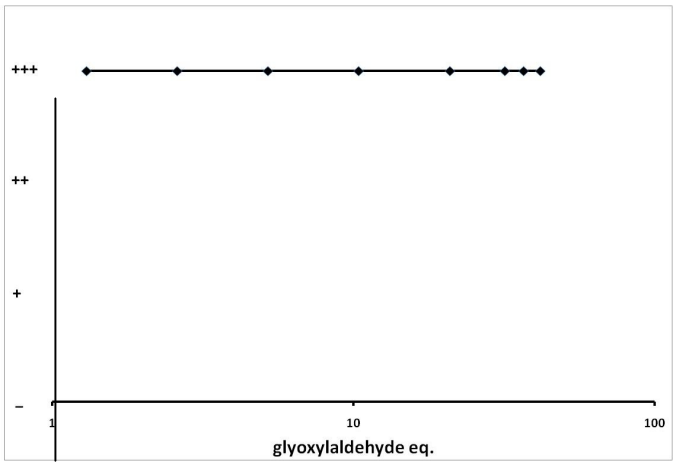
[***] Figure 22 c) shows E192N activity in presence of diamine and 5 eq. propionaldehyde, when the propionaldehyde concentration was varied in presence of the damine **69** the E192N activity was identical at equivalent diamine **69** loadings to that at 5 eq. propionaldehyde. This result was mirrored with the glyoxylaldehyde **1a** in the presence of the diamine **69**.

enzyme activity. However, lower levels of the diamine could be tolerated by the aldolase variant.

a)



b)



c)

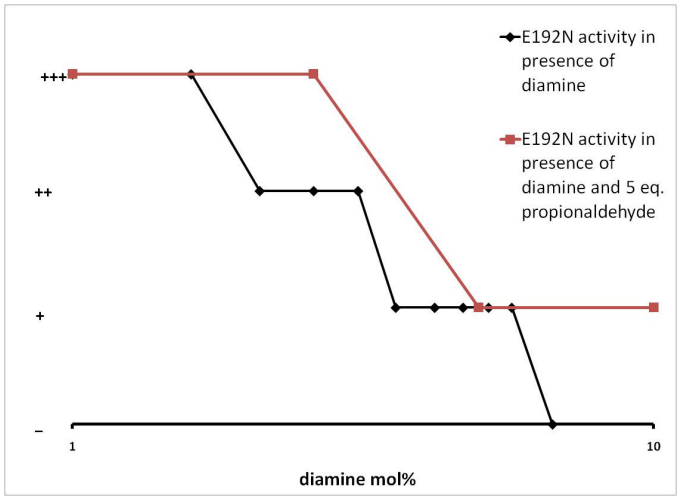
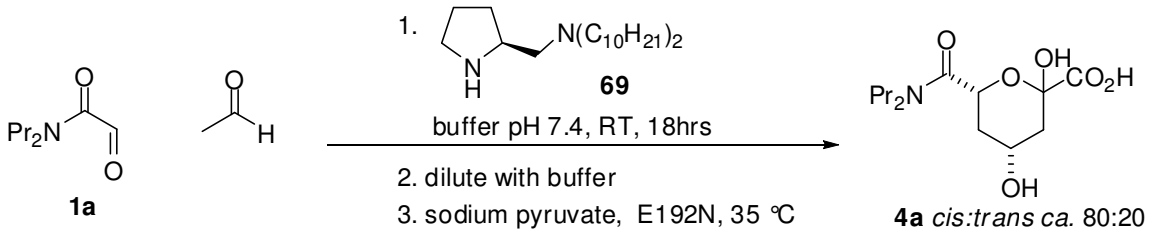


Figure 22 Effect on E192N activity of **a)** propionaldehyde, **b)** the glyoxylaldehyde **1a** and **c)** the diamine **69** and the diamine **69** with 5 eq. propionaldehyde; as determined by the TBA assay where +++, signifies very good E192N activity, equivalent intensity to E192N-catalysed reaction with no additives; ++, signifies good E192N activity; +, signifies little E192N activity; –, no E192N-catalysed reaction. Concentrations of components are all equivalent concentration to the optimised organocatalysed conditions.

3.3 Development of an Organo- and Enzyme-Catalysed One-Pot Reaction

The results from the TBA assay suggested that only low concentrations of the diamine **69** would be tolerated by the E192N variant aldolase. When combining both organo- and enzyme-catalytic steps, the issue of diamine concentration would, therefore, have to be addressed. A simple solution would be to use a dilution approach. The approach would entail performing the diamine-catalysed aldol reaction before diluting the reaction with an appropriate volume of buffer to give a concentration of the diamine **69** that would be tolerated by the E192N aldolase variant. The enzyme and sodium pyruvate would then be added to allow the enzyme reaction to proceed.

The dilution approach was used in several screening reactions and the products were isolated by preparative HPLC (Scheme 49 and Table 12). All other purification methods attempted, including column chromatography and ion exchange chromatography, did not yield the acid **4a** cleanly.



Scheme 49: One-pot reactions with dilution-step (see Table 12). Only the major diastereomer is depicted.

Entry[a]	Diamine 69 (mol%)	Acetaldehyde eq.	Dilution factor[b]	Isolated yield 4a(%)[c,d]	4a *E.r.*[e]
1	10	10	1.7	39	50:50
2	10	10	3.23	36	26:74
3	10	10	5.7	17	36:64
4	10	10	11.1	35	28:72
5	5	10	1.7	40	52:48
6	20	10	1.7	31	52:48
7	50	10	1.7	30	52:48
8	10	5	1.7	37	51:49
9	10	20	1.7	19	16:84
10	10	50	1.7	33	49:51

Table 12: One-pot reactions. [a]All reactions were performed on a 0.0955 mmol scale of the glyoxylaldehyde **1a**, in 20 mM, pH 7.4 potassium phosphate buffer (0.307 mL), with appropriate amount of the diamine **69** and acetaldehyde at RT. After 18 hrs reactions were diluted with buffer (0.115 mL) and E192N (6.98 mg/mL in pH 7.4, 20 mM potassium phosphate buffer) and sodium pyruvate (0.955 mmol) added and reaction incubated at 35 °C for 3 days; [b] Relative to the volume of buffer in reaction at dilution; [c] All products isolated by preparative HPLC; [d]*Cis*:*trans* ca. 80:20 and was determined using 500 MHz ^{1}H NMR spectroscopy ; [e] Enantiomeric ratio, determined by conversion of the acid **4** into the lactone **171** (section 3.3.1.2).

It appeared that the dilution approach was successful, with all reaction conditions screened giving the acid **4a** in isolated yields between 17-40% (Scheme 49 and Table12). The volume of buffer used to dilute the one-pot reactions was varied[†††] along with the diamine catalyst loading and acetaldehyde equivalences. The volume of buffer used to dilute the reactions (dilution factor) was important in governing reaction yield (Table 12, entries 1-4) with the optimum dilution appeared to be a 1.7-fold dilution. The diamine **69** loading also affected the isolated yield of the one-pot reactions (Table 12, entries 1 and 5-7,) with interesting inverse relationship between yield and loading. The observation fits with the findings from the TBA assay with the optimum catalyst loading screened being 5 mol% of the diamine **69**. The isolated yield also varies with the amount of acetaldehyde (Table 12, entries 1 and 8-9) where the optimum appeared to be 10 equivalents.

3.3.1 Stereochemical Outcome of the One-Pot Reactions

Establishing the stereochemical outcome of the one-pot reaction was not trivial as two stereocentres as well as an anomeric centre were formed in the reaction.

3.3.1.1 Diastereoselectivity of the One-Pot Reactions

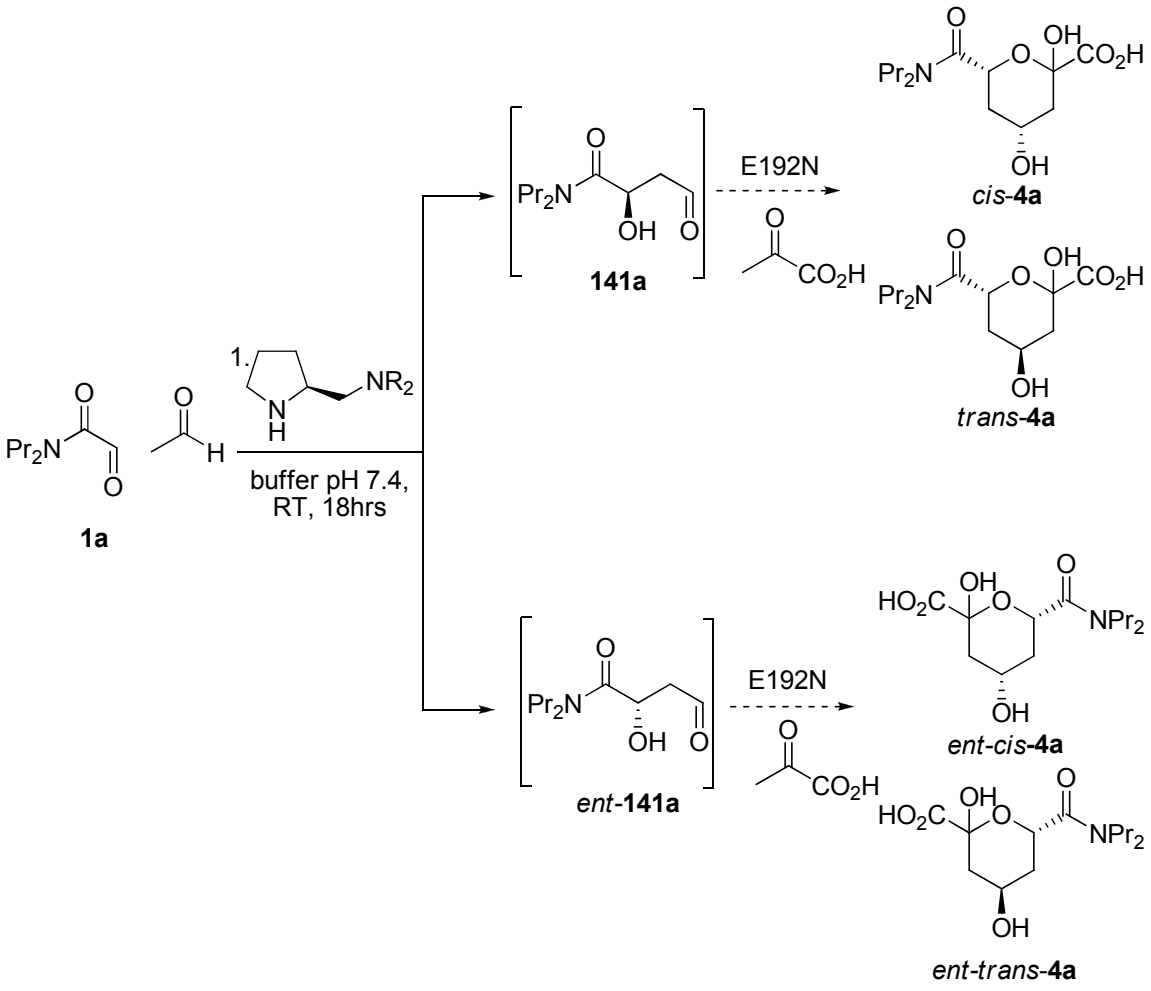
The diastereoselectivity observed for the isolated acid **4a** was determined as described earlier (section 2.5.3). The diastereoselectivity was similar to that observed in the E192N-catalysed aldol reactions of **123** (section 2.5.2) and is likely to be under thermodynamic control.

[†††] The concentration of E192N was kept constant across all reactions.

3.3.1.2 Determination of the Enantioselectivity of the One-Pot Reactions

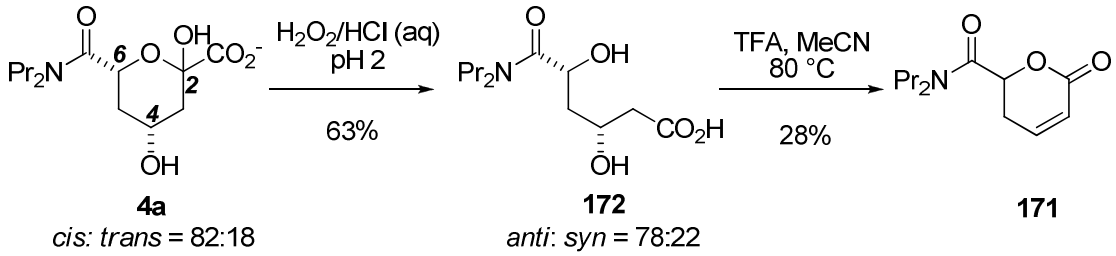
An essential part of the project was the determination of the enantioselectivity of the one-pot reaction. In the one-pot reaction the initial organocatalysed reaction yields the aldol intermediate **141a** as one or a mixture of enantiomers, both of which may be accepted by the E192N aldolase variant to give the acid **4a** and its enantiomer *ent*-**4a**, as a mixtures of diastereomers at the C-4 position (Scheme 50).

It is a fair assessment to assume that the enantioselectivity of the initial organocatalysed aldol reaction, and hence the enantiomeric excess of the intermediate aldehyde **141a**, is controlled by the chiral diamine **69**. However, one-pot reaction conditions which produce the acid **4a** with enantiomeric excess may result from a number of possibilities. The organocatalysed reaction may be stereoselective and produce the aldehyde **141a** in high enantiomeric excess and the E192N-catalysed aldol reaction therefore gives the acid **4a** in high enantiomeric excess. Alternatively, the organocatalysed reaction may not be stereoselective and hence, produce a mixture of the aldehyde **141a** and its enantiomer ent-**141a**. In this case the aldolase may be selective for one of the enantiomers of the aldehyde **141a**, allowing kinetic resolution to occur yielding an enantiomerically enriched acid **4a**. No knowledge existed on whether the E192N aldolase variant could differentiate between enantiomers of enzyme substrates, as all previous studies were performed using single enantiomers of screening compounds.[46, 47, 52, 118, 119]



Scheme 50: Possible substrate-specificity of the E192N aldolase variant in the one-pot reaction.

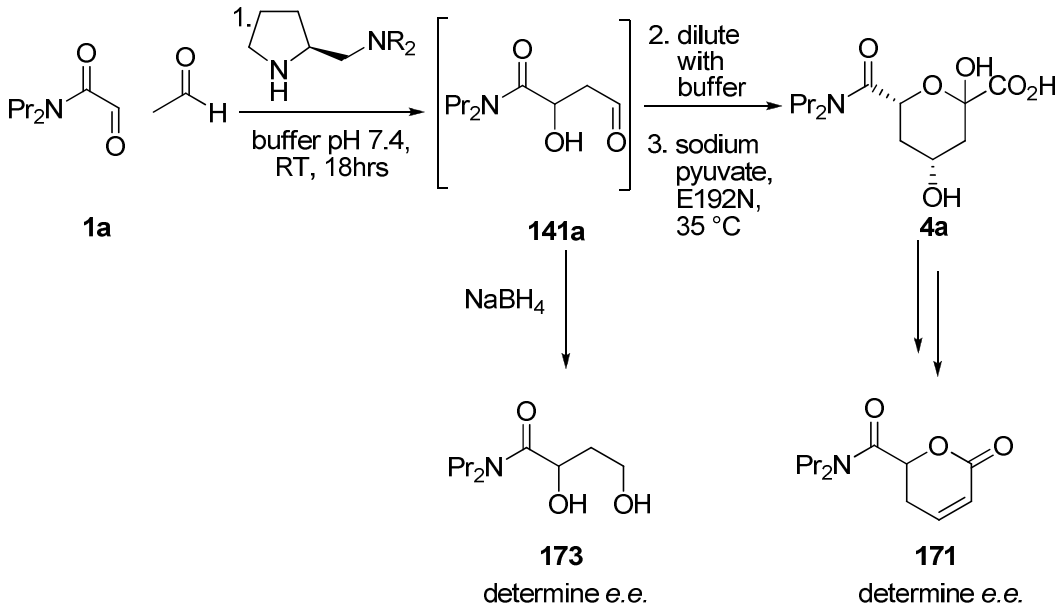
The enantiomeric excess of the acid **4a**, prepared in the one-pot reaction, was determined by conversion into a derivative. The derivative prepared was lactone **171** (Scheme 51), which was synthesised from the acid **4a**. Firstly, the acid **4a** was exposed to hydrogen peroxide under aqueous acidic conditions, which yielded the diol **172**. The diol **172** was treated with trifluoroacetic acid in acetonitrile, which, upon heating, cyclised and dehydrated to give the lactone **171**. A chiral HPLC method was developed for the lactone **171** (Appendix i).



Scheme 51: Preparation of the lactone **171** from the acid **4a**

Upon conversion to the lactone **171**, some of the products **4a** from the one-pot reactions showed low enantiomeric excesses however, most of the reactions were racemic (Scheme 49 and Table 12). Given that the higher enantioselectivities were observed for lower yielding reactions, it was possible that some kinetic resolution of the aldehyde **141a** and *ent*-**141a** was occurring under these reaction conditions.

To establish whether kinetic resolution had occurred under some one-pot reaction conditions, the enantiomeric excess of the aldol intermediate **141a** was determined. If the intermediate aldehyde **141a** was racemic then the enzyme must have processed one enantiomer of the aldehyde **141a** faster than the other. The enantiomeric excess of the intermediate aldehyde **141a** was determined under the same reaction conditions which yielded the acid **4a** with enantiomeric excess (Scheme 52). The enantiomeric excess of the aldol intermediate **141a** was determined by reduction using sodium borohydride to give the diol **173**. Upon analysis, using chiral normal phase chromatography (Appendix ii), the diol **173** was found to be racemic (Table 13 and Scheme 52), whereas the corresponding acid **4a**, prepared under the same one-pot conditions had low enantiomeric excess. This was disappointing as we had believed that the use of the chiral diamine **69** would have led to enantiocontrol in the organocatalysed reaction. However, it was concluded that the E192N aldolase variant must be performing a kinetic resolution under certain one-pot conditions. Under these conditions, the enzyme-catalysed reactions had simply not reached completion and however under most one-pot conditions, in which the acid **4a** was racemic the reactions must have proceeded to completion.



Scheme 52: Strategy used to determine the whether enantiomers of the aldol intermediate **141a** were kinetically resolved under one-pot reaction conditions

Reaction Conditions	***e.r.* of diol 173**	***e.r.* of lactone 171**
10 mol% diamine **69**, 10 eq. acetaldehyde dilution factor 3.23	47:53	26:74

Table 13: Enantiomeric excesses determined for the diol **173** and the lactone **171** in order to establish the source of enantioselectivity observed under some one-pot reaction conditions

3.3.2 Determination of the Substrate Scope of the One-Pot Reaction

With the conditions in place for the one-pot reaction, the scope and limitations of the process were investigated. Six glyoxylaldehydes **1** (Figure 23) were selected as potential substrates for the one-pot reaction and prepared prior to screening.

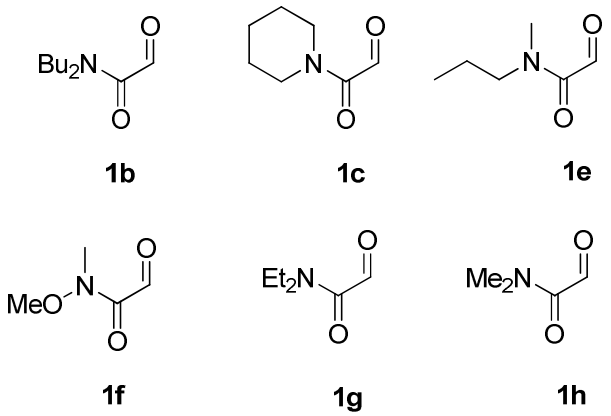
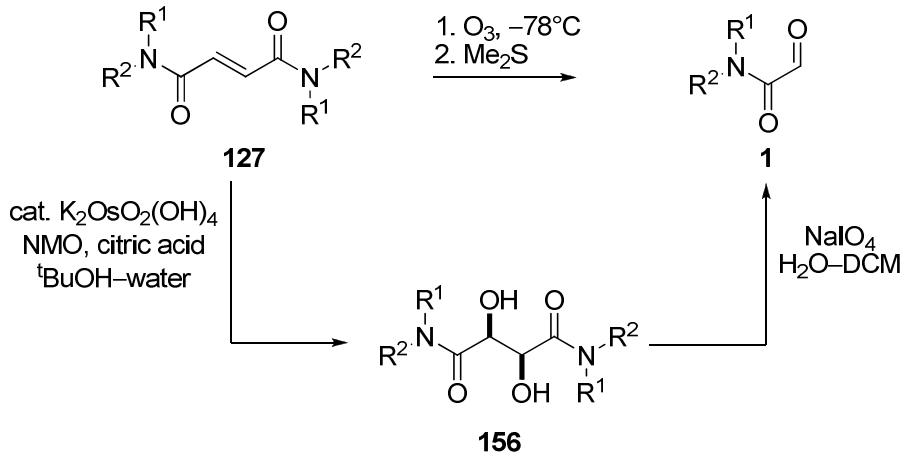


Figure 23: Aldehydes selected as potential substrates in the one-pot reaction

3.3.2.1 Preparation of Glyoxylaldehydes 1

The glyoxylaldehydes **1** were prepared from the amides **127** by ozonolysis or dihydroxylation and periodic cleavage (Scheme 53). Generally, ozonolysis was a higher yielding route, with all yields between 62-79%.



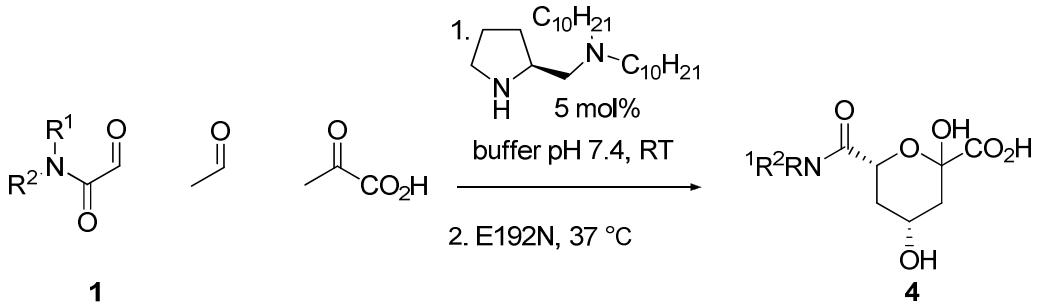
Scheme 53: Preparation of glyoxylaldehyde **1** from amides **127** by ozonolysis of two step dihydroxylation and oxidative cleavage (see Table 14)

Entry	Amide	R^1	R^2	Ozonolysis Yield (%)	Diol	156 Yield (%)	Periodic Cleavage Yield (%)	Glyoxyl-aldehyde
1	**127a**	Pr	Pr	-	**156a**	91	93	**1a**
2	**127b**	Bu	Bu	78	**-**	-	-	**1b**
3	**127c**	$-(CH_2)_5-$		69	**-**	-	-	**1c**
4	**127e**	Me	Pr	79	**-**	-	-	**1e**
5	**127f**	Me	OMe	a	**-**	-	-	**1f**
6	**127g**	Et	Et	62	**-**	-	-	**1g**
7	**127h**	Me	Me	76	**156h**	10	26	**1h**

Table 14: Isolated yields of the ozonolysis of amides **127**, dihydroxylation of amides **127** and periodic cleavage of diols **156** (see Scheme 53). [a] The aldehyde **1f** was used without purification in following reaction.

3.3.2.2 Determination the Substrate Scope of the One-Pot Reaction with Glyoxylaldehydes 1

The glyoxylaldehydes **1** were exposed to the developed one-pot reaction conditions (5 mol% diamine **69**, 10 eq. acetaldehyde and dilution factor of 1.7). Five of the glyoxylaldehydes **1** were good substrates for the one-pot reaction with acetaldehyde, and the acids **4** were obtained in yields between 40-51% (Scheme 54 and Table 15). The result concurs with previous results since the aldol intermediates **141** had previously been shown to be substrates for the E192N aldolase variant (section 2.5.2). The dibutylamide **1b** and the Weinreb amide **1f** were not substrates for the one-pot reaction. This observation is surprising because the full-length dibutylamide **135b** is a substrate for the E192N aldolase variant (section 2.5.2). The lack of reactivity of the dibutylamide **1b** is most likely due to its poor solubility under the aqueous conditions of the one-pot reaction. The failure of the Weinreb amide **1f** to react, however, fits with previous findings that the aldol intermediate **141f** was not a substrate for the E192N aldolase variant.



1 **4**

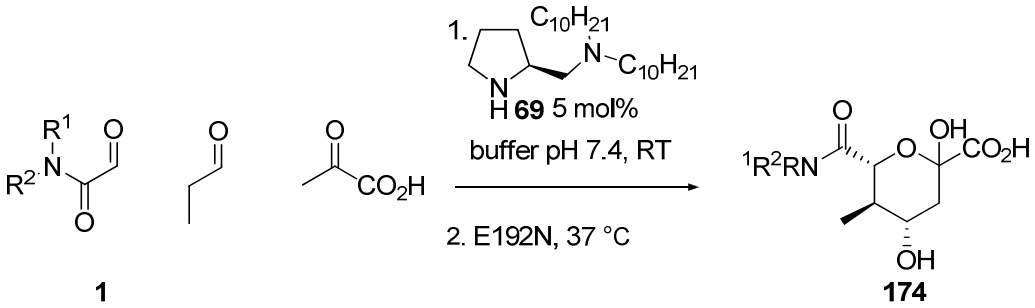
Scheme 54 One-pot reactions with substrates **1** and acetaldehyde.

Entry	Glyoxylaldehyde	R^1	R^2	Product	Yield 4 (%)[a]	*Cis* :*trans* [b]
1	**1a**	Pr	Pr	**4a**	40	78:22
2	**1b**	Bu	Bu	**4b**	No product	-
3	**1c**	$-(CH_2)_5-$		**4c**	48	66:34
4	**1e**	Me	Pr	**4e**	48	57:43
5	**1f**	Me	OMe	**4f**	No product	-
6	**1g**	Me	Me	**4g**	43	71:29
7	**1h**	Et	Et	**4h**	51	61:39

Table 15 One-pot reactions with glyoxylaldehydes **1** with acetaldehyde. [a]All products were isolated by preparative HPLC [b] Diastereoselectivities of isolated products were determined by 500 MHz ^{1}H spectroscopy

The diastereoselectivity of the reactions with the glyoxylaldehydes **1** (Scheme 54 and Table 15) was comparable to that observed with the dipropylglyoxylaldehyde **1a** (section 2.5.3). The diastereoselectivity was established by 500 MHz ^{1}H NMR spectroscopy. When the diagnostic H-6 and H-4 ^{1}H signals of the acids **4** were compared, the chemical shifts and coupling constants were consistent across the series of compounds. The similarities between the 500 MHz ^{1}H spectra of the acids **4** allowed the identification of diastereomers and anomers across the series (Appendix iii)

The donor aldehyde can also be varied. Propionaldehyde was a good substrate in the one-pot reaction with the range of acceptor glyoxylaldehydes 1 (Scheme 55 and Table 16).



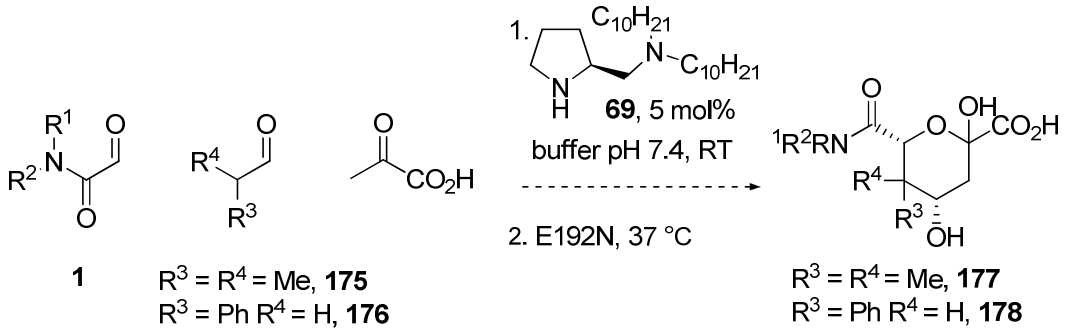
1 **174**

Scheme 55: One-pot reactions with substrates **1** and propionaldehyde. The major product **174** is depicted only

Entry	Glyoxylaldehyde	R^1	R^2	Product	Yield 4 (%)[a]	Diastereoselectivity *Maj*:*minor* [b,c]
1	**1a**	Pr	Pr	**174a**	70	55:45
2	**1b**	Bu	Bu	**174b**	No product	-
4	**1e**	Me	Pr	**174e**	48	56:44
5	**1f**	Me	OMe	**174f**	No product	-
3	**1g**	Et	Et	**174g**	69	74:26

Table 16: One-pot reactions with glyoxylaldehydes **1** with propionaldehyde. [a]all products isolated by preparative HPLC [b] ratio between the major diastereomer (min), as shown in Scheme 55, and all other diastereomers (min); [c] diastereoselectivities of isolated products determined by analysis by 500 MHz ^{1}H NMR spectroscopy

The diastereoselectivity of the one-pot reactions involving propionaldehyde was established by analysis of the isolated products by 500 MHz ^{1}H NMR spectroscopy. The identity of the major product **174** was achieved previously (Chapter 2, section 2.5.4). The diastereoselectivities are quoted as a ratio between the major product **174** (as shown in Scheme 55) and all other diastereomers, and is comparable across the series of compounds. The stereoselectivity of the one-pot reaction is likely to be controlled in the same manner as the one-pot reactions are with acetaldehyde. The organocatalysed reaction is likely to generate a racemic aldol intermediate which is accepted by the E192N aldolase variant. The enzyme-catalysed reaction is likely to be under thermodynamic control with the relative energies of the enzyme products controlling the proportion of products prepared.



Scheme 56: One-pot reactions with *iso*-butyric aldehyde **175** and phenylacetaldehyde **176**

When the donor aldehydes *iso*-butyric aldehyde (**175**) and phenylacetaldehyde (**176**) (Scheme 56) were exposed to the glyoxylaldehydes **1a-h** under the optimised reaction conditions, none of the acid products, **177** and **178,** were observed. The only product observed in these reactions was the intermediate aldol product. The lack of conversion to the E192N product suggests that the aldol intermediates are poor substrates for the E192N aldolase variant.

The absence of E192N activity was unexpected and did not fit with our original model in which, the nature of the substituent in the C-5 position of the enzyme substrate was predicted to have little effect. Further structural studies are underway within the group which may allow a more refined understanding of the enzyme variant's substrate specificity.

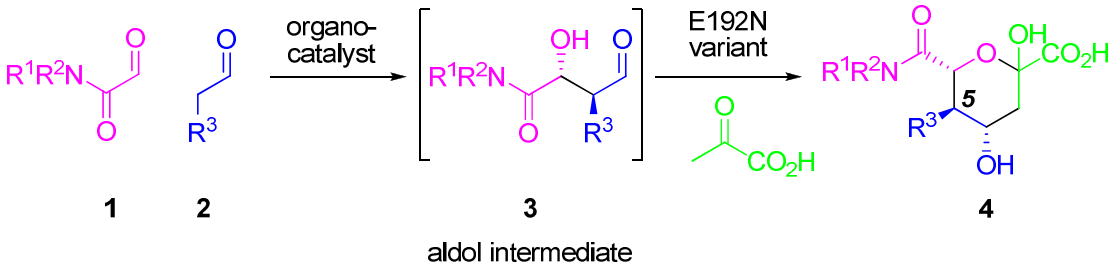
3.4 Chapter 3 Summary

In this chapter, the identity of common conditions under which both organo- and enzyme-catalysed steps could occur was discussed. To start with, the organocatalysed step was investigated. Thereafter, it was ascertained whether the optimised conditions for the organocatalysed step were compatible with the E192N aldolase variant using an enzyme assay. The results from the assay allowed the development of one-pot conditions which allowed both catalytic steps to occur in a one-pot, sequential manner. The reaction conditions were optimised and the scope of the process was investigated

Chapter 4

Summary

A novel, one-pot sequential three-component reaction was developed which combined organo- and enzyme-catalysis in an aldol/aldol cascade for the first time. The feasibility of the approach was first assessed by investigating whether the aldol intermediates **3** were substrates for the E192N aldolase variant. Analysis of the crystal structure of the wild-type NAL enzyme suggested that the aldol intermediates would be substrates for the enzyme due to the solvent exposed nature of the substituents in the C-5 position of the substrates. This theory was tested by the preparation and evaluation of the potential aldolase products **135**. It was concluded that the nature of the substituent at C-5 had little effect on the specificity constants with the E192N aldolase variant, and, crucially the aldol intermediates **3** were enzyme substrates. It was shown that the corresponding aldol condensations were thermodynamically favourable and hence, synthetically useful.



Scheme 57

Once the aldol intermediates were established as substrates for the aldolase, the one-pot reaction was developed. Three organocatalysts were prepared and together with L-proline and L-alanine, were investigated in the crossed aldol reaction between the glyoxylaldehyde **1a** and propionaldehyde. The diamine **69** catalysed the aldol reaction in buffer at a suitable pH for the aldolase; this catalyst was, therefore, selected as the most appropriate catalyst for the one-pot reaction. An assay was developed and used to assess the compatibility of the

diamine **69**, propionaldehyde and the glyoxylaldehyde **1a** with the E192N aldolase variant. The diamine **69** was found to have a substantial detrimental effect on the activity of the enzyme and only low concentrations could be tolerated. Accordingly, it was decided to conduct a one-pot sequential process: after the organocatalysed step, the reaction was diluted and the enzyme along with pyruvate were added; this allowed the acids **4** to be obtained. Despite the use of the chiral diamine **69** in the organocatalytic step, the one-pot reaction was unfortunately not enantioselective when the one-pot reaction was run to completion. This was found to be due to the lack of enantioselectivity in the organocatalysed step. Some kinetic resolution was observed between aldol intermediates **141a** under some one-pot conditions, however this was at the expense of yield. To circumvent this problem of enantioselectivity, further screening of organocatalysts, (including perhaps polymer supported catalysts) would possibly yield an enantioselective one-pot reaction. This approach would possibly avoid incompatibility with the enzyme catalyst and may allow the reaction be performed non-sequentially. The scope of the one-pot reaction was determined using a range of aldehyde acceptors (glyoxylaldehydes **1**) and aldehyde donors (acetaldehyde and propionaldehyde) demonstrating the degree of diversity that can be generated using these systems.

In summary, a novel aldol-aldol sequential cascade reaction, with broad scope, has been developed. The reaction generates molecular complexity rapidly and hence may find synthetic applications such as natural product synthesis or in diversity oriented chemistry.

Chapter 5

Experimental

General Experimental

All non-aqueous reactions were carried out under an atmosphere of nitrogen. Water-sensitive reactions were performed in oven- or flame-dried glassware cooled under nitrogen before use. Solvents were removed under reduced pressure using either a Büchi rotary evaporator and a Vacuubrand PC2001 Vario diaphragm pump, or a Genevac HT-4 evaporation system. Solvents were distilled before use when necessary.

Solvents

Tetrahydrofuran was freshly distilled from sodium, using benzophenone as a self-indicator.
Dichloromethane, and toluene were freshly distilled from calcium hydride.
Anhydrous *N,N*-dimethylformamide was obtained from Sigma–Aldrich. Ether refers to diethyl ether and petrol refers to petroleum spirit (b.p. 40-60 °C) unless otherwise stated.

Reagents

Commercially available starting materials were obtained from Sigma–Aldrich, Fluka, Lancaster, Alfa Aesar, or Novabiochem

Flash Chromatography

Flash column chromatography was carried out using silica (35-70 μm particles). Thin layer chromatography was carried out on commercially available pre-coated glass or aluminium plates (Merck silica 2 8 8 0 Kieselgel 60F254).

Ozonolysis

Ozone was generated using a Welsbach generator (0.4-0.6 psi), and the reaction mixture was purged with oxygen gas (0.4-0.6 psi) for a minimum of 15 minutes before and after ozonolysis.

LCMS

Analytical LC-MS was performed using a Waters X-Terra chiral column (MS C18, 5 µm, 50 × 4.6 mm) with a Waters 2525 pump, Waters 2996 photodiode array detector and a Waters Micromass ZQ mass spectrometer as the detector. or

Analytical LC-MS was performed using an Agilent 1200 series LC system comprising of a Bruker HCT Ultra ion trap mass spectrometer, a high vacuum degasser, a binary pump, a high performance autosampler and micro well plate autosampler, an autosampler thermostat, a thermostated column compartment and a diode array detector. The system used two solvent systems: MeCN/H_2O + 0.1% formic acid with a Phenomenex Luna C18 50 × 2mm 5 micron column or MeCN/H_2O with a Phenomenex Luna C18 50 × 2mm 5 micron column.

Analytical HPLC and normal phase chromatography

Analytical HPLC was performed using a Daicel Chemical Industries LTD chiral column AS-RH Chiralpak, 5 µm, 150 × 4.6 mm or a Daicel Chemical Industries LTD chiral column AD-RH Chiralpak and 5 µm, 150 × 4.6 mm eluting with Linear gradients of 0.1% TFA in MeCN/ H_2O. Normal phase liquid chromatography was performed using a Daicel Chemical Industries LTD chiral column AD-H Chiralpak, 5 µm, 250 × 4.6 mm chiral column with isocratic 5% IPA in hexane. Both were run with a Dionex Analytical HPLC system and diode array detector

Prep HPLC

Preparative HPLC was performed using a Phenomenex Hyperclone column (ODS (C18), 120 A, 5 µm, 250 × 21.2 mm) with a Gilson 333/334 Binary pump, a Gilson 402 syringe pump, a Gilson UV/Vis- 151 single wavelength detector and a Gilson FC 204 fraction collector.

Semi-prep HPLC

Semi preparative HPLC was performed using a Gilson semi-prep HPLC system comprising of 1 × Gilson 215 liquid handler; 1× 811c dynamic mixer; 1 × 806 manometric module; 2 × 306 pumps with 25cc pump heads; 1 × 119 UV/VIS detector. Linear gradients of 0.1% TFA in MeCN/ H_2O and Zorbax-SB-C18 21.2 × 100mm 5uM column.

Semi-prep mass-directed HPLC

Semi-preparative HPLC was performed using a Phenomenex Hyperclone column (ODS (C18), 120 A, 5 µm, 250 × 10 mm) with a Dionex P580 Binary pump, a STH 585 Column oven, a Gynkotek sampler, a Gynktotek UVD340U Detector Dionex semi-preparative HPLC system or a Gilson preparative HPLC system .

NMR

Proton and carbon NMR spectra were recorded on a Bruker Advance DPX 300, Advance 500 or DRX500 spectrophotometer using an internal deuterium lock. Carbon NMR spectra were recorded with composite pulse decoupling using the waltz 16 pulse sequence. DEPT, COSY, HMQC and HMBC pulse sequences were routinely used to aid the assignment of spectra. Chemical shifts are quoted in parts per million downfield of tetramethylsilane, and coupling constants (*J*) are given in Hz. NMR spectra were recorded at 300 K unless otherwise stated.

Melting point

Melting points were determined on a Reichert hot stage microscope and are uncorrected.

IR

Infrared spectra were recorded on a Perkin Elmer spectrum One FT-IR spectrophotometer.

Mass Spectrometry

Nominal mass spectrometry was routinely performed on a Waters-Micromass ZMD spectrometer using electrospray (+) ionization. Isotopic distributions were as expected. Nominal and accurate mass spectrometry using electrospray ionisation was carried out by staff in the School of Chemistry at the University of Leeds, using either a Micromass LCT-KA111 or Bruker MicroTOF mass spectrometer and reserpine as the lock mass and sodium iodide as the standard.. Field Desorption Ionisation mass spectra were acquired on a Waters-Micromass GCT premier spectrometer equipped with a Linden LIFDI probe. All mass spectra quoted to four decimal places are high resolution spectra

Microanalysis

Microanalyses were carried out by staff in the School of Chemistry at the University of Leeds using a Carlo Erba 1108 automatic analyser.

$[\alpha]_D$

Optical activity measurements were recorded at room temperature on an AA-1000 polarimeter; units for $[\alpha]_D$ are 10^{-1} deg cm^2 g^{-1} and are omitted.

Fractional hydrogens in mixtures

For compounds that exist as inseparable mixtures or as mixtures of species in slow exchange (eg anomers, hydrates and rotamers), fractional protons are assigned to ^1H NMR signals. The fractional protons reflect the proportion of the species in the mixture: for example, "5.16 (0.04H, d, *J* 10.3, 6-$H_{trans(maj)}$)" indicates that the major anomer of the *trans* diastereomer forms 4% of the total mixture of species present. The total number of hydrogens in all species sum to 1H for each specified proton signal.

General Procedures

General Procedure A – Aldol reactions

The organocatalyst was added to the glyoxylaldehyde **1a** and propionaldehyde in the given solvent and reaction stirred at the given temperature until judged complete, $NaBH_4$ was added and stirred at the given temperature for the given time. Reaction was quenched with aqueous 2M NH_4Cl solution and extracted with EtOAc. Column chromatography yielded the diols **158**.

General Procedure B – Aldol reactions with tetrazole 67

Propionaldehyde was added to a mixture of the glyoxylaldehyde **1a** and tetrazole catalyst **67** in the given solvent and reaction stirred at the given temperature until judged complete, $NaBH_4$ was added and stirred at the given temperature until judged complete. Reaction was quenched with a 2M aqueous NH_4Cl solution and extracted with EtOAc. Column chromatography yielded the diols **158**.

General Procedure C – Aldol reactions with diamine catalyst 69

Additive was added to a mixture of diamine **69** and stated solvent and stirred for 5 min in a sealed system under air. This mixture was then added to the glyoxylaldehyde **1a** followed by propionaldehyde and reaction stirred for the stated time in a sealed vessel under air atmosphere at room temperature until judged complete. $NaBH_4$ was added reaction stirred until judged complete. Reaction was quenched with aqueous NH_4Cl solution and extracted with EtOAc. Column chromatography yielded the diols **158**.

General Procedure D – Aldol reactions with diamine catalyst 69 in buffer

Glyoxylaldehyde **1a**, the diamine **69** and stated solvent and stirred for 5 min propionaldehyde was added and reaction stirred for the stated time at room temperature until judged complete . $NaBH_4$ was added, reaction stirred until

judged complete. Reaction was quenched with a 2M aqueous NH_4Cl solution and extracted with EtOAc. Column chromatography yielded the diols **158**.

General Procedure E; Dihydroxylation with Potassium Osmate

4-methyl-morpholine-*N*-oxide in water was added to a suspension of diamide**,** citric acid and $K_2OsO_2(OH)_4$ in 1:1 ^{t}BuOH–water which upon addition turned from green to yellow. The reaction was stirred at the given temperature for the given time, and ^{t}BuOH removed by rotary evaporator. The mixture was diluted with 1 M aqueous HCl solution and extracted with EtOAc, dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product as a colourless oil.

General Procedure F; Ozonolysis

Oxygen (15 min, 0.5 psi), ozone (0.5 psi, until reaction mixture turned blue), and oxygen (15 min, 0.5 psi), were bubbled sequentially through a stirred solution of alkene in the given solvent at −78 °C. Dimethyl sulfide was added, and the mixture was allowed to warm to room temperature, stirred until reaction was quenched, as established by testing with starch-iodide paper, and evaporated under reduced pressure to give the aldehyde.

General Procedure G; Allylation with allytrimethylsilane

The aldehyde was dissolved in DCM and $TiCl_4$ added at 0 °C. The reaction was stirred for 15 minutes at this temperature then allyltrimethylsilane added. The reaction was warmed to room temperature and stirred for the allotted time and quenched at 0 °C with a saturated aqueous sodium bicarbonate solution. The phases were separated and the aqueous phase washed with DCM twice, the combined organic layers were dried ($MgSO_4$) and concentrated to give the crude product.

General Procedure H; Indium coupling

Ethyl (bromomethyl)acylate and indium metal were added to the aldehyde in the given solvent, and the reaction stirred for 61 h at room temperature. The reaction was filtered through Celite, washed with EtOAc and the combined organics washed with a saturated aqueous solution of sodium bicarbonate. The organic layer was dried ($MgSO_4$), and concentrated to give the crude product.

General Procedure I; Barium hydroxide hydrolysis

The ester was dissolved in MeOH and was added to a solution of barium hydroxide monohydrate in water and stirred for 18 h at room temperature. The reaction mixture was concentrated under reduced pressure, dissolved in water, ammonium sulphate added and reaction stirred at room temperature for 2 h. The reaction mixture was filtered through Celite and 0.2 µm Whatman syringe filter and the filtrate concentrated under reduced pressure to give the ammonium salt .

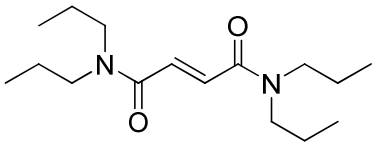
General Procedure J; One-pot reactions

The glyoxylaldehyde was dissolved in buffer and was added to the diamine **69**. Acetaldehyde or propionaldehyde was added to a mixture and reaction was stirred at room temperature for 20 h then diluted with a pH adjusted solution (pH 7.4) of sodium pyruvate in buffer (pH 7.4, 20 mM potassium phosphate) and E192N (in pH 7.4, 20 mM potassium phosphate) and reaction incubated at 35 ºC for 70h then reaction pH lowered to pH 2 with an aqueous solution of 2M formic acid for 5 minutes then pH raised to pH 7 with an aqueous solution of 2M NH_4OH solution. The reaction mixture was filtered through Celite and reaction purified using preparative HPLC.

General Procedure K; E192N catalysed aldol reactions with sodium pyruvate

The alkene was ozonolysed following *General Procedure F* in MeOH. The resultant residue was dissolved in an aqueous potassium phosphate buffer (20 mM, pH 7.4), sodium pyruvate added and pH adjusted to 7.4 using and aqueous solution of 2M NH_4OH. E192N was added in potassium phosphate buffer (20 mM, pH 7.4) and reaction incubated at 35 ºC. After reaction reached completion the pH was lowered to pH 2 with an aqueous solution of 2M formic acid for 5 minutes then pH raised to pH 7 with an aqueous solution of 2M NH_4OH solution and filtered through Celite and reaction purified using preparative HPLC.

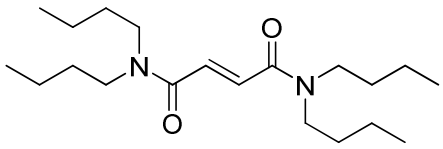
***N*^{1}*,N*^{1}*,N*^{4}*,N*^{4}-Tetrapropylfumaramide 127a**



Dipropylamine (6.62 g, 65.4 mmol) was added dropwise to a vigorously stirred solution of fumaryl chloride (1.77 mL, 16.3 mmol) in ether (200 mL) at 0 ºC. After addition the reaction was allowed to warm to room temperature and stirred for further 2 h. The reaction was filtered to remove precipitate and filtrate was washed with ether (100 mL). The mother liquor was concentrated under reduced pressure to give the crude product. Column chromatography, eluting with 3:7 EtOAc–Petrol, afforded the *title compound* **127a** (3.16 g, 69%) as pale yellow oil, R_f: 0.20 (3:7 EtOAc–Petrol); (Found: C, 60.7; H, 10.25, N, 9.0%; $C_{16}H_{32}N_2O_4$ requires C, 60.7; H, 10.2, N, 8.9%); ν_{max}/cm^{-1} (film) 3534, 2964, 2875, 1723, 1625, 1463 and 1380;δ_H (500 MHz, $CDCl_3$) 7.33-7.31(1H, m, CH), 3.31 (2H, t, *J* 7.7, $N(CH_2)_A$, 3.27 (2H, t, *J* 7.7, $N(CH_2)_B$), 1.62-1.49 (4H, m, $(NCH_2\mathit{CH_2})_{A,B}$), 0.87-0.83 (6H, m, $(CH_2\mathit{CH_3})_{A, B}$); δ_C (75 MHz, $CDCl_3$) 165.3 (CO), 131.7 (CH), 50.2 ($(NCH_2)_A$), 48.9 ($(NCH_2)_B$), 23.4 ($(NCH_2\mathit{CH_2})_A$), 21.4 ($(NCH_2\mathit{CH_2})_B$), 11.8 ($(CH_2\mathit{CH_3})_A$), 11.5 ($(CH_2\mathit{CH_3})_B$); *m/z* (ES^+) 305.2 (60%,

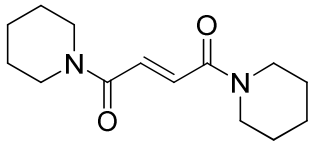
MNa^+) 283.2 (40%, MH^+); HRMS Found MNa^+ 305.2198. $C_{16}H_{30}N_2O_2$ requires MNa^+ 305.2205.

N^1,N^1,N^4,N^4-Tetrabutylfumaramide[142] 127b



Dibutylamine (13.2 mL, 78.4 mmol) was added slowly to a solution of fumaryl chloride (2.12 mL, 19.6 mmol) in dry ether (200 mL) at 0 °C. The reaction was allowed to warm to room temperature and after 17 h reaction was filtered mother liquor evaporated under reduced pressure to give the title compound[142] **127b** (3.32 g, 68%) as pale brown oil*; R_f*: 0.23 (1:9 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3463, 2957 1624 and 1443; δ_H (300 MHz, $CDCl_3$) 7.37 (1H, s, CH), 3.39 (2H, t, *J 7.7*, $(NCH_2)_A$), 3.35 (2H, t, *J 7.9*, $(NCH_2)_B$), 1.65-1.48 (4H, m, $(NCH_2CH_2)_{A,B}$), 1.33 (4H, sextet, *J* 7.5, $(NCH_2CH_2CH_2)_{A,B}$), 0.94 (6H, t, *J* 7.5, $(CH_3)_{A,B}$); δ_C (75 MHz, $CDCl_3$) 165.2 (CO), 131.6 (CH), 48.5 and 47.5($(NCH_2)_{A,B}$), 32.4 and 30.3 ($(NCH_2CH_2)_{A,B}$), 20.7 and 20.4 ($(NCH_2CH_2CH_2)_{A,B}$), 14.2 and 14.2 ($(CH_3)_{A,B}$); *m/z* (EI^+) 339.3 (100%, MH^+); HRMS Found:MH^+: 339.2991, $C_{20}H_{39}N_2O_2$ requires MH^+ 339.3006.

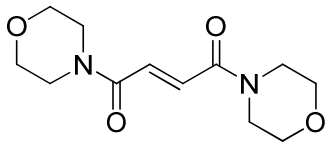
(*E*)-1,4-Di(piperidin-1-yl)but-2-ene-1,4-dione[144] 127c



Piperidine (7.75 mL, 78.4 mmol) was added slowly to a solution of fumaryl chloride (2.12 mL, 19.6 mmol) in dry ether (200 mL) at 0 °C. The reaction was allowed to warm to room temperature, after 17 h the reaction was filtered and mother liquor evaporated under reduced pressure to give the title compound[144] **127c** (3.32 g, 68%) as colourless needles, m.p. 100-103 °C (DCM–petrol) [lit.[144]

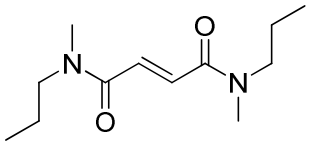
m.p. 104 °C]; R_f: 0.20 (1:1 EtOAc–petrol); v_{max}/cm^{-1} (solid) 3493, 2929, 1627 and 1441; δ_H (300 MHz, $CDCl_3$) 7.14 (1H, s, CH), 3.44 (2H, t, *J* 5.6, $(NCH_2)_A$), 3.36 (2H, t, *J* 5.6, $(NCH_2)_B$), 1.54-1.33 (6H, m, CH_2); δ_C (75 MHz, $CDCl_3$) 164.1 (1-C), 131.4 (CH), 47.3 and 43.5 $(NCH_2)_{A,B}$), 26.9, 26.9 and 24.7 (CH_2); *m/z* (ES^+) 251.2 (100%, MH^+); HRMS Found MH^+: 251.1759, $C_{14}H_{23}N_2O_2$ requires MH^+ 251.1759.

(*E*)-1,4-Dimorpholinobut-2-ene-1,4-dione[145]127d



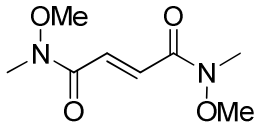
Morpholine (5.74 mL, 65.2 mmol) was added slowly to a solution of fumaryl chloride (1.77 mL, 163. mmol) in dry ether (100 mL) at 0 °C. Reaction was allowed to warm to room temperature and after 45 minutes reaction diluted with water (100 mL). The reaction was washed with 1-butanol (3× 100 mL) and the combined organics dried ($MgSO_4$) and evaporated under reduced pressure to give the title compound[145] **127d** (1.04g, 25%) as colourless needles, m.p. 188.8-192.2 °C (DCM–petrol) [lit. [145] m.p. 195-200 °C]; R_f: 0.38 (9:1 EtOAc–MeOH); v_{max}/cm^{-1} (solid) 3484, 2871, 1626 and 1288; δ_H (500 MHz, $CDCl_3$) 7.4 (1H, s, CH), 3.76-3.58 (8H, m, $(NCH_2)_{A,B}$ and $(NCH_2CH_2)_{A,B}$); δ_C (75 MHz, $CDCl_3$) 164.1 (CO), 131.4 (CH), 67.0, 42.8 and 42.1 ($(NCH_2)_{A,B}$ and $(NCH_2CH_2)_{A,B}$); m/z (ES^+) 255.1 (60%, MH^+), 277.1 (40%, MNa^+); HRMS Found MNa^+: 277.1154, $C_{12}H_{18}N_2O_4$ requires MNa^+ 277.1159.

***N*[1],*N*[4]-Dimethyl-*N*[1],*N*[4]-dipropylfumaramide 127e**



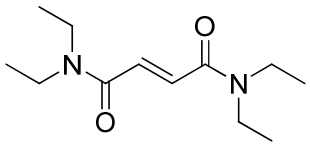
N-Methylpropylamine (12.6 mL, 136 mmol) was added slowly to a solution of fumaryl chloride (3.67 mL, 34.2 mmol) in dry ether (100 mL) at 0 °C. Reaction was allowed to warm to room temperature and after 1 h reaction was filtered and mother liquor evaporated under reduced pressure to give the *title compound* **127e** (7.90 g, 100%) as pale yellow oil; R_f: 0.30 (EtOAc); ν_{max}/cm^{-1} (film); 3501. 2964, 1620, 1455 and 1143; δ_H (400 MHz, $CDCl_3$) 7.39 (2H, s, CH), 3.46-3.34 (4H, m, (NCH_2)), 3.12 (3H, s, NCH_3), 3.03 (3H, s, NCH_3), 1.70-1.53 (4H, m, NCH_2CH_2), 0.92 (6H, t, *J* 7.4, $NCH_2CH_2CH_3$); δ_C (100 MHz, $CDCl_3$) 167.6 and 165.7 (CO), 132.3 and 131.5 (CH), 52.1 and 50.2 (NCH_2), 35.9 and 34.5 (NCH_3), 22.6 and 20.7 (NCH_2CH_2), 11.6 and 11.3 (CH_3); *m/z* (ES^+) 227.2 (100%, MH^+); HRMS Found MH^+: 227.1750, $C_{12}H_{22}N_2O_2$ requires MH^+ 227.1754.

N^1, N^4-Dimethoxy- N^1, N^4-dimethylfumaramide[143] 127f



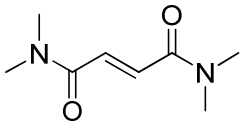
Dry pyridine (42 mL) was added at dropwise to a suspension of *N*,*O*-dimethylhydroxylamine hydrochloride (3.37g, 34.4 mmol) and fumaryl chloride (931 µL, 8.6 mmol) and DCM (20 mL) at 0°C. The reaction was then warmed to room temperature and stirred for 17 h. The reaction was cooled to 0°C and diluted and with aqueous saturated of NH_4Cl (50 mL), washed with DCM (3 × 75 mL) and the combined organic layers dried ($MgSO_4$) and evaporated under reduced pressure to give the title compound[143] **127f** (1.40 g, 81%) as colourless needles; m.p. 120.9-123.6 °C (DCM–petrol) [lit.[143] m.p. 129.5-134.5 °C]; R_f: 0.63 (9:1 EtOAc–MeOH); ν_{max}/cm^{-1} (solid) 2971, 1642, 1463 and 1390; δ_H (300 MHz, $CDCl_3$) 7.49 (1H, s, CH), 3.76 (3H, s, CH_3), 3.30 (3H, s, CH_3); *m/z* (ES^+) 203.1 (70%, MH^+), 225.1 (30%, MNa^+); HRMS Found MH^+: 203.1027, $C_8H_{15}N_2O_4$ requires MH^+ 203.1026.

N[1],*N*[1],*N*[4],*N*[4]-Tetraethylfumaramide[141] 127g



Diethylamine (27 mL, 263 mmol) was added slowly to a solution of fumaryl chloride (7.08 mL, 65.8 mmol) in dry ether (200 mL) at 0 °C. Reaction was allowed to warm to room temperature and after 2h the reaction was filtered and the mother liquor evaporated under reduced pressure to give the title compound[141] **127g** (14.9 g, 100%) as colourless needles m.p. 88-90 °C, [lit.[141] m.p. 90.5°C]; R_f: 0.19 (EtOAc); v_{max}/cm^{-1} (solid); 3436, 1978, 1614, 1463 and 1314; δ_H (500 MHz, $CDCl_3$) 6.91 (1H, s, CH), 3.08-2.95 (4H, m, $(NCH_2)_{A,B}$), 0.78 (3H, t, *J* 7.2, $(CH_3)_A$), 0.71 (3H, t, *J* 7.2, $(CH_3)_B$); δ_C (75 MHz, $CDCl_3$) 166.2 (CO), 131.4 (CH), 42.4 and 40.9 ($(NCH_2)_{A,B}$), 15.2 and 12.9 ($(CH_3)_{A,B}$); *m/z* (ES^+) 227.2 (100%, MH^+); HRMS Found MH^+: 227.1761, $C_{12}H_{22}N_2O_2$ requires MH^+ 227.1754.

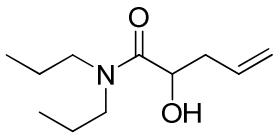
N[1],*N*[1],*N*[4],*N*[4]-Tetramethylfumaramide[141] 127h



Dry triethylamine (10 mL) was added in two portions to a mixture of dimethylamine. hydrogen chloride (5.32 g, 65.2 mmol) and fumaryl chloride (1.77 mL, 16.3 mmol) and DCM (25 mL) at 0 °C. After 1 h further dry triethylamine (10 mL) was added followed by DCM (25 mL) and reaction allowed to warm to room temperature. The reaction was diluted with NH_4Cl (50 mL) after 22 h, washed with EtOAc (3 × 75 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give the title compound [141] **127h** (1.77 g, 46%) as colourless needles; m.p. 120.3-122.9 °C (DCM–petrol) [lit.[141] m.p. 135°C]; R_f: 0.38 (8:2 EtOAc–MeOH); v_{max}/cm^{-1} (solid) 2938, 1621, 1503, 1397 and 1271; δ_H (300 MHz, $CDCl_3$) 7.39 (1H, s, CH), 3.15 (3H, $(NCH_3)_A$), 3.05 (3H, $(NCH_3)_B$);

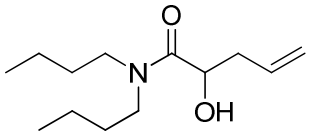
δ_C (75 MHz, $CDCl_3$) 165.6 (CO), 131.4 (CH), 37.7 and 36.1 ($(NCH_3)_A$ and $(NCH_3)_B$); *m/z* (EI^+) 170.1 (30%, M^+), 127.1 (70%, $[M–N(CH_3)_2]^+$), 98.1 (100%, $[M–CON(CH_3)_2]^+$), 72 (30%, $[M–(CH)_2CON(CH_3)_2]^+$); HRMS Found M^+: 170.1056, $C_8H_{14}N_2O_2$ requires M^+170.1055.

2-Hydroxy-*N,N*-dipropylpent-4-enamide 123a



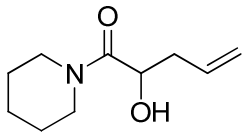
Titanium (IV) chloride (4.91 mL, 44.5 mmol) was added to a solution of the glyoxylaldehyde **1a** (3.5 g, 22.3 mmol) in DCM (100 mL) at 0 ºC and stirred at for 15 minutes. Allyltrimethylsilane (17.7 mL, 111 mmol) was added the reaction warmed to room temperature. After 17 h the reaction was cooled to 0 ºC and diluted with a saturated aqueous solution of sodium bicarbonate (50 mL) and phases separated. The aqueous layer was washed with DCM (2 x 75 mL) and the combined organic layers were dried ($MgSO_4$), and concentrated to give the crude product. Column chromatography, eluting with 7:3 isohexanes–EtOAc, afforded the *title compound* **123a** (4.04 g, 91%) as a colourless oil; R_f: 0.3 (19:1, $CHCl_3$–EtOAc); ν_{max}/cm^{-1} (film) 3411, 2965, 1639 and 1466; δ_H (500 MHz, $CDCl_3$) 5.88-5.76 (1H, m, 4-H), 5.10 (1H, d, *J* 5.1, 5-H_A), 5.07 (1H, br s, 5-H_B), 4.37-4.32 (1H, m, 2-H), 3.70 (1H, d, *J* 8.12, OH), 3.62-6.54 (1H, m, $(NCH_A)_A$), 3.24-3.16 (1H, m, $(NCH_B)_A$), 3.06-2.96 (2H, m, $(NCH_{A,B})_B$), 2.42-2.34 (1H, m, 3-H_A), 2.29-2.20 (1H, m, 3-H_B), 1.65-1.46 (4H, m, $(NCH_2CH_2)_{A,B}$), 0.89 (3H, t, *J* 7.3, $(CH_3)_A$), 0.86 (3H, t, *J* 7.3, $(CH_3)_B$); δ_C (75 MHz, $CDCl_3$) 176.1 (1-C), 135.9 (4-C), 120.7 (5-C), 70.4 (2-C), 51.1 and 50.2 ($(NCH_2)_{A,B}$), 42.3 (3-C), 24.7 and 23.5 ($(NCH_2CH_2)_{A,B}$), 14.1 and 13.9 ($(CH_3)_{A,B}$); *m/z* (ES^+) 222.1 (30%, MNa^+), 200.0 (5%, MH^+), 186.2 (65%, $M\text{-}H_2O^+$); HRMS Found MNa^+: 222.1465, $C_{11}H_{21}NO_2$ requires MNa^+ 222.1457.

2-Hydroxy- *N,N*-dibutylpent-4-enamide 123b



The amide **127b** (276 mg, 0.814 mmol) was ozonolysed following *General Procedure F* in DCM (5 mL) quenching with DMS (5 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (2 mL), with $TiCl_4$ (1M in DCM, 3.26 mL, 3.26 mmol) and allyltrimethylsilane (1.29 mL, 8.15 mmol) for 18 h. Column chromatography, eluting with 1:9 EtOAc–petrol, afforded the *title compound* **123b** (170 mg, 46%) as a colourless oil; R_f: 0.15 (1:9 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3412, 2959, 1639 and 1467; δ_H (300 MHz, $CDCl_3$) 5.87 (1H, ddt, *J* 17.4, 9.7 and 6.91, 4-H), 5.17-5.12 (1H, m, 5-H_A), 5.11-5.08 (1H, m, 5-H_B), 4.36 (1H, ddd, *J* 7.9, 7.1 and 3.8, 2-H), 3.72 (1H, d, *J* 7.9, OH), 3.64 (1H, dt, *J* 14.4 and 7.7, $(NCH_A)_A$), 3.27 (1H, dt, *J* 13.8 and 7.7, $(NCH_B)_A$), 3.14-3.01 (2H, m, $(NCH_{A,B})_B$), 2.47-2.36 (1H, m, 3-H_A), 2.33-2.22 (1H, m, 3-H_B), 1.64-1.49 (4H, m, $(NCH_2\mathit{CH_2})_{A,B}$), 1.40-1.24 (4H, m, $(NCH_2CH_2\mathit{CH_2})_{A,B}$), 0.95 (3H, t, *J* 6.7, $(CH_3)_A$), 0.93 (3H, t, *J* 6.7, $(CH_3)_B$); δ_C (75 MHz, $CDCl_3$) 173.6 (1-C), 133.7 (4-C), 118.3 (5-C), 68.9 (2-C), 47.0 and 46.0 ($(NCH_2)_{A,B}$), 40.5 (3-C), 31.3 and 30.1 ($(NCH_2\mathit{CH_2})_{A,B}$), 20.6 and 20.4 ($(NCH_2CH_2\mathit{CH_2})_{A,B}$), 14.2 and 14.1 ($(CH_3)_{A,B}$); m/z ($EI^+$) 227 (15%, M^+), 156 (100%, $[M-C_4H_8O]^+$); HRMS Found M^+: 227.1891, $C_{13}H_{25}NO_2$ requires M^+ 227.1885.

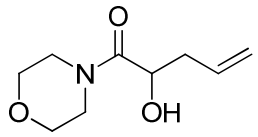
2-Hydroxy-1-(piperidin-1-yl)pent-4-en-1-one 123c



The amide **127c** (279 mg, 1.11 mmol) was ozonolysed following *General Procedure F* in DCM (5 mL) quenching with DMS (5 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (2 mL), with $TiCl_4$ (1M in DCM, 4.44 mL, 4.44 mmol) and allyltrimethylsilane (1.76 mL, 11.1

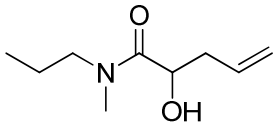
mmol) for 18 h. Column chromatography, eluting with 3:7 EtOAc–petrol, afforded the *title compound* **123c** (122 mg, 30%) as a colourless oil; R_f: 0.24 (3:7 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3410, 1938, 2938, 1638, 1445, 1339 and 1257; δ_H (300 MHz, $CDCl_3$) 5.87 (1H, ddt, *J* 17.4, 9.3 and 7.6, 4-H), 5.17-5.13 (1H, m, 5-H_A), 5.12-5.08 (1H, m, 5-H_B), 4.47-4.38 (1H, m, 2-H), 3.89 (1H, d, *J* 6.9, OH), 3.59 (4H, t, *J* 5.4, ($(NCH_2)_A$), 3.34 (4H, t, *J* 5.4, $(NCH_2)_B$), 2.47-2.37 (1H, m, 3-H_A), 2.32-2.19 (1H, m, 3-H_B), 1.74-1.56 (6H, m, $(NCH_2CH_2)_{A,B}$ and $NCH_2CH_2CH_2$); δ_C (75 MHz, $CDCl_3$) 173.2 (1-C), 133.6 (4-C), 118.3 (5-C), 67.8 (2-C), 46.3 and 44.1 ($(NCH_2)_{A,B}$), 40.1 (3-C), 26.6 and 25.9 ($(NCH_2CH_2)_{A,B}$), 24.8 ($NCH_2CH_2CH_2$); *m/z* (ES^+) 184.1 (100%, MH^+); HRMS Found MH^+: 184.1339, $C_{10}H_{18}NO_2$ requires MH^+184.1332.

2-Hydroxy-1-morpholinopent-4-en-1-one 123d



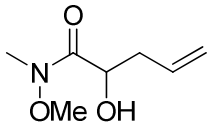
The amide **127d** (207 mg, 0.815 mmol) was ozonolysed following *General Procedure F* in MeOH (2 mL) and DCM (4 mL) quenching with DMS (5 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (5 mL), with $TiCl_4$ (1M in DCM, 1.63 mL, 1.63 mmol) and allyltrimethylsilane (647 µL, 707 mmol) for 18 h. Column chromatography, eluting with 1:1 EtOAc–petrol, afforded the *title compound* **123d** (180 mg, 60%) as a colourless oil; R_f: 0.17 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film); 3418, 2919, 1642, 1439 and 1273; δ_H (500 MHz, $CDCl_3$) 5.86-5.76 (1H, m, 4-H), 5.11 (1H, br s, 5-H_A), 5.09-5.07 (1H, m, 5-H_B), 4.41-4.35 (1H, m, 2-H), 3.75-3.33 (9H, m, $(NCH_2)_{A,B}$, $(NCH_2CH_2)_{A,B}$, and OH), 2.42-2.34 (1H, m, 3-H_A), 2.29-2.20 (1H, m, 3-H_B); δ_C (75 MHz, $CDCl_3$) 75.1 (1-C), 135.7 (4-C), 121 (5-C), 70.3 (2-C), 69.6, 69.2, 48.3 and 45.6 ($(NCH_2)_{A,B}$ and $(NCH_2CH_2)_{A,B}$,), 42.4 (3-C); *m/z* (EI^+) 185.1 (20%, M^+), 144.1 (100%, $[M\text{-}CH_2CHCH_2]^+$); HRMS Found M^+: 185.1053, $C_9H_{15}NO_3$ requires M^+ 185.1052.

2-hydroxy-*N*-methyl-*N*-propylpent-4-enamide 123e



The amide **127e** (3.00 g, 13.3 mmol) was ozonolysed following *General Procedure F* in DCM (75 mL) quenching with DMS (75 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (40 mL), with $TiCl_4$ (11.7 mL, 106 mmol) and allyltrimethylsilane (21.1 mL, 133 mmol) for 17 h. Column chromatography, eluting with 7:3 EtOAc–petrol, afforded the *title compound* **123e** (1.00 g, 22%, *ca*. 6:5 mixture of rotomers) as a colourless oil; R_f: 0.42 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3414, 2965, 1639 and 1388; δ_H (500 MHz, $CDCl_3$) 5.95-5.75 (1H, m, 4-H), 5.19-5.12 (1H, m, 5-H_A), 5.10 (1H, br s, 5-H_B), 4.44 (1H, td, *J* 7.2 and 3.6, 2-H), 3.58-3.08 (2H, m, NCH_2), 2.95 (3H, s, NCH_3^{rotA}), 2.93 (s, NCH_3^{rotB}), 2.51-2.37 (1H, m, 3-H_A), 2.37-2.23 (1H, m, 3-H_B), 1.73-1.48 (2H, m, $NCH_2\mathit{CH_2}$), 0.98-0.85 (3H, m, $NCH_2CH_2\mathit{CH_3}$); δ_C (75 MHz, $CDCl_3$) 174.0 (1-C^{rotA}), 173.8 (1-C^{rotB}), 133.6 (4-C^{rotA}), 133.5 (4- C^{rotB}), 118.5 (5-C), 68.1 (2-C), 50.7 (NCH_2^{rotA}), 50.5 (NCH_2^{rotB}), 40.3 (NCH_3^{rotA}), 39.6 (NCH_3^{rotB}), 35.0 (3-C^{rotA}), 33.9 (3-C^{rotB}), 21.8 ($NCH_2\mathit{CH_2}^{rotA}$), 30.6 ($NCH_2\mathit{CH_2}^{rotB}$), 11.6 ($NCH_2CH_2\mathit{CH_3}^{rotA}$) and 11.4 ($NCH_2CH_2\mathit{CH_3}^{rotB}$); *m/z* ($ES^+$) 172.1 (100%, MH^+); HRMS Found MH^+: 172.1336, $C_9H_{17}NO_2$ requires MH^+ 171.1332.

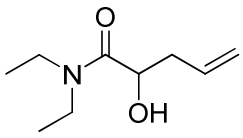
2-Hydroxy-*N*-methoxy-*N*-methylpent-4-enamide 123f



The amide **127f** (156 mg, 0.772 mmol) was ozonolysed following *General Procedure F* in DCM (5 mL) quenching with DMS (5 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (5 mL), with $TiCl_4$ (1M in DCM, 3.08 mL, 3.08 mmol) and allyltrimethylsilane (1.22 mL, 7.72 mmol) for 16 h. Column chromatography, eluting with 6:4 EtOAc–petrol, afforded the *title compound* **123f** (82 mg, 34%) as a colourless oil; R_f: 0.33 (1:1

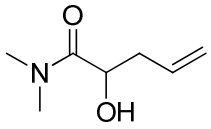
EtOAc–petrol); ν_{max}/cm^{-1} (film) 3435, 1941 and 1658; δ_H (500 MHz, $CDCl_3$) 5.91-5.80 (1H, m, 4-H), 5.14 (1H, d, *J* 7.69, 5-H_A), 5.11 (1H, br s, 5-H_B), 4.48 (1H, br s, 2-H), 4.73 (3H, s, CH_3), 3.33 (1H, d, *J* 8.12, OH), 3.25 (3H, s, CH_3), 2.56-2.48 (1H, m, 3-H_A), 2.36 (1H, dt, *J* 14.5 and 6.4, 3-H_B); δ_C (75 MHz, $CDCl_3$) 171.6 (1-C), 133.7 (4-C), 118.4 (5-C), 68.7 (2-C), 61.7 (CH_3), 39.4 (CH_3), 32.9 (3-C); *m/z* (EI^+) 160.1 (20%, M^+), 118 (80%, $[M-CH_2CHCH_2]^+$); HRMS Found M^+, 160.0970, $C_7H_{14}NO_3$ requires M^+ 160.097.

2-Hydroxy-*N,N*-diethylpent-4-enamide 123g



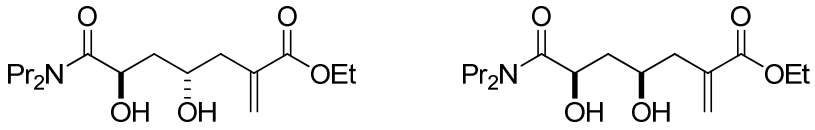
The amide **127g** (5.00 g, 22.1mmol) was ozonolysed following *General Procedure F* in DCM (100 mL) quenching with DMS (100 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (50 mL), with $TiCl_4$ (19.5 mL, 177 mmol) and allyltrimethylsilane (35.1 mL, 221 mmol) for three days. Column chromatography, eluting with a gradient elution of 1:1 to 7:3 EtOAc–petrol, afforded the *title compound* **123g** (1.66 g, 37%) as a colourless oil; R_f: 0.36 (1: 1 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3409, 2977, 1634, 1435 and 1310; δ_H (300 MHz, $CDCl_3$) 5.95-5.75 (1H, m, 4-H), 5.15 (1H, d, *J* 4.1, 5-H_A), 5.10 (1H, br s, 5-H_B), 4.74 (1H, br s, 2-H), 3.81 (1H, br s, OH), 3.68-3.64 (4H, m, $(NCH_2)_{A,B}$), 2.49-2.36 (1H, m, 3-H_A), 2.29 (1H, dt, *J* 14.3 and 7.2, 3-H_B), 1.21 (3H, t, *J* 7.2, $(NCH_2\mathit{CH_3})_A$), 1.14 (3H, t, *J* 8.2, $(NCH_2\mathit{CH_3})_B$);δ_C (75 MHz, $CDCl_3$) 173.2 (1-C), 133.6 (4-C), 118.4 (5-C), 68.0 (2-C), 41.4 and 40.4 ($(NCH_2)_{A,B}$), 40.6 (3-C), 14.6 and 13.3 ($(NCH_2\mathit{CH_3})_{A,B}$); *m/z* ($ES^+$) 172.1 (50%, MH^+), 194.1 (50%, MNa^+); HRMS Found MH^+: 172.1335, $C_9H_{17}NO_2$ requires MH^+ 172.1332.

2-Hydroxy-*N,N*-dimethylpent-4-enamide 123h



The amide **127h** (171 mg, 1.01 mmol) was ozonolysed following *General Procedure F* in DCM (5 mL) quenching with DMS (5 mL). The resultant aldehyde was allylated following *General Procedure G* in DCM (5 mL), with $TiCl_4$ (1M in DCM, 2.02 mL, 2.02 mmol) and allyltrimethylsilane (799 µL, 5.03 mmol) for 18 h. Column chromatography, eluting with 7:3 EtOAc–petrol, afforded the *title compound* **123h** (86 mg, 30%) as a colourless oil; R_f: 0.17 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3412, 2937, 1642 and 1381; δ_H (300 MHz, $CDCl_3$) 5.93-5.74 (1H, m, 4-H), 5.16-5.18 (1H, m, 5-H_A), 5.06 (1H, br s, 5-H_B), 4.46-4.35 (1H, m, 2-H), 4.73 (1H, d, *J* 7.4, OH), 2.98 (6H, s, $(CH_3)_{A,B}$), 2.48-2.33 (1H, m, 3-H_A), 2.26 (1H, dt, *J* 14.8 and 7.4, 3-H_B); δ_C (75 MHz, $CDCl_3$) 174 (1-C), 133.6 (4-C), 118.3 (5-C), 68.1 (2-C), 39.6 (3-C), 36.8 and 36.3 ($(CH_3)_{A,B}$); *m/z* (EI^+) 43.1 (30%, M^+), 102 (100%, $[M\text{-}CH_2CHCH_2]^+$); HRMS Found M^+: 143.0939, $C_7H_{13}NO_2$ requires M^+ 143.0946.

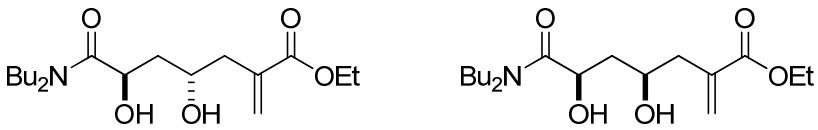
(4*S,6*R**)-Ethyl 7-(dipropylamino)-4,6-dihydroxy-2-methylene-7-oxoheptanoate and (4*R**,6*R**)-ethyl 7-(dipropylamino)-4,6-dihydroxy-2-methylene-7-oxoheptanoate 122a**



The alkene **123a** (2.1 g, 10.6 mmol) was ozonolysed following *General Procedure F* in DCM (25 mL) and was quenched with DMS (25 mL). The crude aldehyde was alkylated following *General Procedure H* in 1:1 THF–water (160 mL), with ethyl (bromomethyl) acrylate (1.91 mL, 13.3 mmol), THF (40 mL) and indium metal (1.40 g, 12.2 mmol), washed with EtOAc (4 × 125 mL) and saturated aqueous solution of sodium bicarbonate (3 × 100 mL). Column chromatography, eluting with 8:2 to 1:1 EtOAc–petrol, afforded the *title*

compound **122a** (811 mg, 24%, 70:30 *anti:syn*) as a colourless oil; R_f: 0.37 (7:3 EtOAc–petrol); v_{max}/cm^{-1} (film) 3413, 2965, 1714, 1632 and 1466 ; δ_H (500 MHz, $CDCl_3$) 6.25 (1H, s, 2-C-CH_A), 5.68 (1H, s, 2-C-CH_B), 4.61 (0.70 H, td, *J* 9.2 and 2.8, 6-H_{anti}), 4.52 (0.30 H, td, *J* 8.5 and 2.7, 6-H_{syn}),4.21 (2H, q, *J* 7.0, OCH_2), 4.12 (1H, br s, 4-H), 3.85 (1H, d, *J* 7.7, OH), 3.64-3.46 (1H, m, $(NCH_A)_A$), 3.39-3.00 (3H, m, $(NCH_B)_A$ and $(NCH_{A,B})_B$), 2.77-2.37 (2H, m, 3-H), 1.70-1.46 (6H, m, $(NCH_2CH_2)_{A,B}$ and 5-H), 1.30 (3H, t, *J* 7.0, OCH_2CH_3), 0.91 (3H, t, *J* 6.8, $N(CH_2CH_2CH_3)_A$), 0.89 (3H, t, *J* 7.2, $N(CH_2CH_2CH_3)_B$); δ_C (75 MHz, $CDCl_3$, *anti* isomer only) 174.7 and 168.2 (1-C and 7-C), 137.8 and 129.3 (2-C and 2-C-CH_2), 67.1 and 65.8 (4-C and 6-C), 61.5 (OCH_2), 48.8 and 48.0 ($(NCH_2)_{A,B}$), 42.9 and 40.8 (3-C and 5-C), 22.3 and 21.0 ($(NCH_2CH_2)_{A,B}$), 14.5 (OCH_2CH_3), 11.6 and 11.4 ($(NCH_2CH_2CH_3)_{A,B}$); *m/z* (ES^+) 338.2 (100%, MNa^+); HRMS Found MNa^+: 338.1933, $C_{16}H_{29}NO_5$ requires MNa^+338.1938. The ratio of the *anti* and *syn* diastereomers were determined by the integration of the signals at 4.61 ppm (*anti*), 4.52 ppm (*syn*).

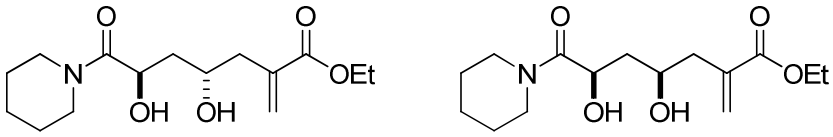
(4*S,6*R**)-Ethyl 7-(dibutylamino)-4,6-dihydroxy-2-methylene-7-oxoheptanoate and (4*R**,6*R**)-ethyl 7-(dibutylamino)-4,6-dihydroxy-2-methylene-7-oxoheptanoate 122b**



The alkene **123b** (6.26 g, 24.3 mmol) was ozonolysed following *General Procedure F* in DCM (150 mL) and was quenched with DMS (150 mL). The crude aldehyde was alkylated following *General Procedure H* in 1:1 water–THF (200 mL), with ethyl (bromomethyl) acrylate (4.74 mL, 33.1 mmol) and indium metal (3.49 g, 30.4 mmol), washed with EtOAc (4 × 250 mL) and saturated aqueous solution of sodium bicarbonate (3 × 100 mL). Column chromatography, eluting with 8:2 to 4:6 petrol–EtOAc afforded the *title compound* **122b** (275 mg, 12%, 80:20 *anti:syn*) as a colourless oil*;* R_f: 0.58 (3:7 EtOAc–petrol); δ_H (500 MHz, $CDCl_3$) 6.26 (1H, s, 2-C-CH_A), 5.67 (1H, s, 2-C-CH_B), 4.60 (0.80 H, td, *J* 9.3 and 2.4, 6-H_{anti}), 4.54-4.49 (0.20 H, m, 6-H_{syn}), 4.22

(2H, t, *J* 7.7, OCH_2), 4.15-4.07 (1H, m, 4-H), 3.76 (1H, d, *J* 7.7, OH), 3.66-3.54 (1H, m, $(NCH_A)_A$), 3.37-3.01 (3H, m, $(NCH_B)_A$, $(NCH_A)_B$ and $(NCH_B)_B$), 2.96-2.89 (1H, m, OH), 2.73-2.37 (2H, m, 3-H), 1.84-1.26 (12H, m, 5-H, $(NCH_2\mathit{CH_2},)_{A,B}$, $(NCH_2CH_2\mathit{CH_2})_{A,B}$ and $OCH_2\mathit{CH_3}$), 1.35-1.27 (6H, m, $(NCH_2CH_2CH_2\mathit{CH_3})_{A,B}$); δ_C (75 MHz, $CDCl_3$, *anti* isomer only) 174.6 and 169.1(7-C and 1-C), 137.8 and 128.2 (2-C-CH_2 and 2-C), 67.9 (4-C), 65.8 (6-C), 61.5 (OCH_2), 46.7 and 46.2 ($(NCH_2)_{A,B}$), 43.1 and 40.8 (5-C and 3-C), 31.2 and 30.0 ($(NCH_2\mathit{CH_2})_{A,B}$), 20.5 and 20.3 ($(NCH_2CH_2\mathit{CH_2})_{A,B}$), 14.6 ($OCH_2\mathit{CH_3}$), 14.3 and 14.2 ($(NCH_2CH_2CH_2\mathit{CH_3})_{A,B}$); *m/z* ($ES^+$) 344.2 (70%, MH^+), 366.2 (30%, MNa^+); HRMS Found MH^+: 344.2433, $C_{18}H_{33}NO_5$ requires MH^+ 344.2431. The ratio of the *anti* and *syn* diastereomers were determined by the integration of the signals at 4.60 ppm (*anti*), 4.54-4.49ppm (*syn*).

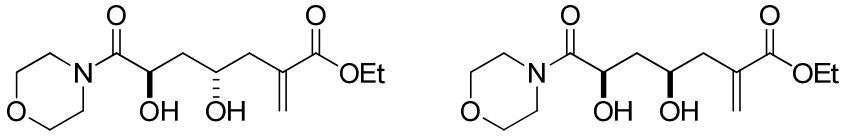
(4*S,6*R**)-Ethyl 4,6-dihydroxy-2-methylene-7-oxo-7-(piperidin-1-yl)heptanoate and (4*R**,6*R**)-ethyl 4,6-dihydroxy-2-methylene-7-oxo-7-(piperidin-1-yl)heptanoate 122c**



The alkene **123c** (857 mg, 4.68 mmol) was ozonolysed following *General Procedure F* in DCM (25 mL) and was quenched with DMS (15 mL). The crude aldehyde was alkylated following *General Procedure H* in water (72 mL), with ethyl (bromomethyl) acrylate (0.775 mL, 5.61 mmol) and indium metal (592 mg, 5.15 mmol), washed with EtOAc (4 × 100 mL) and saturated aqueous solution of sodium bicarbonate (3 × 75 mL). Column chromatography, eluting with 8:2 to 5:95 petrol–EtOAc, afforded the *title compound* **122c** (565 mg, 33%, 68:32 *anti*:*syn*) as a colourless oil*; R_f*: 0.19 (7:3 EtOAc–petrol); v_{max}/cm^{-1} (film) 3401, 2938, 1712, 1631 and 1446; δ_H (400 MHz, $CDCl_3$) 6.25 (1H, s, 2-C-CH_A), 5.66 (1H, s, 2-C-CH_B), 4.67-4.61 (0.68 H, m, 6-H_{anti}), 4.67-4.61 (0.32 H, m, 6-H_{syn}), 4.25-4.18 (2H, m, OCH_2), 4.15-4.08 (1H, m, 4-H), 3.89 (1H, d, *J* 7.8, 6-OH), 3.71-3.59 (1H, m, $(NCH_A)_A$), 3.57-3.41 (1H, m, $(NCH_B)_A$), 3.42-3.36 (2H, m, $(NCH_{A,B})_B$), 2.90 (1H, d, *J* 4.7, 4-OH), 2.62-2.41 (2H, m, 3-H), 1.72-1.49 (8H, m,

($NCH_2CH_2)_{A,B,}$ NCH_2 CH_2CH_2, and 5-H), 1.30 (3H, t, *J* 7.2, CH_3); δ_C (100 MHz, $CDCl_3$, *anti* isomer only) 172.7 (C=O), 172.0 (C=O), 137.3 and 127.6 (2-C-CH_2 and 2-C), 67.3 (4-C), 65.2 (6-C), 60.9 (OCH_2), 45.7 and 43.8 ($(NCH_2)_{A,B}$), 42.2 and 40.2 (5-C and 3-C), 26.2 and 25.5 ($(NCH_2CH_2)_{A,B}$), 25.4 ($NCH_2CH_2CH_2$), 14.2 (CH_3); *m/z* (ES^+) 322.2 (100%, MNa^+); HRMS Found MNa^+: 322.1625, $C_{15}H_{25}NO_5$ requires MNa^+ 322.1625. The ratio of the *anti* and *syn* diastereomers were determined by the integration of the signals at 4.67-4.61 ppm (*anti*), 4.67-4.61 ppm (*syn*).

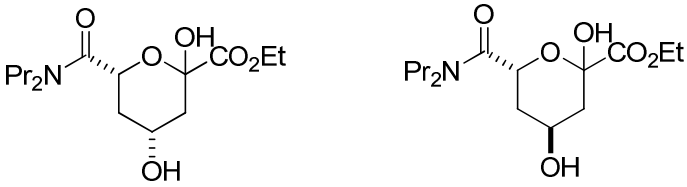
(4*S,6*R**)-Ethyl 4,6-dihydroxy-2-methylene-7-morpholino-7-oxoheptanoate and (4*R**,6*R**)-ethyl 4,6-dihydroxy-2-methylene-7-morpholino-7-oxoheptanoate 122d**



The alkene **123d** (4.50 g, 24.3 mmol,) was ozonolysed following *General Procedure F* in DCM (100 mL) and was quenched with DMS (100 mL). The crude aldehyde was alkylated following *General Procedure H* in 1:1 water–THF (200 mL), with ethyl (bromomethyl) acrylate (4.17 mL, 29.2 mmol) and indium metal (3.07 g, 26.7 mmol), washed with EtOAc (4 × 200 mL) and saturated aqueous solution of sodium bicarbonate (3 × 100 mL). Column chromatography, eluting with 1:1 petrol–EtOAc to 6% MeOH in EtOAc, afforded the *title compound* **122d** (3.75 g, 18%, 74:26 *anti*:*syn*) as a colourless oil*;* R_f: 0.18 (3% MeOH in EtOAc); v_{max}/cm^{-1} (film) 3413, 2978, 1712, 1634 and 1442; δ_H (500 MHz, $CDCl_3$, *anti* isomer only) 6.26 (1H, s, 2-C-CH_A), 5.67 (1H, s, 2-C-CH_B), 4.64 (0.74 H, br s, 6-H_{anti}), 4.59 (0.24 H, br s, 6-H_{syn}), 4.22 (2H, q, *J* 7.3, OCH_2), 4.15-4.06 (1H, m, 4-H), 3.80-3.41 (8H, m, $(NCH_2)_{A,B}$ and $(NCH_2CH_2)_{A,B}$), 3.09 (1H, br s, OH), 2.55 (1H, dd, *J* 13.3 and 4.3, 3-H_A), 2.44 (1H, dd, *J* 14.1 and 8.1, 3-H_B), 1.61-1.55 (2H, m, 5-H), 1.31 (3H, t, *J* 7.3, OCH_2CH_3); δ_C (100 MHz, $CDCl_3$, *anti* isomer only) 173.3 and 167.8 (1-C and 7-C), 137.4 and 127.8 (2-C-CH_2 and 2-C), 69.8 (4-C), 66.7 and 66.4 ($(NCH_2)_{A,B}$), 67.8 (6-C), 61.2 (OCH_2CH_3), 45.5 and 42.7 ($(NCH_2CH_2)_{A,B}$), 42.8 (5-C), 40.9 (3-C), 14.2

(CH_3); *m/z* (ES^+) 324.1 (100%, MNa^+); HRMS Found *MNa*$^+$: 324.1402, $C_{14}H_{23}NO_6$ requires *MNa*$^+$ 324.1418. The ratio of the *anti* and *syn* diastereomers were determined by the integration of the signals at 4.64 ppm (*anti*), 4.59 ppm (*syn*).

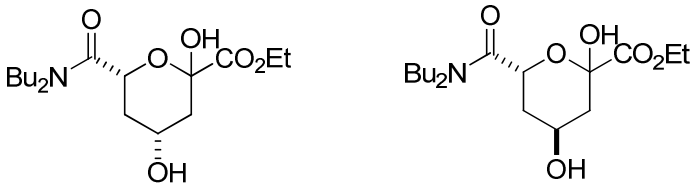
(4*R,6*R**)-ethyl 6-(dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate and (4*S**,6*R**)-ethyl 6-(dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate 134a**



The alkene **122a** (710 mg, 2.25 mmol, 70:30 *anti:syn*) was ozonolysed following *General Procedure F* in DCM (15 mL) and was quenched with DMS (15 mL). Column chromatography eluting with 9:1 to 7:3 DCM–acetone followed by 95:5 EtOAc–MeOH, afforded the *title compound* **134a** (418 mg, 59%, 95:5 *cis*:*trans*) as a colourless oil; R_f: 0.18 (8:2 DCM–acetone); ν_{max}/cm^{-1} (film) 3368, 2966, 1743, 1634 and 1466; δ_H (500 MHz, $CDCl_3$) 4.72 (1H, dd, *J* 12.0 and 2.4, 6-H), 4.60 (1H, d, *J* 9.4, 6-H), 4.37-4.10 (4H, m, 4-H, OCH_2 and OH), 3.37-3.00 (4H, m, $(NCH_2)_{A,B}$), 2.19-2.09 (2H, m, 5-H_A and 3-H_A), 1.99-1.85 (1H, m, 3-H_B and 5-H_B), 1.67-1.47 (4H, m, $(NCH_2\mathit{CH_2})_{A,B}$), 1.32 (3H, t, *J* 7.3, $OCH_2\mathit{CH_3}$), 0.94-0.83 (6H, m, $(NCH_2CH_2\mathit{CH_3})_{A,B}$); δ_C (75 MHz, $CDCl_3$, *major* isomer only) 170.4 and 168.8 (amide and 1-C), 96.2 (2-C), 68.5 and 64.5 (6-C and 4-C), 63.4 (OCH_2), 49.6 and 48.1 $(NCH_2)_{A,B}$), 39.9 and 37.0 (5-C and 3-C), 22.7 and 21.0 $(NCH_2\mathit{CH_2})_{A,B}$), 14.3 ($OCH_2\mathit{CH_3}$), 11.7 and 11.6 $(NCH_2CH_2\mathit{CH_3})_{A,B}$); *m/z* ($ES^+$) 318.2 (60%, MH^+), 340.2 (40%, MNa^+); HRMS Found *MH*$^+$: 318.1904, $C_{15}H_{28}NO_6$ requires *MH*$^+$ 318.1911.

Full interpretation of the anomeric species of the *cis* and *trans* diastereomers was not achieved by analysis by 500 MHz ^1H NMR of the esters **134a-d** but was achieved for the corresponding ammonium salts **135a-d**.

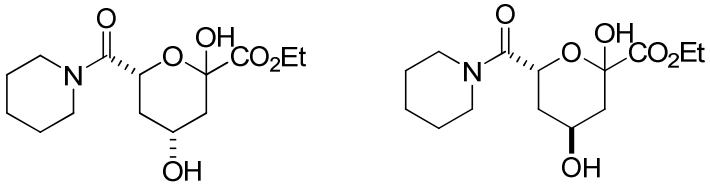
(4*R,6*R**)-ethyl 6-(dibutylcarbamoyl)-2,4-dihydroxytetrahydro-2H-pyran-2-carboxylate and (4*S**,6*R**)-ethyl 6-(dibutylcarbamoyl)-2,4-dihydroxytetrahydro-2H-pyran-2-carboxylate 134b**



The alkene **122b** (1.00 g, 2.91 mmol, 80:20 *anti:syn*) was ozonolysed following *General Procedure F* in DCM (20 mL) and was quenched with DMS (25 mL). The crude product was purified with column chromatography, eluting with 1:1 ether–DCM, to give the *title compound* **134b** (620 mg, 62%, 95:5 *cis*:*trans*) as a colourless film; R_f: 0.14 (1:1 ether–DCM); ν_{max}/cm^{-1} (film) 3368, 2959, 1744, 1633, 1467, 1370 and 1274; δ_H (400 MHz, $CDCl_3$) 4.72 (1H, d, *J* 11.2, 6-H), 4.37-4.13 (4H, m, 6-H, 4-H and OCH_2), 3.40-2.97 (4H, m, $(NCH_2)_{A,B}$), 2.17-1.78 (4H, m, 5-H and 3-H), 1.63-1.43 (4H, m, $(NCH_2\textit{CH}_2)_{A,B}$), 1.38-1.23 (7H, m, $(NCH_2CH_2\textit{CH}_2)_{A,B}$ and $O\textit{CH}_2CH_3$), 0.93 (3H, t, *J* 7.0, $(NCH_2CH_2CH_2\textit{CH}_3)_A$), 0.90 (3H, t, *J* 7.4, $(NCH_2CH_2CH_2\textit{CH}_3)_B$); δ_C (75 MHz, $CDCl_3$, *major* isomer only); 170.4 and 169.5 (amide and 1-C), 96.3 (2-C), 68.5 (6-C), 62.6 (4-C), 62.4 (OCH_2), 46.3 and 45.9 ($(NCH_2)_{A,B}$), 39.9 and 36.4 (5-C and 3-C), 31.3 and 31.0 ($(NCH_2\textit{CH}_2)_{A,B}$), 20.6 and 20.3 ($(NCH_2CH_2\textit{CH}_2)_{A,B}$), 14.5 and 14.1 ($(NCH_2CH_2CH_2\textit{CH}_3)_{A,B}$ and $OCH_2\textit{CH}_3$); *m/z* (ES^+) 346.2 (70%, MH^+), 368.2 (30%, MNa^+); HRMS Found MH^+: 346.2233, $C_{17}H_{31}NO_6$ requires MH^+ 346.2224.

Full interpretation of the anomeric species of the *cis* and *trans* diastereomers was not achieved by analysis by 500 MHz ^1H NMR of the esters **134a-d** but was achieved for the corresponding ammonium salts **135a-d**.

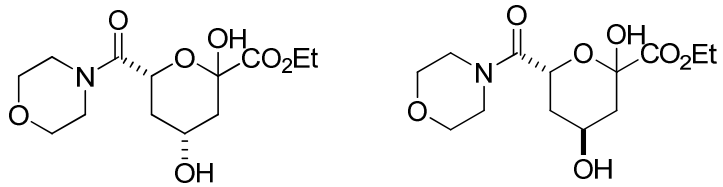
(4*R,6*R**)-ethyl 2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate and (4*S**,6*R**)-ethyl 2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate 134c**



The alkene **122c** (410 mg, 1.37 mmol, 68:32 *anti*:*syn*) was ozonolysed following *General Procedure F* in DCM (10mL) and was quenched with DMS (10 mL). The crude product was purified with column chromatography, eluting with 9:1 EtOAc–toluene, to give the *title compound* **134c** (112 mg, 27%, 88:12 *cis*:*trans*) as a colourless film; R_f: 0.18 (9:1 EtOAc–toluene); ν_{max}/cm^{-1} (film) 3368, 2936, 1742, 1630 and 1447; δ_H (500 MHz, $CDCl_3$) 5.07 (1H, dd, *J* 12.9 and 2.5, 6-H), 4.71 (1H, dd, *J* 11.8 and 2.3, 6-H), 4.73-4.13 (3H, m, 4-H and OCH_2), 3.83-3.27 (4H, m, $(NCH_2)_{A,B}$), 2.20-2.01 (1H, m, 5-H_A), 1.87 (1H, m, 3-H_A), 1.70-1.48 (8H, m, 5-H_B, 3-H_B, $(NCH_2CH_2)_{A,B}$ and $NCH_2CH_2CH_2$), 1.31 (3H, t, *J* 7.6, CH_3); δ_C (75 MHz, $CDCl_3$, *major* isomer only) 170.3 and 167.7 (1-C and amide), 96.4 (2-C), 69.3 ,64.2 and 62.5 (6-C, 4-C and OCH_2), 46.1 and 44.1 ($(NCH_2)_{A,B}$), 39.0 and 36.7 (5-C and 3-C), 26.8 and 25.9 ($(NCH_2CH_2)_{A,B}$), 24.7 ($NCH_2CH_2CH_2$), 14.3 (CH_3); *m/z* (ES^+) 302.2 (60%, MH^+), 324.1 (50%, MNa^+); HRMS Found MNa^+: 324.1413, $C_{14}H_{23}NO_6$ requires MNa^+ 324.1418.

Full interpretation of the anomeric species of the *cis* and *trans* diastereomers was not achieved by analysis by 500 MHz ^1H NMR of the esters **134a-d** but was achieved for the corresponding ammonium salts **135a-d**.

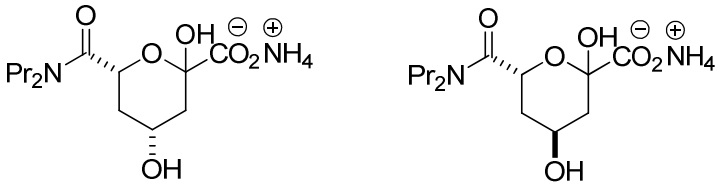
(4*R,6*R**)-ethyl 2,4-dihydroxy-6-(morpholine-4-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate and (4*S**,6*R**)-ethyl 2,4-dihydroxy-6-(morpholine-4-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate 134d**



The alkene **122d** (1.20 g, 3.99 mmol, 74:26 *cis*:*trans*) was ozonolysed following *General Procedure F* in DCM (20 mL) and was quenched with DMS (25 mL). The crude product was purified with column chromatography, eluting with 2% MeOH in EtOAc, to give the *title compound* **134d** (598 mg, 49%, 78:22 *cis*:*trans*) as a colourless film; R_f: 0.17 (2% MeOH in EtOAc); v_{max}/cm^{-1} (film) 3400, 2978, 1740, 1643, 1471 and 1274; δ_H (500 MHz, $CDCl_3$) 5.05 (1H, d, *J* 11.5, 6-H), 4.71 (1H, d, *J* 12.0, 6-H), 4.35-4.08 (3H, m, 4-H and O*CH*$_2$CH$_3$), 3.81-3.24 (8H, m, $(NCH_2)_{A,B}$ and $(NCH_2CH_2)_{A,B}$), 2.12-1.60 (4H, m, 5-H and 3-H), 1.31 (3H, t, *J* 7.3, CH_3); δ_C (75 MHz, $CDCl_3$, *major* isomer only) 170.5 and 169.1 (amide and 1-C), 96.5 (2-C), 67.1 and 62.9 (6-C and 4-C), 67.2 and 67.0 ($(NCH_2CH_2)_{A,B}$), 60.8 (O*CH*$_2$CH$_3$), 43.1, 39.0, 34.0 and 34.3 (5-C, 3-C and $(NCH_2)_{A,B}$), 14.5 (CH_3); *m/z* (ES^+) 326.1 (70%, MNa^+), 304.1 (30%, MH^+); HRMS Found MNa^+: 326.1214, $C_{13}H_{21}NO_7$ requires MNa^+326.1210.

Full interpretation of the anomeric species of the *cis* and *trans* diastereomers was not achieved by analysis by 500 MHz ^{1}H NMR of the esters **134a-d** but was achieved for the corresponding ammonium salts **135a-d**.

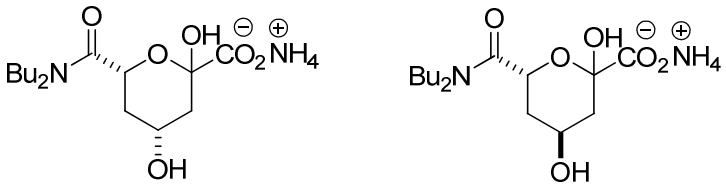
Ammonium (4*R,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate and ammonium and (4*S**,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate 135a**



The *ammonium salt* **135a** was prepared following *General Procedure I* from ester **134a** (258 mg, 0.813 mmol, 95:5 *cis*:*trans*) in MeOH (4 mL) with barium hydroxide monohydrate (77.0 mg, 0.406 mmol) in water (20 mL). The reaction was stirred for 16.5 h at room temperature then further barium hydroxide monohydrate (35.0 mg, 0.203 mmol) and stirred for further 8 h and reaction mixture concentrated and was dissolved in water (5 mL), ammonium sulphate

(122 mg, 0.591 mmol) added giving the *ammonium salt* **135a** (218 mg, 100%, 95:5 *cis*:*trans*) as an amorphous solid; ν_{max}/cm^{-1} (solid) 3233, 2964, 1764, 1625 and 1458; δ_H (500 MHz, D_2O) 5.18 (0.04H, d, *J* 11.5, 6-$H_{trans(maj)}$), 5.18 (0.02H, d, *J* 11.2, 6-$H_{trans(min)}$), 4.81 (0.84H, d, *J* 11.5, 6-$H_{cis(maj)}$), 4.65-4.57 (0.13H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.33-4.22 (0.04H, m, 4-$H_{trans(maj)}$), 4.15-4.05 (0.84H, m, 4-$H_{cis(maj)}$), 4.03-3.94 (0.11H, m, 4-$H_{cis(min)}$), 3.45-2.95 (4H, m, $(NCH_2)_{A,B}$), 2.11-2.00 (2H, m, 5-H_{eq} and 3-H_{eq}), 1.64 (1H, t, *J* 12.0, 3-H_{ax}), 1.59-1.40 (5H, m, 5-H_{ax} and $(NCH_2CH_2)_{A,B}$), 0.81 (3H, t, *J* 7.3, $(CH_3)_A$), 0.77 (3H, t, *J* 7.7, $(CH_3)_B$); δ_C (75 MHz, D_2O,*cis(maj)* only) 175.5 and 172.2 (amide and 1-C), 97.9 (2-C), 69.3 and 63.7 (6-C and 4-C), 49.7 and 48.4 ($(NCH_2)_{A,B}$), 41.9 and 38.2 (5-C and 3-C), 22.0 and 20.5 ($(NCH_2CH_2)_{A,B}$), 11.0 and 10.7 ($(CH_3)_{A,B}$)); *m/z* (ES^-) 288.1 (100%, $[M–NH_4]^-$); HRMS Found *$[M–NH_4]^-$:* 288.1456, $C_{13}H_{26}N_2O_6$ requires *$[M–NH_4]^-$* 288.1453. Correlation with the acid **4a** allowed the identification the *cis* and *trans* isomers and the *maj* and *min* anomers of each of the isomers. The ratio of the species were determined by the integration of the signals at 5.18 ppm (*trans(maj)*), 5.18 ppm (t*rans(min)*), 4.14-4.04 ppm (*cis(maj)*), 4.03-3.94 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 95:5 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer exists as a 88:12 mixture of anomers; the *trans* isomer exists as a 70:30 mixture of anomers.

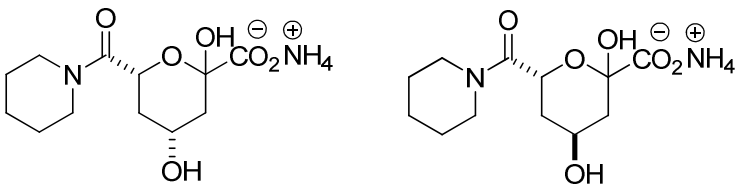
Ammonium (4*R,6*R**)-6-(dibutylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate and ammonium and (4*S**,6*R**)-6-(dibutylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylate 135b**



The ester **134b** (211 mg, 0.612 mmol, 95:5 cis trans) was hydrolysed following *General Procedure I* in MeOH (2 mL) with barium hydroxide monohydrate (74.1 mg, 0.391 mmol) in water (5 mL). The reaction was concentrated and dissolved in water (3 mL) and ammonium sulphate (59.5 mg, 0.391 mmol) added and

gave the *title compound* **135b** (234 mg, 100%, 95:5 cis trans) as an amorphous solid; ν_{max}/cm^{-1} (solid) 3326, 2958, 1632, 1455 and 1005; δ_H (500 MHz, D_2O) 5.16 (0.04H, d, *J* 10.3, 6-$H_{trans(maj)}$), 4.92 (0.02H, d, *J* 10.7, 6-$H_{trans(min)}$), 4.78 (0.86H, d, *J* 11.5, 6-$H_{cis(maj)}$), 4.65-4.56 (0.11H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.32-4.26 (0.04H, m, 4-$H_{trans\ (maj)}$), 4.16-4.05 (0.86H, m, 4-$H_{cis(maj}$), 4.02-3.94 (0.09H, m, 4-$H_{cis(min)}$), 3.51-3.00 (4H, m, $(NCH_2)_{A,B}$), 2.09-1.59 (4H, m, 5-H and 3-H), 1.57-1.37 (4H, m, $(NCH_2\textit{CH}_2)_{A,B}$), 1.29-1.14 (4H, m, $(NCH_2CH_2\textit{CH}_2)_{A,B}$), 0.87-0.78 (6H, m, $(CH_3)_{A,B}$); δ_C (75 MHz, D_2O, *cis(maj)* only) 176.5 and 171.4 (amide and 1-C), 97.8 (2-C), 67.7and 63.8 (6-C and 4-C), 48.1 and 46.4 ($(NCH_2)_{A,B}$), 40.1 and 36.2 (5-C and 3-C), 31.0 and 29.4 ($(NCH_2\textit{CH}_2)_{A,B}$), 20.0 and 19.8 ($(NCH_2CH_2\textit{CH}_2)_{A,B}$), 13.6 and 13.6 ($(CH_3)_{A,B}$); *m/z* ($ES^-$) 316.2 (100%, $[M–NH_4]^-$); HRMS Found $\textit{[M–NH}_4\textit{]}^-$: 316.1757, $C_{15}H_{30}N_2O_6$ requires $\textit{[M–NH}_4\textit{]}^-$ 316.1766. Correlation with the acid **4a** allowed the identification the *cis* and *trans* isomers and the *maj* and *min* anomers of each of the isomers. The ratio of the species were determined by the integration of the signals at 5.16 ppm (*trans(maj)*), 4.92 ppm (t*rans(min)*), 4.16-4.05 ppm (*cis(maj)*), 4.02-3.94 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 95:5 mixture of *cis* and *trans* diastereomers was present and the *cis* isomer exists as a 91:9 mixture of anomers; the *trans* isomer exists as a 67:33 mixture of anomers.

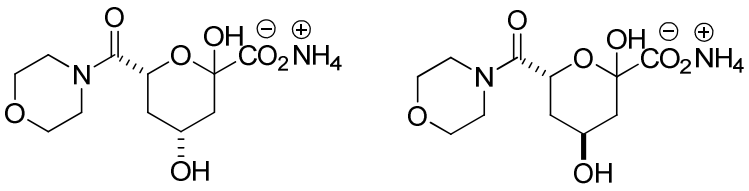
Ammonium (4*R,6*R**)-2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate and ammonium (4*S**,6*R**)-2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate and 135c**



The ester **134c** (108 mg, 0.358 mmol, 88:12 cis trans) was hydrolysed following *General Procedure I* in MeOH (2 mL) with barium hydroxide monohydrate (35.0 mg, 0.185 mmol) in water (5 mL). The reaction was concentrated and dissolved in water (3 mL) and ammonium sulphate (24.5 mg, 0. 185 mmol) added and

gave the *title compound* **135c** (104 mg, 100%, 88:12 *cis :trans*) as a yellow film; ν_{max}/cm^{-1} (film) 3226, 2506, 1759, 1600 and 1447, δ_H (500 MHz, D_2O) 5.31-5.20 (0.12H, m, 6-$H_{trans(maj)}$ and 6-$H_{trans(min)}$), 4.87 (0.77H, d, *J* 12.0, 6-$H_{cis(maj)}$), 4.70-4.61 (0.14H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.33-4.25 (0.09H, m, 4-$H_{trans\ (maj)}$), 4.18-4.06 (0.77H, m, 4-$H_{cis(maj)}$), 4.02-3.93 (0.11H, m, 4-$H_{cis(min)}$), 3.54-3.30 (4H, m, $(NCH_2)_{A,B}$), 2.15-1.98 and 1.68-1.36 (10H, m, 5-H, 3-H, $(NCH_2\mathit{CH_2})_{A,B}$ and $NCH_2CH_2\mathit{CH_2}$); δ_C (75 MHz, D_2O, *cis(maj)* only) 180.6 and 174.5 (amide and 1-C), 101.9 (2-C), 71.9 and 68.0 (6-C and 4-C), 51.2 and 48.3 ($(NCH_2)_{A,B}$), 44.0 and 39.8 (5-C and 3-C), 30.2, 29.6 and 28.1 ($(NCH_2\mathit{CH_2})_{A,B}$ and $NCH_2CH_2\mathit{CH_2}$); *m/z* (ES^-) 272.1 (100%,$[M–NH_4]^-$); HRMS Found $\mathit{[M–NH_4]^-}$: 272.1129, $C_{12}H_{22}N_2O_6$ requires $\mathit{[M–NH_4]^-}$ 272.1140. Correlation with the acid **4a** allowed the identification the *cis* and *trans* isomers and the *maj* and *min* anomers of each of the isomers. The ratio of the species were determined by the integration of the signals at 5.31-5.20 (*trans(maj)* and t*rans(min)*), 4.61 (t*rans(min)* and (*cis(min)*) *and* 4.18-4.06 ppm (*cis(maj)*), 4.02-3.93 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 88:12 mixture of *cis* and *trans* diastereomers was present and the *cis* isomer exists as a 88:12 mixture of anomers; the *trans* isomer exists as a 78:22 mixture of anomers.

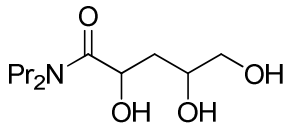
Ammonium (4*R,6*R**)-2,4-dihydroxy-6-(morpholine-4-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate and ammonium (4*S**,6*R**)-2,4-dihydroxy-6-(morpholine-4-carbonyl)tetrahydro-2*H*-pyran-2-carboxylate 135d**



The ester **134d** (299 mg, 0.970 mmol, 78:22 *cis*:*trans*) was hydrolysed following *General Procedure I* in MeOH (2 mL) with barium hydroxide monohydrate (91.0 mg, 0.485 mmol) in water (10 mL). The reaction was concentrated and re-dissolved in water (3 mL) and ammonium sulphate (73.8 mg, 0.485 mmol) added and gave the *title compound* **135d** (264 mg, 93%, 78:22 *cis*:*trans*) as a colourless solid; ν_{max}/cm^{-1} (solid) 3392, 2524, 2159, 1627 and 1444; δ_H (500

MHz, D_2O) 5.22 (0.14H, d, *J* 9.8, 6-$H_{trans(maj)}$), 5.04 (0.08H, d, *J* 11.5, 6-$H_{trans(min)}$), 4.85 (0.67H, d, *J* 11.5, 6-$H_{cis(maj)}$), 4.65-4.57 (0.19H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.27-4.19 (0.14H, m, 4-$H_{trans\ (maj)}$), 4.13-4.03 (0.67H, m, 4-$H_{cis(maj)}$), 4.01-3.94 (0.11H, m, 4-$H_{cis(min)}$), 3.67-3.14 (8H, m, $(NCH_2)_{A,B}$ and $(NCH_2\mathit{CH_2})_{A,B}$), 2.11-1.37 (4H, m, 5-H and 3-H); δ_C (75 MHz, D_2O, *cis(maj)* only) 176.7 and 171.5 (amide and 1-C), 98.2 (2-C), 68.2 and 63.5 (6-C and 4-C), 66.7, 66.5, 46.1, 42.7, 40.3 and 36.0 (5-C, 3-C, $(NCH_2)_{A,B}$ and $(NCH_2\mathit{CH_2})_{A,B}$); *m/z* ($ES^-$) 274.1 (100%,$[M–NH_4]^-$); HRMS Found $[M–NH_4]^-$: 274.0931, $C_{11}H_{20}N_2O_7$ requires $[M–NH_4]^-$ 274.0961. Correlation with the acid **4a** allowed the identification the *cis* and *trans* isomers and the *maj* and *min* anomers of each of the isomers. The ratio of the species were determined by the integration of the signals at 5.22 ppm (*trans(maj)*), 5.04 ppm (t*rans(min)*), 4.13-4.03 ppm (*cis(maj)*), 4.01-3.94 ppm (*cis(min)*). Analysis by 500 MHz ^{1}H NMR revealed that a 78:22 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer exists as a 86:14 mixture of anomers; the *trans* isomer exists as a 65:35 mixture of anomers.

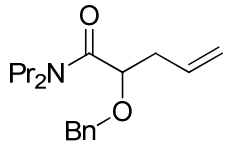
2,4,5-Trihydroxy-*N,N*-dipropylpentanamide 142



Following *General Procedure E* a spatula tip of $K_2OsO_2(OH)_4$, citric acid (2.01 mmol, 386 mg), the amide **123a** (200 mg, 1.00 mmol) and NMO (150 mg, 1.10 mmol) in ^{t}BuOH (2 ml) and water (2.4 ml) were stirred at room temperature for 21 h extracted with EtOAc (3 × 25 mL). Column chromatography, eluting with 3:97 MeOH–EtOAc afforded the *title compound* **142** (180 mg, 77%, 3:2 mixture of diastereomers) as a colourless oil; R_f: 0.33 (5% MeOH–EtOAc); ν_{max}/cm^{-1} (film) 3391, 2965, 2877, 1632, 1463 and 1098; δ_H (400 MHz, $CDCl_3$) 4.62 (1H, dd, *J* 9.7 and 2.1, 2-H_{maj}) and 4.60 (1H, dd , *J* 9.3 and 2.6, 2-H_{min}), 4.09-4.02 (1H, m, 4-H), 3.71-3.48 (3H, m, 5-H and $(NCH_A)_A$), 3.33-3.20 (1H, m, $NCH_B)_A$), 3.13-3.02 (2H, m, $(NCH_{A,B})_B$), 1.86-1.50 (6H, m, $(NCH_2\ \mathit{CH_2})_{A,B}$ and 3-H), 0.96-0.87 (6H, m, $(NCH_2CH_2\ \mathit{CH_3})_{A,B}$); δ_C (100 MHz, $CDCl_3$) 174.5 and 174.0 (1-C), 70.5 and 69.4 (2-C), 67.2 and 66.6 (5-C), 67.1 and 65.8 (4-C), 48.8 and 48.7

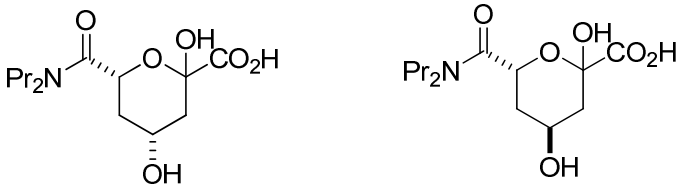
and 47.8 and 47.8 ($(NCH_2)_{A,B}$), 38.8 and 38.6 (3-C), 22.4 and 22.3 and 21.1 and 21.0 ($(NCH_2\textit{CH}_2)_{A,B}$), 11.7 and 11.5 ($(NCH_2CH_2\textit{CH}_3)_{A,B}$); *m/z* ($ES^+$) 256.2 (80%, MNa^+), 234.2 (20%, MH^+); HRMS Found $\textit{MNa}^+$: 256.1512, $C_{11}H_{23}NONa$ requires $\textit{MNa}^+$ 256.1519.

2-(Benzyloxy)-*N,N*-dipropylpent-4-enamide 143



The alkene **123a** (0.502 mmol, 100 mg) in THF (12.0 mL) was added *via* a cannula to a suspension of NaH (60% in mineral oil, 36.1 mg, 1.51 mmol) in DMF (12 ml) at 0 ℃. The mixture was stirred at this temperature for 10 minutes then cooled to –10 ℃ and benzyl bromide (60 µL, 0.502 mmol) added, the reaction was stirred at this temperature for 30 minutes then allowed to warm to room temperature. The reaction was stirred overnight for 17 hthen cooled to 0 ℃ and saturated aqueous sodium bicarbonate solution added and the reaction mixture was washed with DCM (3 × 75 mL). The combined the organic layers were washed with water (3 × 50 mL), dried ($MgSO_4$) and concentrated to give the crude product. Column chromatography, eluting with 3:1 EtOAc–isohexane, afforded the *title compound* **143** (112 mg, 79%) as a colourless oil; R_f: 0.37 (1:1 EtOAc–heptane); ν_{max}/cm^{-1} (film) 1650, 1454 and 1098; δ_H (400 MHz, $CDCl_3$) 7.34-7.27 (5H, m, Ar-H), 5.92-5.82 (1H, m, 4-H), 5.16-5.12 (1H, m, 5-H_A), 5.10-5.08 (1H, m, 5-H_B), 5.64 (1H, d, *J* 11.8, CH_APh), 4.39 (1H, d, *J* 11.8, CH_BPh), 4.24 (1H, dd, *J* 7.9 and 5.5, 2-H), 3.39-3.34 (1H, m, $(NCH_A)_A$), 3.27-3.18 (3H, m, $(NCH_B)_A$ and $(NCH_{A,B})_B$), 2.64-2.57 (1H, m, 3-H_A), 2.52-2.45 (1H, m, 3-H_B), 1.67-1.46 (4H, m, $(NCH_2\ \textit{CH}_2)_{A,B}$), 0.90 (3H, t, *J* 7.4, $(CH_3)_A$), 0.82 (3H, t, *J* 7.4, $(CH_3)_B$); δ_C (100 MHz, $CDCl_3$) 170.4 (1-C), 137.8, 128.4, 127.8 and 127.7 (Ar-C), 133.9 (4-C); 117.6 (5-C), 76.4 (2-C), 70.5 (CH_2Ph), 48.6 and 47.7 $((NCH_2)_{A,B})$, 36.9 (3-C), 22.4 and 20.8 ($(NCH_2\textit{CH}_2)_{A,B}$), 11.4 and 11.1 ($(CH_3)_{A,B}$); m/z ($ES^+$) 290.2 (80%, MH^+), 312.2 (20%, MNa^+); HRMS Found $\textit{MH}^+$: 290.2116, $C_{18}H_{28}NO_2$ requires $\textit{MH}^+$ 290.2115.

(4*R,6*R**)-6-(Dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid and (4*S**,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid 4a**



The *title compound* **4a** can be prepared from the alkene **123a** (60.0 mg, 0.301 mmol) following *General Procedure K* in MeOH (5 mL) quenching with DMS (5 mL). Sodium pyruvate (331 mg, 3.01 mmol) was added to residue in potassium phosphate buffer (1 mL, 20 mM, pH 7.4) followed by E192N (6.98 mg/mL in 20 mM, pH 7.4 potassium phosphate buffer (1 mL) incubating at 35 ºC for 16 h. The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (63.4 mg, 73%, 70:30 *cis*:*trans*) as a colourless film;

*The title compound **4a** can also be prepared using the one-pot reaction*

10 mol% diamine, 10 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 1

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a**(10.8 mg, 39%; *ca*. 80:20 *cis*:*trans*) as a colourless film;

10 mol% diamine, 10 eq. acetaldehyde, dilution factor 3.23, Table 12, entry 2

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (877 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (9.9 mg, 36%; *ca* 80:20 *cis*:*trans*) as a colourless film;

10 mol% diamine, 10 eq. acetaldehyde, dilution factor 5.7, Table 12, entry 3

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (1626 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (4.7 mg, 17%; *ca* 80:20 *cis*:*trans*) as a colourless film;

10 mol% diamine, 10 eq. acetaldehyde, dilution factor 11.1, Table 12, entry 4

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (3300 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The

reaction gave the *title compound* **4a** (9.7 mg, 35%; *ca* 80:20 *cis*:*trans*) as a colourless film;

5 mol% diamine, 10 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 5

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (11.1 mg, 40%; *ca* 80:20 *cis*:*trans*) as a colourless film

20 mol% diamine, 10 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 6

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (7.24mg, 19.0 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (8.6 mg, 31%; *ca* 80:20 *cis*:*trans*) as a colourless film;

50 mol% diamine, 10 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 7

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (18.1mg, 47.5 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC

eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (8.3 mg, 30%; *ca* 80:20 *cis*:*trans*) as a colourless film;

10 mol% diamine, 5 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 8

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (27µL, 0.478 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (10.3 mg, 37%; *ca* 80:20 *cis*:*trans*) as a colourless film;

10 mol% diamine,20 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 9

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (108µL, 1.91 mmol), diamine **69** (3.62 mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (5.3 mg, 19%; *ca* 80:20 *cis*:*trans*) as a colourless film;

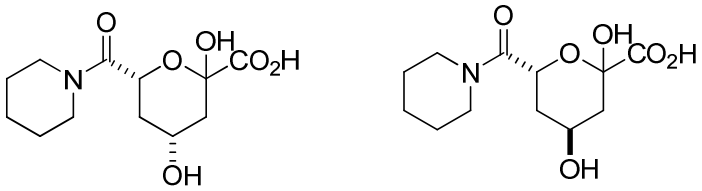
10 mol% diamine, 50 eq. acetaldehyde, dilution factor 1.7, Table 12, entry 10

Glyoxylaldehyde **1a** (15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (270µL, 4.78 mmol), diamine **69** (3.62mg, 9.55 µmol), diluted with sodium pyruvate (105 mg, 1.91 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM

potassium phosphate). The product was purified using preparative HPLC eluting with 15-30% (1% TFA in H_2O–1% TFA in MeCN) over 23 minutes. The reaction gave the *title compound* **4a** (9.2 mg, 33%; *ca* 80:20 *cis*:*trans*) as a colourless film;

ν_{max}/cm^{-1} (film) 3233, 2964, 1764, 1625 and 1458; δ_H (500 MHz, D_2O) 5.18 (0.15H, dd, *J* 9.5 and 3.3, 6-$H_{trans(maj)}$), 5.10 (0.05H, dd, *J* 6.7 and 4.9, 6-$H_{trans(min)}$), 4.83 (0.74H, d, *J* 10.5, 6-$H_{cis(maj)}$), 4.69-4.63 (0.11H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.30 (0.15H, br s, 4-$H_{trans\ (maj)}$), 4.17-4.08 (0.74H, m, 4-$H_{cis(maj)}$), 4.01-3.93 (0.06H, m, 4-$H_{cis(min)}$), 3.64-2.85 (4H, m, NCH_2), 2.16-1.58 (4H, m, H-5 and H-3), 1.58-1.35 (4H, m, NCH_2CH_2), 0.78 (3H, t, *J* 6.4, CH_3), 0.76 (3H, t, *J* 7.3, CH_3);δ_C (125 MHz, D_2O, *cis(maj)* only); 173 and 171 (amide and 1-C), 67.2 (6-C), 62.8 (4-C), 49.4 and 48.1 (NCH_2), 39.1 and 35.5 (5-C and 3-C), 21.6 and 20.0 (NCH_2CH_2), 10.4 and 10.2 (CH_3); *m/z* (ES^-) 288.1 (100%, $[M–H]^-$); HRMS Found $[M–H]^-$: 288.1456, $C_{13}H_{23}NO_6$ requires $[M–H]^-$ 288.1453. TOCSY and NOESY analysis allowed the identification of the major (*maj)* and minor (*min*) anomers of the *cis* and *trans* diastereoisomers. The ratio of the species was determined by the integration of the signals at 5.18 ppm (*trans(maj)*), 5.10ppm (*trans(min)*), 4.17-4.08 ppm (*cis(maj)*) and 4.01-3.93 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 70:30 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer existed as a 92:8 mixture of anomers and the *trans* isomer existed as a 75:25 mixture of anomers.

(4*R,6*R**)-2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylic acid and (4*S**,6*R**)-2,4-dihydroxy-6-(piperidine-1-carbonyl)tetrahydro-2*H*-pyran-2-carboxylic acid 4c**



The *title compound* **4c** can be prepared following *General Procedure K* with alkene **123c** (11.6 mg, 0.0633 mmol) in MeOH(1 mL) quenching with DMS (1 mL). Sodium pyruvate (70 mg, 0.633 mmol) was added to residue in potassium

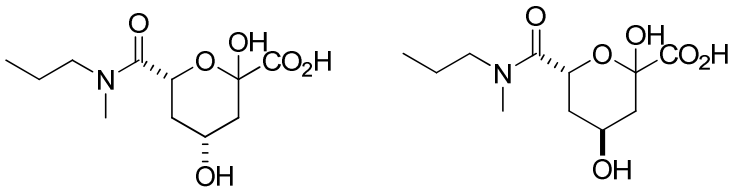
phosphate buffer (0.250 mL, 20 mM, pH 7.4) followed by E192N (6.98 mg/mL in potassium phosphate buffer (0.250 mL, 20 mM, pH 7.4)) incubating for 66 h. The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes.. The reaction gave the *title compound* **4c** (9.1 mg, 53%, 74:26 *cis*:*trans*) as a colourless film.

The *title compound* **4c** can also be prepared using the one-pot reaction Glyoxylaldehyde **1c** (12.3 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol) diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **4c** (11.9 mg, 48%, 66:34 *cis*:*trans*) as a colourless film.

The data presented is for the product prepared using general procedure *K and subsequent E192N-catalysed aldol reaction.*
ν_{max}/cm^{-1} (film)3247, 2941, 1755, 1675, 1632, 1447 and 1202; δ_H (500 MHz, D_2O) 5.13 (0.16H, dd, *J* 9.2 and 3.4, 6-$H_{trans(maj)}$), 5.07 (0.07H, dd, *J* 6.4 and 4.2, 6-$H_{trans(min)}$), 4.84 (0.63H, dd, *J* 11.7 and 1.6, 6-$H_{cis(maj)}$), 4.56-4.51 (0.18H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.48-4.38 (0.16H, m, 4-$H_{trans\ (maj)}$,4.14-4.04 (0.63H, m, 4-$H_{cis(maj)}$), 3.94-3.84 (0.11H, m, 4-$H_{cis(min)}$), 3.50-3.23 and (3H, m, NCH), 3.01 (1H, t, *J* 6.0, NCH), 2.25-1.66 (2H, m, H-5 and H-3), 1.65-1.33 (6H, m, NCH_2CH_2 and $NCH_2CH_2CH_2$); δ_C (125 MHz, D_2O, *cis(maj)* only) 175.8 and 172.1 (amide and 1-C), 70.1 and 65.8 (6-C and 4-C), 49.9, 49.5, 47.4, 46.8, 40.7 and 38.2 (5-C, 3-C and NCH_2), 28.8, 28.0 and 26.5 (NCH_2CH_2 and $NCH_2CH_2CH_2$); *m/z* (ES^-) 272.1 (100%, $[M–H]^-$); HRMS Found *$[M–H]^-$*: 272.1142, $C_{12}H_{19}NO_6$ requires *$[M–H]^-$* 272.1140. Correlation of the ^1H NMR spectrum with that of the acid **4a** allowed the identification of the anomers of the *cis* and *trans* diastereoisomers. The ratio of the species was determined by the integration of the signals at 5.13 ppm (*trans(maj)*), 5.07 ppm (t*rans(min)*), 4.14-4.04 ppm (*cis(maj)*), 3.94-3.84 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 74:26 mixture of *cis* and *trans* diastereomers was present and

that the *cis* isomer existed as an 85:15 mixture of anomers and the *trans* isomer existed as a 63:27 mixture of anomers.

(4*R,6*R**)-2,4-dihydroxy-6-(methyl(propyl)carbamoyl)tetrahydro-2*H*-pyran-2-carboxylic acid and (4*S**,6*R**)-2,4-dihydroxy-6-(methyl(propyl)carbamoyl)tetrahydro-2*H*-pyran-2-carboxylic acid 4e**



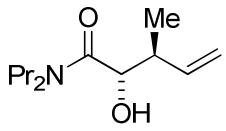
The *title compound* **4e** can be prepared following general procedure *K* with alkene **123e** (30mg, 0.175 mmol) in MeOH (1 mL) quenching with DMS (2 mL). Sodium pyruvate (193 mg, 1.75 mmol) was added to residue in potassium phosphate buffer (0.580 mL, 20 mM, pH 7.4) followed by E192N (6.98 mg/mL in potassium phosphate buffer (0.580 mL, 20 mM, pH 7.4)) incubating for 66 h. The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **4e** (4.75 mg, 19%, 76:24 *cis*:*trans*) as a colourless film.

The *title compound* **4e** can also be prepared using the one-pot reaction Glyoxylaldehyde **1e** (12.3 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol), diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **4e** (11.9 mg, 48%, 57:43 *cis*:*trans*) as a colourless film.

The data presented is for the product prepared using general procedure *K and subsequent E192N-catalysed aldol reaction*

v_{max}/cm^{-1} (film) 3368, 2968, 1744, 1635, 1632, 1439, 1201 and 1142;δ_H (500 MHz, D_2O) 5.23-5.02 (0.26H, m, 6-$H_{trans(maj)}$ and 6-$H_{trans(min)}$), 4.85 (0.65H, br d, *J* 12.0, 6-$H_{cis(maj)}$), 4.62-4.53 (0.20H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.33-4.26 (0.17H, m, 4-$H_{trans\ (maj)}$), 4.17-4.08 (0.65H, m, 4-$H_{cis(maj)}$), 4.00-3.90 (0.11H, m, 4-$H_{cis(min)}$), 3.36-3.11 (2H, m, NCH_2), 3.05-2.96 (3H, m, NCH_3^{rotA}), 2.85-2.81 (4H, m, NCH_3^{rotB}), 2.25-1.59 (4H, m, 5-H and 3-H), 1.59-1.41 (2H, m, NCH_2CH_2), 0.89-0.72 (3H, m, CH_3); δ_C (100 MHz, D_2O, *cis(maj)* only) 171.5, 163.2 and 163.1 (amide and 1-C), 67.7, 67.4, 63.2 and 63.1 (6-C and 4-C), 51.0 and 50.4 (NCH_2), 39.4, 35.6, 35.4, 35.2, 34.1 and 32.8 (3-C, 5-C and NCH_3), 19.7 and 19.3 (NCH_2CH_2), 10.6 and 10.4 ($NCH_2CH_2CH_3$); *m/z* (ES^+) 284.1 (80%, MH^+), 306.1 (20%, MNa^+); HRMS Found MH^+: 284.1111, $C_{11}H_{19}NO_6$ requires MH^+ 284.1105. Correlation of the ^1H NMR spectrum with that of the acid **4a** allowed the identification of the anomers of the *cis* and *trans* diastereoisomers. The ratio of the species were determined by the integration of the signals at 5.23-5.02 ppm *(trans(maj) and (trans(min*)), 4.62-4.53 ppm *(trans(min) and cis(min)),* 4.17-4.08 ppm *(cis(maj)),* 4.00-3.90 ppm *(cis(min)).* Analysis by 500 MHz ^1H NMR revealed that a 76:24 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer exists as a 88:12 mixture of anomeris and as a and as a *ca*. 1:1 mixture of rotomers ; the *trans* isomer exists as a 67:33 mixture of anomers.

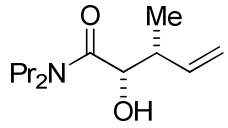
(2*S,3*S**)-2-Hydroxy-3-methyl-*N,N*-dipropylpent-4-enamide 146**



Trans-crotylboronic acid pinacol ester (721 µl, 3.50 mmol) in DCM (5 mL) was added to the glyoxylaldehyde **1a** (500mg, 3.18 mmol) in DCM (15 mL)at –78 ℃. The reaction was stirred overnight at –78 ℃. After 23 hrs the reaction was diluted with water (5 mL) and the aqueous layer was washed with DCM(3 x 20 mL) and the combined the organiclayers was dried ($MgSO_4$) and concentrated to give the crude product. Column chromatography, eluting with 8:2 petrol–EtOAc, afforded the *title compound* **146** (468 mg, 70%) as a colourless oil; R_f:

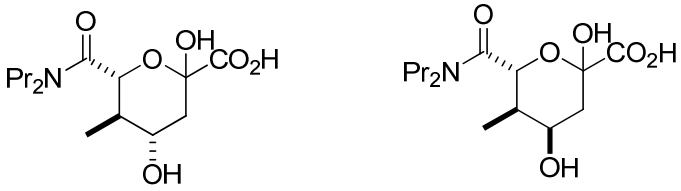
0.29 (1:1, EtOAc–Heptane); ν_{max}/cm^{-1} (film) 2966, 1637, 1450; δ_H (500 MHz, $CDCl_3$) 5.81-5.72 (1H, m, 4-H), 5.04 (1H, d, *J* 10.4, 5-H_A), 4.99 (1H, d, *J* 17.2, 5-H_B), 4.26 (1H, dd, *J* 7.7 and 3.0, 2-H), 3.74-3.61 (2H, m, OH and $(NCH_A)_A$), 3.34-3.22 (1H, $(NCH_A)_B$), 3.16-3.07 (1H, m, $(NCH_B)_A$), 3.00-2.90 (1H, m, $(NCH_B)_B$), 2.46-2.38 (1H, m, 3-H), 1.67-1.52 (4H, m, $(NCH_2$ ($CH_{A,B}$))$_{A,B}$), 1.9 (3H, d, *J* 6.9, 3-CH_3), 0.93 (3H, t, *J* 7.4, (NCH_2 CH_2 CH_3)$_A$), 0.90 (3H, t, *J* 7.4, (NCH_2 CH_2 CH_3)$_B$); δ_C (75 MHz, $CDCl_3$) 173.3 (1-C), 138.0 (4-C), 116.4 (5-C), 72.1 (2-C), 49.0 and 47.9 (NCH_2), 42.4 (3-C), 22.4 and 21.3 (NCH_2 CH_2), 17.6 (3-CH_3), 11.8 and 11.6 (NCH_2 CH_2 CH_3); *m/z* (ES^+) 236.2 (60%, MNa^+), 214.2 (40%, MH^+); HRMS Found MNa^+: 236.1618, requires $C_{12}H_{23}NNaO_2$ MNa^+ 236.1621.

(2*S,3*R**)-2-hydroxy-3-methyl-*N*,*N*-dipropylpent-4-enamide**



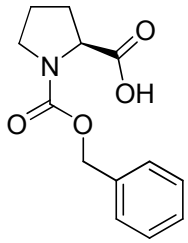
Prepared in an identical manner the *alkene* **146** (shown above) using *cis*-crotylboronic acid pinacol ester (721 µl, 3.50 mmol) and column chromatography, eluting with 8:2 petrol–EtOAc, afforded the *title compound* (394 mg, 59%) as a colourless oil; R_f: 0.29 (1:1 EtOAc–heptane); ν_{max}/cm^{-1} (film) 3100, 2966, 1636 and 1461; δ_H (400 MHz $CDCl_3$) 5.93 (1H, ddd, *J* 17.4, 10.6 and 7.6, 4-H), 5.10 (1H, d, *J* 17.6, 5-H_A), 5.07 (1H, d, *J* 10.6, 5-H_B), 4.31 (1H, dd, *J* 7.9 and 3.4, 2-H), 3.69-3.64 (2H, m, $(NCH_A)_A$ and OH), 3.27 (1H, dt, *J* 14.6 and 7.0, $(NCH_A)_B$), 3.10-2.96 (2H, m, $(NCH_B)_{A,B}$), 2.41-2.36 (1H, m, 3-H), 1.66-1.56 (4H, m, (NCH_2 ($CH_{A,B}$))$_{A,B}$), 0.97 (3H, d, *J* 6.8, 3-CH_3), 0.92 (3H, t, *J* 7.5, NCH_2 CH_2 CH_3)$_A$), 0.90 (3H, t, *J* 7.5, NCH_2 CH_2 CH_3)$_A$) δ_C (100 MHz, $CDCl_3$) 173.5 (1-C), 141.1 (4-C), 115.1 (5-C), 71.5 (2-C), 49.0 and 47.9 (NCH_2), 42.4 (3-C), 22.4 and 21.2 (NCH_2 CH_2), 13.3 (3-CH_3), 11.8 and 11.5 (NCH_2 CH_2 CH_3); m/z (ES^+) 236.2 (75%, MNa^+), 214.2 (25%, MH^+); HRMS Found MNa^+: 236.1216, requires $C_{12}H_{23}NNaO_2$ MNa^+ 236.1621.

(4*S,5*R**,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxy-5-methyltetrahydro-2*H*-pyran-2-carboxylic acid and (4*R**,5*R**,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxy-5-methyltetrahydro-2*H*-pyran-2-carboxylic acid 147**



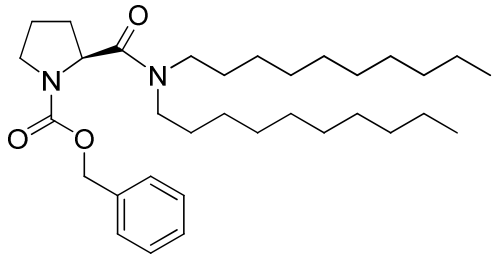
Alkene **146** (50 mg, 0.235 mmol) was reacted following *General Procedure K* in MeOH (10 mL) quenching with DMS (10 mL). Sodium pyruvate (258 mg, 2.35 mmol) was added to residue in potassium phosphate buffer (0.778 mL, 20 mM, pH 7.4) followed by E192N (6.98 mg/mL in 20 mM, pH 7.4 potassium phosphate buffer (0.778 mL) incubating at 35 ºC for 3 days. The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **147**(53 mg, 74%, 85:15, 4*S*:4*R*) as a colourless film; v_{max}/cm^{-1} (film) 3391, 2966, 1743, 1632 and 1458; δ_H (500 MHz, D_2O) 4.95(0.06H, d, *J* 8.55, 6-H^{4R}_{min}), 4.86(0.09H, d, *J* 10.6, 6-H^{4R}_{maj}), 4.53 (0.71H, d, *J* 10.5, 6-H^{4S}_{maj}), 4.22 (0.14H, d, *J* 10.4, 6-H^{4S}_{min}), 4.06-4.01 (0.15H, m, 4-H^{4R}_{min} and 4-H^{4R}_{maj}), 3.72 (0.71H, td, *J* 11.5 and 4.6, 4-H^{4S}_{maj}), 3.55 (0.14H, td, *J* 11.5 and 4.4, 4-H^{4S}_{min}), 3.49-2.85 (4H, m, $(NCH_{A,B})_{A,B}$) 2.17-2.10 (1H, m, 3-H_A), 1.82-1.70 (2H, m, 3-H_B and 5-H)1.65-1.41 (4H, m, $(NCH_2CH_2)_{A,B}$), 0.86 (1H, d, *J* 6.5, 5-C-CH_3), 0.83-0.72 (6H, m, $(NCH_2CH_2CH_3)_{A,B}$) δ_C (125MHz, CD_3OD) 171.6 (C=O), 170.6 (C=O), 97.8 (2-C), 73.6 (6-C), 69.9 (4-C), 50.7 and 49.5 (NCH_2), 41.5 (3-C), 41.3 (5-C), 23.9 and 21.8 (NCH_2CH_2), 13.3 (5-C-CH_3), 11.8 and 11.5 ($NCH_2CH_2CH_3$); *m/z* (ES^+) 302.2 (100%, MH^+); HRMS Found MH^+: 302.1620, $C_{14}H_{24}NO_6$ requires MH^+ 302.1609. TOCSY and NOESY analysis allowed the identification of the anomers of the *4S* and *4R* diastereomers. The ratio of the species were determined by the integration of the signals at: 4.95 ppm (4*R-min*), 4.86 ppm (*4R-maj*), 3.72 ppm (*4S-maj*), 3.55 ppm (4*S-min*). Analysis by 500 MHz ^1H NMR revealed that a 85:15 mixture of 4*S* and 4*R* diastereomers was present and that the 4*S* isomer existed as an 83:17 mixture of anomers and the 4*R* isomer existed as a 63:27 mixture of anomers.

1-[(Benzyloxy)carbonyl]-L-proline[128] 148



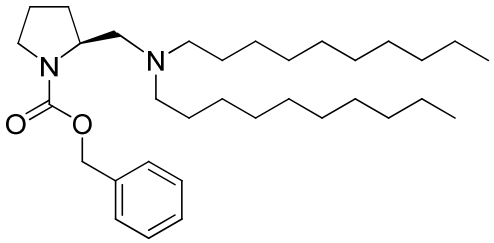
L -Proline (11.6 g, 0.10 mol) was added to a vigorously stirred solution of 2M NaOH (50 mL) at –5 °C followed by addition of benzyl chloroformate (20.5 g, 0.120 mol) and aqueous 4M NaOH (35 mL) simultaneously over 1 h with temperature maintained between –5 °C and 0 °C. The reaction was stirred for 1 h, warmed to room temperature and washed with diethyl ether (2 × 20 mL). The organic layer was then re-extracted with aqueous 2M NaOH solution (3 × 20 mL) and the aqueous phase acidified to pH 2, saturated with NaCl and extracted with EtOAc (3 × 20 mL). The aqueous phase was acidified to pH 2 with aqueous 6M HCl, saturated with NaCl and extracted with EtOAc (3 × 50 mL) and combined with other organic fractions, dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product as a colourless oil. Crystallisation afforded the title compound[128] **148** (16.79 g, 67%) as colourless needles (from EtOAc–Petrol), m.p. 55-57 °C (lit. m.p. 69-74 °C [128]); R_f: 0.57 (EtOAc); $[\alpha]_D^{23}$ –55.2 (c =1.0, MeOH) [lit. [128] $[\alpha]_D^{22}$ –39.9 (c = 1.0, EtOH)]; ν_{max}/cm^{-1} (solid) 3478, 3023, 2629, 1758, 1657, 1437 and 1337; δ_H (500 MHz, $CDCl_3$, ca. 3:2 mixture of rotomers) 9.50 (1H, br s, COOH) 7.43-7.22 (5H, m, Ar-H), 5.25-5.07 (2H, m, Ar-$CH_{A,B}$), 4.48-4.33 (1H, m, 2-H), 3.68-3.42 (2H, m, 5-$H_{A,B}$), 2.34-1.85 (4H, m, 3-$H_{A,B}$ and 4-$H_{A,B}$); δ_C (75 MHz, $CDCl_3$, mixture of rotomers) 178.7 and 177.1 (COOH), 156.2 and 154.9 (carbamate CO) 136.9 and 136.7 (Ar), 128.9 and 128.8 (Ar), 128.6 and 128.4 (Ar), 128.3 and 128.1 (Ar), 67.9 and 67.6 ($ArCH_2$), 59.7 and 59.1 (2-C), 47.3 and 47.1 (5-C), 31.3 and 29.8 (3-C), 24.7 and 23.9 (4-C); *m/z* (ES^+) 250.2 (100%, MH^+).

Benzyl (2S)-2-(didecylcarbamoyl)pyrrolidine-1-carboxylate [86] 149



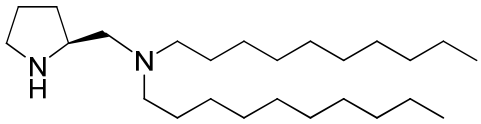
Didecylamine (8.36 g, 28.1 mmol) was added in one portion to a mixture of the acid **148** (7.00 g, 28.1 mmol), 1-[3-(dimethylaminopropyl]-3-ethylcarbodiimide hydrogen chloride (5.30 g, 28.1 mmol) and 4-(dimethylamino)pyridine (3.43 g, 28.1 mmol) in DCM (100 mL) and reaction stirred at room temperature for 23 h. The reaction was washed with 1 M aqueous HCl solution (3 × 30 mL) followed by saturated $NaHCO_3$ solution (3 × 30 mL) the organic phase was then was dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product. Column chromatography, eluting with 2:98 MeOH–DCM, afforded the title compound[86] **149** (11.3 g, 76%) as a colourless oil; R_f: 0.17 (2:98 MeOH–DCM); $[\alpha]_D^{23}$ –17.6 (*c* = 1.0, $CHCl_3$) [lit. [86] $[\alpha]_D^{24}$ –32.5 (*c* =1.0, EtOH)]; ν_{max}/cm^{-1} (film) 2925, 2854, 1712, 1652, 1422, 1415 and 1352; δ_H (500 MHz, $CDCl_3$ ca.1:1 mixture of rotomers) 7.38-7.25 (5H, m, Ar-H), 5.18 (2H, d, *J* 13.3, $ArCH_2$ [rotA]) and 5.07 (2H, d, *J* 16.2, $ArCH_2$ [rotB]) 4.65-4.61(1H, m, 2-H [rotA]) and 4.48(1H, dd, *J* 5.1 and 3.4, 2-H [rotB]), 3.75-3.00 (6H, m, 5-$H_{A,B}$ and 2 × NCH_2), 2.21-2.08 (2H, m, 3-$H_{A,B}$), 1.92-1.83 (2H, m, 4-$H_{A,B}$), 1.55 (32H, m, 16 × CH_2) 0.92-0.84 (6H, m, 2 × CH_3); δ_C (75 MHz, $CDCl_3$, ca.1:1 mixture of rotomers) 172.4 ($(C_{10}H_{21})_2NCO^{rotA}$), 172.3 ($(C_{10}H_{21})_2NCO^{rotB}$), 155.2 (pyrro-$CO^{rotA}$), 154.6 (pyrro-$CO^{rotB}$),137.3 ($Ar^{rotA}$), 137.0 ($Ar^{rotB}$) 129.0, 128.8, 128.7, 128.5, 128.4, 128.2, 128.1 (Ar), 67.6 and 67.2 ($ArCH_2$) 57.5 and 56.8 (2-C^{rotB}), 48.3, and 48.0 (5-C), 47.8 and 47.2(CH_2), 47.1 and 47.0(CH_2), 32.3, 31.6, 31.3, 31.0, 30.1, 30.0, 29.9, 29.8, 29.8, 29.7, 29.7, 29.4, 28.1, 28.1, 27.4, 27.2, 24.7, 23.9, 23.1, 22.4, 14.6 (2 ×CH_3); *m/z* (ES^+) 551.4 (60%, MNa^+) 529.4 (40%, MH^+).
Full assignment of the carbon spectrum was not possible due to rotomers.

Benzyl (2S)-2-[(didecylamino)methyl]pyrrolidine-1-carboxylate [86] 150



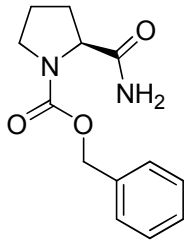
1 M Borane.THF complex in THF (37.8 mL, 37.6 mmol) was added dropwise to the amide **149** (10.0 g, 18.1 mmol) at 0 °C and allowed to return to room temperature slowly before heating to reflux under argon for 4 h. The reaction was quenched at 0 °C with 1 M aqueous HCl solution (5 mL), neutralised with 2M aqueous NaOH solution and extracted with ether (3 × 70 mL). The combined organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product. Column chromatography, eluting with 10:90 raising to 30:70 EtOAc–Petrol, afforded the title compound [86] **150** (7.76 g, 80%) as a colourless oil; R_f: 0.54 (70:30 EtOAc–Petrol); $[\alpha]_D^{23}$ –71.2 (*c* = 1.0, $CHCl_3$) [lit. [86] $[\alpha]_D^{24}$ –5.17 (*c* = 1.0, EtOH)]; ν_{max}/cm^{-1} (film) 2924, 2854, 2388, 2313, 1704, 1455, 1408 and 1355; δ_H(500 MHz, $CDCl_3$ 7.39-7.29 (5H, m Ar-H) 5.24-5.07 (2H, m, Ar-CH_2) 4.31-4.24 (1H, m, 2-H) 3.48-3.33 (2H, m, 5-$H_{A,B}$) 3.06-2.05 (8H, m, 3-$H_{A,B}$ and 3 × NCH_2) 1.90-1.79 (2H, m, 4-$H_{A,B}$) 1.75-1.13 (32H, m, 16 × CH_2) 0.88 (6H, t, *J* 6.8, 2 × CH_3); δ_C (75 MHz, $CDCl_3$, ca. 3:2 mixture of rotomers) 155.3 (CO^{rotA}), 155.0 (CO^{rotB}) 137.1 (Ar) 128.9 (Ar) 128.4 (Ar) 128.2 (Ar) 67.2 ($ArCH_{2\ rotA}$), 65.2 ($ArCH_2{}^{rotB}$) 64.1 ($CH_2{}^{rotA}$) 62.0 ($CH_2{}^{rotB}$) 58.6, 54.3 (2-C^{rotA}), 53.8 (2-C^{rotB}), 46.7, 33.6, 32.9, 32.3, 30.0, 29.9, 29.8, 27.8, 24.2, 23.5, 23.3, 23.1, 14.6 (2 × CH_3); *m/z* (ES^+) 515.5 (70%, MH^+) 551.5 (30%, MNa^+); HRMS Found MH^+, 515.4583. $C_{13}H_{15}N_5O_2$ requires MH^+ 515.4571. Full assignment of the carbon spectrum was not possible due to rotomers.

_N_-decyl-_N_-[(2_S_)-Pyrrolidin-2-ylmethyl]decan-1-amine [86] 69



The amine **150** (7.40 g, 4.38 mmol) in ethanol (100 mL) with 5% Pd/C (0.75 g) was hydrogenated for 29 h, filtered through a bed of Celite and concentrated under reduced pressure. Column chromatography, eluting with 10:90 EtOAc–hexanes raising to 10:90 NEt_3–EtOAc, afforded the title compound[86] **69** (4.04 g, 73%) as a pale yellow oil; R_f 0.1 (9:1 EtOAc– hexanes); $[\alpha]_D^{23}$ + 15.25 (c = 1.0, $CHCl_3$) [lit. $^{86}[\alpha]_D^{24}$ +17.22 (c = 1.0, $CHCl_3$)]; ν_{max}/cm^{-1} (film) 2924, 2853, 1465 and 1403; δ_H (500 MHz, $CDCl_3$) 3.22-3.15 (1H, m, 2-H) 2.98-2.92 (1H, m, NHCHCH_AHN) 2.87-2.81 (1H, m, NHCHCH_BN) 2.48-2.45 (2H, m, 5-$H_{A,B}$) 2.38-2.25 (4H, m, 2 × NCH_2) 1.90-1.69 (4H, m, 3-$H_{A,B}$ and 4-$H_{A,B}$) 1.44-1.20 (32H, m, 16 × CH_2) 0.88 (6H, t, J 6.8, 2 × CH_3) δ_C (75 MHz, $CDCl_3$) 60.1 (NCH_2CH), 56.8 (2-C), 55.0 (CH_2) 46.0 (CH_2), 32.3 (CH_2), 30.1 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 28.0 (CH_2), 27.6 (CH_2), 25.2 (CH_2), 23.1 (CH_2), 14.6 (2 × CH_3); m/z (ES^+) 381.4 (100%, MH^+); HRMS Found MH^+: 381.4204. $C_{25}H_{52}N_2$ requires MH^+ 381.4203.

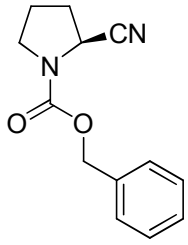
Benzyl (2_S_)-2-carbamoylpyrrolidine-1-carboxylate[129] 151



The acid **148** (5.00 g, 20.1 mmol) was added to a solution of ditertbutyldicarbonate (5.70 g, 26.1 mmol) and ammonium bicarbonate (1.91 g, 24.1 mmol) in MeCN (20 mL). Anhydrous pyridine (0.953 g, 12.0 mmol) was added and stirred for 5 h at room temperature and the reaction concentrated to approximately 5% of volume. The reaction was diluted with water (50 mL), extracted with EtOAc (3 × 50 mL), dried ($MgSO_4$) and concentration to gave the

crude product. Column chromatography eluting with EtOAc afforded the title compound[129] **151** (4.25 g, 85%) as a colourless oil; R_f: 0.23 (EtOAc); $[\alpha]_D^{23}$ –75.7 (c =1.4, $CHCl_3$) [lit. $^{129}[\alpha]_D^{25}$ –82.8 (c = 0.5, $CHCl_3$)]; ν_{max}/cm^{-1} (film) 3390, 3196, 2955, 1692, 1682, 1498, 1417, and 1358;δ_H (500 MHz, $CDCl_3$, ca. 4:1 mixture of rotomers) 7.43-7.25 (5H, app s, Ar-H), 6.70 (app s), 6.02 (app s), 5.89-5.64 (2H, m, NH_2), 5.20-5.08 (2H, m, Ar-CH_2), 4.39-4.25 (1H, m, 2-H), 3.60-3.38 (2H, m, 5-$H_{A,B}$), 2.39-1.83 (4H, m, 3-$H_{A,B}$ and 4-$H_{A,B}$); δ_C (75 MHz, $CDCl_3$ mixture of rotomers) 175.8 and 175.0 ($CONH_2$), 156.5 and 156.3 (COOCH$_2$) 136.7 and 136.4 (Ar), 129.0 and 128.7 (Ar), 128.6 and 128.3 (Ar), 128.3 and 128.1 (Ar), 68.1 and 67.8 (ArCH$_2$), 61.1 and 60.8 (NCH), 47.9 and 47.4 (NCH) 47.3 (NCH$_2$), 31.5 and 29.0 (NCHCH_2), 24.9 and 24.1 (NCH$_2CH_2$); m/z (ES^+) 271.1 (100%, MNa^+); HRMS Found MNa^+: 271.1055. $C_{13}H_{16}N_2O_3$ requires MNa^+271.1053.

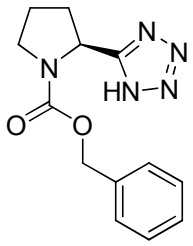
Benzyl (2*S*)-2-cyanopyrrolidine-1-carboxylate [129] 152



The amide **151** (4.26 g, 17.2 mmol) was stirred in dry DMF (45 mL) at 0 °C for 30 min and cyanuric acid (2.06 g, 11.2 mmol) was added in one portion. The reaction was stirred for 1 h and allowed to warm to room temperature and stirred for further 17 h. The reaction mixture was diluted with water (60 mL), ether (60 mL) added and the phases separated, and the aqueous layer extracted with EtOAc (4 × 50 mL). The combined organic fractions were washed with brine (3 × 50 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product. Column chromatography, eluting with DCM to give the title compound[129] **152** (3.67 g, 92%) as a yellow oil; R_f 0.44 (DCM); $[\alpha]_D^{23}$ –108.4 (c = 1, $CDCl_3$) [lit. $^{129}[\alpha]_D^{25}$ –89.0 (c = 1, $CHCl_3$)]; ν_{max}/cm^{-1} (film) 2958, 2889, 2240, 1708, 1411 and 1358; δ_H (500 MHz, $CDCl_3$, ca. 1:1 mixture of rotomers) 7.44-7.30 (5H, m, Ar-H), 5.25-5.13 (2H, m, Ar-CH_2), 4.59 (1H, d, J

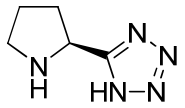
6.0, 2-H^{rotA}) and 4.53 (1H, d, *J* 6.0, 2-H^{rotA}), 3.66-3.53 (1H, m, 5-H_A), 3.50-3.36 (1H, m, 5-H_B), 2.34-2.00 (4H, m, 3-$H_{A,B}$ and 4-$H_{A,B}$); δ_C (75 MHz, $CDCl_3$ mixture of rotomers) 154.7 and 154.0 (CON), 136.5 and 136.4 (Ar), 129.0 (Ar), 128.7 (Ar) 128.6 (Ar), 119.3 and 119.2 (NCH*CN*) 68.2 and 68.1 ($ArCH_2$), 47.9 and 47.4 (2-C), 46.8 and 46.4 (5-C), 32.1 and 31.2 (3-C) 25.1 and 24.2 (4-C); *m/z* (ES^+) 253.1 (100%, MNa^+); HRMS Found MNa^+: 253.0950 $C_{13}H_{16}N_2O_2$ requires, MNa^+ 253.0947.

Benzyl (2*S*)-2-(1′*H*-tetrazol-5′-yl)pyrrolidine-1-carboxylate [129] 153



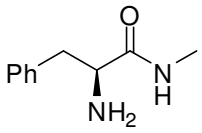
Sodium azide (757 mg, 11.7 mmol) was added in one portion to a suspension of the nitrile **152** (2.00 g, 8.69 mmol) and triethylamine hydrogen chloride (1.49 g, 10.8 mmol) in toluene (15 mL) and heated to 90 ℃ for 3 days. The reaction was cooled to room temperature and diluted with water (20 mL) and the phases separated. The aqueous phase was cooled to 0 ℃ and quenched with a 20% *w/w* aqueous solution of sodium nitrate (7 mL) followed by 20% *w/w* aqueous solution of sulfuric acid (6 mL), extracted with EtOAc (3 × 20 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give the title compound[129] **153** (1.95g, 83%) as a colourless oil, which was used in the next step without purification; R_f: 0.54 (EtOAc); $[\alpha]_D^{23}$ –137.6 (*c* = 1, $CHCl_3$) [lit. $^{129}[\alpha]_D^{25}$ –91.3 (*c* = 1.27, $CHCl_3$)]; ν_{max}/cm^{-1} (film) 3000, 2400, 1703, 1418 and 1358; δ_H (500 MHz, $CDCl_3$) 7.41-7.20 (5H, m, Ar-H), 5.38-4.98 (3H, m, Ar-CH_2 and 2-H), 3.66-3.48 (2H, m, 5-$H_{A,B}$), 2.86-2.74 (1H, m, 3-H_A), 2.50-1.84 (3H, m, 3-H_B and 4-$H_{A,B}$); δ_C (75 MHz, $CDCl_3$ ca. 5:1 mixture of rotomers) 156.8 (CO and 5′-C), 136.0 (Ar), 129.0 (Ar), 128.8 (Ar), 128.3 and 128.2 (Ar), 68.5 and 68.3 ($ArCH_2$), 53.1 and 51.8 (2-C), 47.7 and 47.5 (5-C), 33.4 and 29.9 (3-C), 25.0 and 23.9 (4-C); *m/z* (ES^+) 296.1 (100%, MNa^+); HRMS Found MNa^+: 296.1121. $C_{13}H_{15}N_5O_2$ requires, MNa^+ 296.1118.

5′-[(2*S*)-pyrrolidin-2-yl]-1′*H*-tetrazole [129] 67



A solution of the tetrazole **153** (2.87 g, 10.5 mmol) and 10% Pd/C in AcOH–water (9:1, 140 mL) was hydrogenated at 1 atm for 84 h. The reaction mixture was filtered through a bed of Celite and the solvent removed azetropically with toluene to give the crude product which was crystallised from MeOH–toluene to afford the title compound[129] **67** (1.23 g, 85%) as an off-white solid; R_f: 0.57 (3:3:1 ^{t}BuOH–H_2O–AcOH); $[\alpha]_D^{23}$ –12.1 (*c* = 0.63, $CHCl_3$) [lit. [129] $[\alpha]_D^{25}$ –10.5 (*c* = 0.63, $CHCl_3$)];δ_H (500 MHz, DMSO-d_6) 9.17 (1H, br s, NH), 4.75 (1H, t, *J* 7.7, 2-H), 3.33-3.20 (2H, m, 5-H), 2.39-2.30 (1H, m, 3-H_A), 2.21-2.12 (1H, m, 3-H_B), 2.10-1.97 (2H, m, 4-$H_{A,B}$); δ_C (75 MHz, DMSO-d_6) 158.2 (5′-C), 55.4 (2-C), 45.0 (5-C), 30.3 (3-C), 23.5 (4-C); ν_{max}/cm^{-1} (solid) 3054, 2446, 2159, 2023, 1977 and 1636; *m/z* (ES^+) 139.1 (100%, M^+); HRMS Found M^+, 139.0852. $C_5H_9N_5$ requires, M^+ 139.0858.

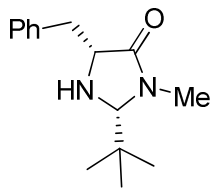
D-Phenylalanine *N*-methyl amide[131] 155



Triethylamine (6.06 mL, 43.9 mmol) was added dropwise to a suspension of D-phenylalanine methyl ester hydrogen chloride (9.46 g, 43.9 mmol) in THF (100 mL) and reaction stirred at room temperature for 1 h. The reaction was filtered and the mother liquor concentrated under reduced pressure. 8M Ethanolic methylamine solution (150 mL) was added immediately and reaction stirred for 24 h at room temperature and concentrated under reduced pressure to give the crude product as a yellow oil. Crystallisation from DCM–pentane afforded the title compound **155**[131] (7.44 g, 95%) as pale yellow needles; m.p. 60-63 °C; R_f: 0.14 (EtOAc); $[\alpha]_D^{23}$ –24.3 (*c* = 1.2, MeOH); ν_{max}/cm^{-1} (solid) 3294, 2944, 2530, 2154, 2029, 1976, 1644 and 1400; δ_H (500 MHz, $CDCl_3$) 7.34-7.18 (5H, m, Ar-

H), 3.61 (1H, dd, *J* 9.5 and 3.9, $PhCH_A$), 3.29 (1H, dd, *J* 13.7 and 3.9, $PhCH_B$), 2.82 (3H, d, *J* 4.7, CH_3), 2.68 (1H, dd, *J* 13.7 and 9.5, *CH*(NH_2)), 1.44 (2H, br s, NH_2) δ_C (75 MHz, $CDCl_3$) 175.3 (CO), 138.4 (Ar-C), 129.7 (Ar-CH), 129.1 (Ar-CH), 127.2 (Ar-CH), 56.9 (NH_2CH), 41.4 ($ArCH_2$), 26.3 (CH_3); *m/z* (ES^+) 179.1 (60%, MH^+) 201.1 (40%, MNa^+); HRMS Found *MH*$^+$, 179.1184. $C_{10}H_{14}N_2O$ requires, *MH*$^+$179.1179.

(2*R*,5*R*)-5-Benzyl-2-*tert*-butyl-3-methylimidazolidin-4-one [79, 132] 68



Method A

A mixture of trimethylacetaldehyde (73 µL, 0.67 mmol) and amide **155** (124 mg, 0.674 mmol) in toluene (4 mL) was added *via* a cannular to a dry flask fitted with Dean–Stark apparatus containing $FeCl_3$ (22 mg, 0.14 mmol) and reaction refluxed for 26 h. The mixture was cooled to room temperature, diluted with brine (10 mL) and extracted with EtOAc (2 × 50 mL) and organic phase was dried ($MgSO_4$) and concentrated under reduced pressure to give the crude *cis* and *trans* products in 1:1 ratio. Column chromatography, eluting with 7:3 Petrol–EtOAc gave the title compound [132] **68** (75 mg, 45%) as a pale yellow film.

Method B

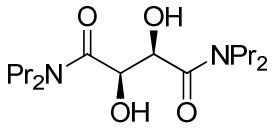
The *trans product can be recycled as follows*

(2*R*,5*S*)-5-Benzyl-2-*tert*-butyl-3-methylimidazolidin-4-one hydrogen chloride (10.4 g, 48.5 mmol) in ether (150 mL) was washed with saturated $NaHCO_3$ solution (150 mL) then concentrated under reduced pressure and dissolved in THF (75 mL) and $FeCl_3$ (1.57 g, 9.70 mmol) and reaction stirred at room temperature for 5 days. The reaction was diluted with DCM (200 mL) and washed with brine (200 mL) followed by H_2O (3 × 200 mL) and organic layer was dried ($MgSO_4$) and concentrated under reduced pressure Column

chromatography, eluting with 3:7 EtOAc–Petrol, afforded the title compound[132] **68** (949 mg, 8%) as pale yellow needles (from DCM–Petrol),

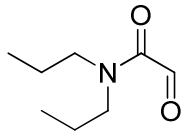
m.p. 76-80 °C; R_f: 0.06 (3:7 EtOAc–Petrol); $[\alpha]_D^{27}$ −12.2 (*c* =1.0 , CH_2Cl_2) [lit. [132]$[\alpha]_D^{25}$ –10.1 (*c* = 1.0, CH_2Cl_2)]; ν_{max}/cm^{-1} (film) 2962, 1745, 1701, 1454, 1397 and 1365; δ_H (300 MHz, $CDCl_3$,) 7.33-7.18 (5H, m, ArH), 4.06 (1H, s, 2-H), 3.75-3.65 (1H, m, 5-H), 3.16 (1H, dd, *J* 13.8 and 4.1, $PhCH_A$), 2.94 (1H, dd, *J* 13.8 and .7.4, $PhCH_B$), 2.91 (3H, m, NCH_3), 0.83 (9H, s, $C(CH_3)_3$; δ_C (75 MHz, $CDCl_3$) 175.3(4-C) , 137.9, 129.6, 128.6, 126.7 (Ar), 82.5, 59.4, 38.2, 35.1, 30.7 and 25.0; *m/z* (ES^+) 247.1 (100%, MH^+).

(2*R,3*R**)-Dihydroxy-*N*^{1},*N*^{1},*N*^{4},*N*^{4}-tetrapropyl-succinamide 156a**



The amide **127a** (3.27 g, 11.5 mmol) was reacted following *General Procedure E* with NMO in water (1.48 g, 12.7 mmol in 1.5 mL), citric acid (4.42 g, 23.0 mmol), $K_2OsO_2(OH)_4$ (9 mg**,** 0.023 mmol), in 1:1 ^{t}BuOH–water (14 mL) for 25 h. Column chromatography eluting with 1:1 EtOAc–DCM gave the *title compound* **156a** (3.32 g, 91%) as a colourless plates; m.p. 108-113°C (from IPA); R_f: 0.30 (1:1 EtOAc–DCM); ν_{max}/cm^{-1} (solid) 3390, 2969, 2874, 1632, 1472 and 1408; δ_H (500 MHz, $CDCl_3$) 4.59 (2H, s, CH), 4.21 (2H, br s, OH) 3.59-3.43 (4H, m, $N(CH_2)_A$), 3.28-3.11 (4H, m, $N(CH_2)_B$), 1.69-1.53 (8H, m, $(NCH_2\mathit{CH_2})_{A, B}$), 0.93 (6H, t, *J* 7.7, $(CH_3)_A$), 0.91 (6H, t, *J* 7.7, $(CH_3)_B$); δ_C (75 MHz, $CDCl_3$) 171.0 (CO), 70.5 (CH), 49.5 ($N(CH_2)_A$) 48.5 ($N(CH_2)_B$), 22.5 (($NCH_2\mathit{CH_2}$)$_A$), 21.1 (($NCH_2\mathit{CH_2}$)$_B$), 11.8 ((CH_3)$_A$), 11.6 ((CH_3)$_B$); *m/z* (ES^+) 339.2 (80%, MNa^+) 317.2 (20%, MH^+); HRMS Found MNa^+: 339.2257. $C_{16}H_{32}N_2O_4$ requires MNa^+ 339.2254.

2-oxo-*N,N*-dipropylacetamide 1a



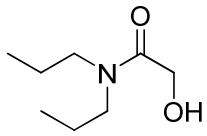
Water (2.8mL) was added dropwise to a vigorously stirred suspension of sodium periodate (948 mg, 4.42mmol) and diol **156a** (700 mg, 2.21 mmol) in DCM (11.2 mL) and reaction stirred for 3 h at room temperature. Sodium sulphate was added to the mixture and the reaction filtered and evaporated under reduced pressure to give the crude *title compound* as a colourless film which was used in all organocatalysed aldol reaction without any further purification.

In all one-pot chemistry the title compound was prepared using an alternative method

Water (25mL, 20% by volume) was added dropwise to a vigorously stirred suspension of sodium periodate (6.76 g, 31.6mmol) and diol **156a** (5.00 g, 15.8 mmol) in DCM (100 mL) and reaction stirred for 20 h at room temperature. The reaction was filtered through a hydrophobic frit, silica added and evaporated under reduced pressure. Column chromatography, eluting with 6:4 EtOAc–hexane, afforded the *title compound* **1a** (4.62 mg, 93%) as colourless oil; R_f: 0.33 (4:6 EtOAc–petrol).

the *title compound* **1a** was characterised as the hydrate; ν_{max}/cm^{-1} (film) 3370, 2965, 1654, 1466, and 1412; δ_H (300 MHz, D_2O) 5.56 (1H, s, hydrate), 3.32 (2H, t, *J* 7.2, $(NCH_2)_A$), 3.27 (2H, t, *J* 7.0, $(NCH_2)_B$), 1.65-1.45 (4H, m, $(NCH_2CH_2)_{A,B}$), 0.85-0.79 (6H, m, $(CH_3)_{A,B}$); δ_C (75 MHz, D_2O) 170.5 (amide), 84.1 (hydrate), 49.1 and 48.2 ($(NCH_2)_{A,B}$), 21.8 and 20.4 ($(NCH_2CH_2)_{A,B}$), 10.9 and 10.6 ($(CH_3)_{A,B}$); *m/z* (ES^+) 158.1 (100% MH^+); HRMS Found MH^+: 158.1177, $C_8H_{15}NO_2$ requires MH^+158.1176.

2-Hydroxy-*N,N*-dipropylacetamide[146] 157

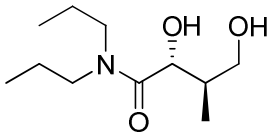


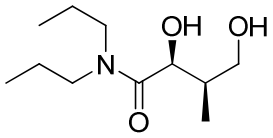
The glyoxylaldehyde **1a** (115 mg, 0.737 mmol) was dissolved in DCM (5 mL) and $NaBH_4$ (279 mg, 7.37 mmol) added and reaction stirred at room temperature for17 h. The reaction was cooled to 0 ºC and quenched with a 2M aqueous solution of NH_4Cl. The reaction was extracted with EtOAc (3 ×50 mL) and organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afforded the title compound **157** (83 mg, 70%) as a colourless film.

Alternatively the title compound can be prepared as follows

Sodium methoxide 0.162 g, 2.44 mmol) was added in one portion to a solution of 2-(dipropylamino)-2oxoethyl acetate (2.48 g, 12.2 mmol) in MeOH (20 mL) and reaction stirred at room temperature for 17 h. The reaction mixture was then diluted with water (10 mL), extracted with EtOAc (3 × 20 mL) and the combined organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure to give the alcohol[146] **157** (1.90 g, 98%) as a colourless oil; R_f: 0.52 (1:1 EtOAc–Petrol); (Found: C, 60.4; H, 10.85, N 8.6%; $C_8H_{17}NO_2$ requires C, 60.4; H, 10.76, N 8.8.%); ν_{max}/cm^{-1} (film) 3409, 2965, 2876, 1645, 1465 and 1404; δ_H (500 MHz, $CDCl_3$, mixture of rotomers, *ca*. 1:1) 4.15 (2H, s, CH_2O), 3.72-3.68 (1H, m, OH), 3.35 (2H, t, *J* 7.7, $N(CH_2)_A$), 3.04 (2H, t, *J* 7.7, $N(CH_2)_B$), 1.64-1.54 (4H, m, $(NCH_2CH_2)_{A, B}$), 0.91 (3H, t, *J* 7.7, $(CH_2CH_3)_A$), 0.89 (3H, t, *J* 7.3, $(CH_2CH_3)_B$); δ_C (75 MHz, $CDCl_3$) 171.2 (CO), 60.1, (CH_2OH), 48.0 $(NCH_2)_A$), 47.8 (($NCH_2)_B$), 22.1 (($NCH_2CH_2)_A$), 21.1 (($NCH_2CH_2)_B$), 11.8 (($CH_2CH_3)_A$), 11.6 (($CH_2CH_3)_B$); *m/z* (EI^+) 159.1 (100%, M^+); HRMS Found M^+, 159.1252. $C_8H_{17}NO_2$ requires, M^+ 159.1259.

(2*R,3*R**)-2,4-dihydroxy-3-methyl-*N*,*N*-dipropylbutanamide *anti*-158 and (2*R**3*S**)-2,4-dihydroxy-3-methyl-*N*,*N*-dipropylbutanamide *syn*-158**





***L*-proline-catalysed aldol reactions**

50 mol% L-proline; 1 eq propionaldehyde; DCM; Table 9 entry 1

Following *General Procedure A*, with L-proline (72 mg, 0.63 mmol) the glyoxylaldehyde **1a** (199 mg, 1.27 mmol), propionaldehyde (92 µL, 1.27 mmol), in DCM (5 mL) the reaction was performed at room temperature for 68 h. $NaBH_4$ (232 mg, 6.13 mmol) was added and the reaction stirred for 1 h 45 min at room temperature. Column chromatography eluting with 1:1 DCM–Ether gave the *title compound* **158** as a colourless film (66 mg, 25%, 62:38 *syn*:*anti*)

50 mol% L-proline; 82 eq propionaldehyde; DCM ; Table 9 entry 2

Following *General Procedure A*, with L-proline (36 mg, 0.316 mmol) the glyoxylaldehyde **1a** (99 mg, 0.632 mmol), propionaldehyde (4 mL, 55.4 mmol), in DCM (25 mL) the reaction was performed at room temperature for 98 h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and the reaction stirred for 1 h 45 min at room temperature. Column chromatography eluting with 9:1 EtOAc–Tol gave the *title compound* **158** as a colourless film (4 mg, 3%, 84:16 *syn*:*anti*)

Tetrazole 67-catalysed aldol reactions

*20 mol% tetrazole **67**; 82 eq propionaldehyde; DMF; Table 9 entry 12*

Following *General Procedure B* propionaldehyde (4 mL, 55.4 mmol), the glyoxylaldehyde **1a** (99 mg, 0.632 mmol), and tetrazole **67** (18 mg, 0.13 mmol) were reacted in DMF (25 mL) for 145 h at +4 ºC. $NaBH_4$ (1.06 g, 27.7 mmol) was added and reaction stirred for 21 h at room temperature. Column

chromatography eluting solvent with 9:1 EtOAc–Tol gave the *title compound* **158** as a colourless film (8 mg, 6%, 40:60 *syn*:*anti*)

*20 mol% tetrazole **67**; 82 eq propionaldehyde; DMSO; Table 9 entry 13*

Following *General Procedure B* propionaldehyde (4 mL, 55.4 mmol), the glyoxylaldehyde **1a** (99 mg, 0.632 mmol), and tetrazole **67** (18 mg, 0.13 mmol) were reacted in DMSO (25 mL) for 74 h at RT. $NaBH_4$ (1.20 g, 31.6 mmol) was added and reaction stirred for 21 h at room temperature. Column chromatography with 7:3 EtOAc–Tol gave the *title compound* **158** as a colourless film (27 mg, 20%, 78:22 *syn*:*anti*)

Diamine 69-catalysed aldol reactions

*20 mol% diamine **69**; 2eq propionaldehyde; TFA additive; H_2O; Table 9 entry 14*

Following *General Procedure C,* TFA (5 µL, 0.06 mmol) as an additive with the diamine **69** (24 mg, 0.063 mmol), glyoxylaldehyde **1a** (99 mg, 0.63 mmol) and propionaldehyde (91 µL, 1.3 mmol) in H_2O (1.25 mL) the reaction was stirred at room temperature for 142 h. $NaBH_4$ (481 mg, 6.32 mmol) was added and stirred at room temperature for 7.5 h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (3 mg, 0.2%, 31:69 *syn*:*anti*)).

*20 mol% diamine **69**; 2eq propionaldehyde; TFA additive; Phosphate buffer pH 7.4; Table 9 entry 15*

Following *General Procedure C,* TFA (5 µL, 0.06 mmol) as an additive with the diamine **69** (24 mg, 0.063 mmol), glyoxylaldehyde **1a** (99 mg, 0.63 mmol) and propionaldehyde (91 µL, 1.3 mmol) in 20 mM Phosphate buffer pH 7.4 (1.25 mL) the reaction was stirred at room temperature for 142 h. $NaBH_4$ (481 mg, 6.32 mmol) was added and stirred at room temperature for 7.5 h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* as colourless film (16 mg, 19%, 64:36 *syn*:*anti*).

*20 mol% diamine **69**; 2eq propionaldehyde; Sc(OTf)$_3$ additive; H_2O; Table 10 entry 2*

Following *General Procedure C,* Sc(OTf)$_3$ (31 mg, 0.063 mmol) as an additive with the diamine **69** (24 mg, 0.063 mmol), glyoxylaldehyde **1a** (99 mg, 0.63 mmol) and propionaldehyde (91 µL, 1.3 mmol) in H_2O (1.25 mL) the reaction was stirred at room temperature for 158 h. $NaBH_4$ (481 mg, 6.32 mmol) was added and stirred at room temperature for 20h. Column chromatography eluting with 6:4 Ether–DCM gave the *title compound* **158** as colourless film (2 mg, 0.2%, 12:88 *syn*:*anti*).

*20 mol% diamine **69**; 2eq propionaldehyde; Sc(OTf)$_3$ additive; Phosphate buffer pH 7.4; Table 10 entry 3*

Following *General Procedure C,* Sc(OTf)$_3$ (31 mg, 0.063 mmol) as an additive with the diamine **69** (24 mg, 0.063 mmol), glyoxylaldehyde **1a** (99 mg, 0.63 mmol) and propionaldehyde (91 µL, 1.3 mmol) in 20 mM Phosphate buffer pH 7.4 (1.25 mL) the reaction was stirred at room temperature for 206h. $NaBH_4$ (481 mg, 6.32 mmol) was added and stirred at room temperature for 20h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound***158** as colourless film (11 mg, 8%.24:76:*syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde TFA additive; Phosphate buffer pH 7.4; Table 10 entry 5*

Following *General Procedure C,* TFA (5 µL, 0.063 mmol) as an additive with the diamine **69** (24 mg, 0.063 mmol), glyoxylaldehyde **1a** (99 mg, 0.63 mmol) and propionaldehyde (91 µL, 6.3 mmol) in 20 mM Phosphate buffer pH 7.4 (1.25 mL) the reaction was stirred at room temperature for 26h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 20h. Column chromatography eluting with 3% MeOH in DCM gave the *title compound* **158** as colourless film (62 mg, 45%, 68:32 *syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde; 100 mM Phosphate buffer pH 6.0; Table 11 entry 1*

Following *General Procedure D,* the glyoxylaldehyde **1a** (115 mg, 0.737 mmol) and propionaldehyde (531µL, 7.37 mmol) diamine **69** (28mg, 0.0737 mmol), in 100 mM Phosphate buffer pH 6 (1.53 mL) the reaction was stirred at room temperature for 25h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 137h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (47 mg, 41%, 62:38 *syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde; 100 mM Phosphate buffer pH 6.4; Table 11 entry 2*

Following *General Procedure D, the* glyoxylaldehyde **1a** (115 mg, 0.737 mmol) and propionaldehyde (531µL, 7.37 mmol) diamine **69** (28 mg, 0.0737 mmol), in 100 mM Phosphate buffer pH 6.4 (1.53 mL) the reaction was stirred at room temperature for 25h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 137h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (28 mg, 26%, 82:18 *syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde; 100 mM Phosphate buffer pH 7.0; Table 11 entry 3*

Following *General Procedure D, the* glyoxylaldehyde **1a** (115 mg, 0.737 mmol) and propionaldehyde (531µL, 7.37 mmol) diamine **69** (28mg, 0.0737 mmol), in 100 mM Phosphate buffer pH 7.0 (1.53 mL) the reaction was stirred at room temperature for 25h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 137h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (37 mg, 33%, 68:33 *syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde; 100 mM Phosphate buffer pH 7.4; Table 11 entry 4*

Following *General Procedure D, the* glyoxylaldehyde **1a** (115 mg, 0.737 mmol) and propionaldehyde (531µL, 7.37 mmol) diamine **69** (28mg, 0.0737 mmol), in

100 mM Phosphate buffer pH 7.4 (1.53 mL) the reaction was stirred at room temperature for 25h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 137h. Colum chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (37 mg, 33%,75:25 *syn*:*anti*).

*10 mol% diamine **69**; 10eq propionaldehyde; 100 mM Phosphate buffer pH 8.0; Table 11 entry 5*

Following *General Procedure D, the* glyoxylaldehyde **1a** (115 mg, 0.737 mmol) and propionaldehyde (531µL, 7.37 mmol) diamine **69** (28mg, 0.0737 mmol), in 100 mM Phosphate buffer pH 8.0 (1.53 mL) the reaction was stirred at room temperature for 25h. $NaBH_4$ (1.20 g, 31.6 mmol) was added and stirred at room temperature for 137h. Column chromatography eluting with 7:3 EtOAc–Tol gave the *title compound* **158** as colourless film (37 mg, 33%, 81:19 *syn*:*anti*).

*The anti diastereomer can also be prepared from the alkene **146***

The alkene **146** (40 mg, 0.186 mmol) was ozonolysed following *General Procedure F* in MeOH (5 mL) with DMS(5 mL). The residue was dissolved in EtOH (5 mL) and $NaBH_4$ added (282 mg, 7.44 mmol) and stirred at room temperature for 2h 45 min the reaction was cooled to 0ºC and 1 M aqueous hydrochloric acid added. The reaction mixture was extracted with EtOAc (3 × 20 mL) and the combined organics were dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product. Column chromatography, eluting with 9:1 EtOAc–Petrol, afforded the *title compound anti*-**158** (4 mg, 13%) as colourless film.

Syn diastereomer **158**

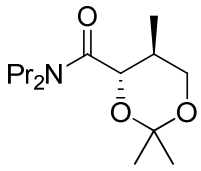
Colourless film; R_f: 0.37 (1:1 DCM–Ether); δ_H (300 MHz, $CDCl_3$,) 4.66 (1H, dd, *J* 7.4 and 2.1, 2-H), 3.85 (1H, d, *J* 7.4, 2-OH), 3.72-3.61 (3H, m, $N(CH_A)_A$ and 4-H), 3.32 (1H, dt, *J* 14.5 and 7.9, $(NCH_A)_B$), 3.13-2.95 (2H, m, $(NCH_B)_A$ and $(NCH_B)_B$), 2.21 (1H, br s, 4-OH), 1.97-1.87 (1H, m, 3-H), 1.69-1.50 (4H, m, $(CH_2\mathit{CH_2})_{A,B}$), 0.93 (3H, t, *J* 7.4 , $(CH_2\mathit{CH_3})_A$,), 0.90 (3H, t, *J* 7.4 , $(CH_2\mathit{CH_3})_B$), 0.78 (3H, t, *J* 6.9 , $(CH\mathit{CH_3})$); δ_H (75 MHz, $CDCl_3$) 173.3 (CO), 68.2 (2-C), 65.6

(4-C) 48.2 ($(NCH_2)_A$), 47.3 ($(NCH_2)_B$), 38.3 (3-C), 21.9 ($(NCH_2\mathit{CH_2})_A$), 20.7 ($(NCH_2\mathit{CH_2})_B$), 11.3 ($(CH_2\mathit{CH_3})_A$), 11.1 ($(CH_2\mathit{CH_3})_B$) 9.1 ($CH\mathit{CH_3}$);□$_{max}$/cm^{-1} (film) 3400, 2965, 2935, 2876, 1632, 1466 and 1401; *m/z* (EI^+) 240.2 (80%, MNa^+) 218.2 (20%, MH^+); HRMS Found MH^+, 218.15757,. $C_{11}H_{23}NO_3$ requires, MH^+ 218.1751.

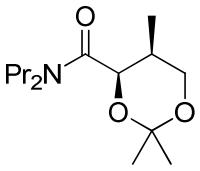
Anti diastereomer **158**

Colourless film; R_f: 0.30 (1:1 DCM–Ether);□$_{max}$/cm^{-1} (film) 3391, 2964, 2932, 2876, 1626, 1458 and 1381; δ_H (500 MHz, $CDCl_3$,) 4.53 (1H, dd, *J* 7.3, 3.5, 2-H), 3.76-3.70 (1H, m, 4-H), 3.69-3.59 (2H, m, 2-OH and $(NCH_A)_A$), 3.54-3.47 (1H, m, 4-H), 3.35-3.21 (1H, m, $(NCH_A)_B$), 3.15-3.00 (2H, m, $(NCH_B)_A$ and $(NCH_B)_B$), 2.01-1.92 (1H, m, 3-H), 1.69-1.53 (4H, m, $(CH_2\mathit{CH_2})_{A,\,B}$), 1.04 (3H, d, *J* 6.8, 3-CH_3), 0.93 (3H, t, *J* 7.3 , $(CH_2\mathit{CH_3})_{A,}$), 0.91 (3H, t, *J* 7.3 , $(CH_2\mathit{CH_3})_B$); δ_C (75 MHz, $CDCl_3$) 174.6 (CO), 72.0 (C-2), 63.8 (C-4) 48.8 ($(NCH_2)_A$), 47.9 ($(NCH_2)_B$), 39.8 (C-3), 22.0 ($(NCH_2\mathit{CH_2})_A$), 20.6 ($(NCH_2\mathit{CH_2})_B$), 14.8 ($CH\mathit{CH_3}$)11.4 ($(CH_2\mathit{CH_3})_A$), 11.2 ($(CH_2\mathit{CH_3})_B$); *m/z* (EI^+) 218.0 (100%, MH^+); HRMS Found MH^+, 218.15757,. $C_{11}H_{23}NO_3$ requires, MH^+ 218.1751.

(4*S,5*S**)-2,2,5-trimethyl-*N,N*-dipropyl-1,3-dioxane-4-carboxamide163**
(4*R,5*S**)-2,2,5-trimethyl-*N,N*-dipropyl-1,3-dioxane-4-carboxamide 164**



163



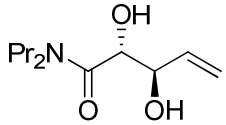
164

PPTS (6 mg, 2.39 µmol) was added to a 8:3 mixture of alcohols *anti*- and *syn*-**158** (18.6 mg, 82.8 µmol) in a 1:1 mixture of acetone/2,2-dimethylpropane (4 mL) and heated to 56 ℃ for 2 days. The reaction was cooled to room temperature and dry NEt_3 (75 µL, 0.497 mmol) stirred for 5 minutes then diluted with water (20 mL), extracted with EtOAc (3 × 20 mL) and the combined organics were dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product. Column chromatography, eluting with 7:3 EtOAc–Petrol,

afforded the *title compound* **163** (14.5 mg, 68%) as colourless film**;** R_f: 0.24 (3:7 EtOAc–petrol); v_{max}/cm^{-1} (film) 2963, 1652, 1456 and 1382; δ_H (500 MHz, $CDCl_3$) 4.18 (1H, d, *J* 10.7, 4-H), 3.81 (1H, dd, *J* 11.6 and 5.4, 6-H_{eq}), 3.56 (1H, t, *J* 11.6, 6-H_{ax}), 3.39 (1H, ddd, *J* 14.8, 10.2 and 5.5, (NCH_A)$_A$), 3.32-3.21 (2H, m, ($NCH_{A,B}$)$_B$), 3.16 (1H, ddd, *J* 14.8, 10.2 and 5.5, (NCH_B)$_A$), 2.41-2.30 (1H, m, 5-H), 1.72-1.52 (4H, m, NCH_2*CH_2*), 1.47 (3H, s, 2-C-CH_3), 1.40 (3H, s, 2-C-CH_3), 0.92 (3H, t, *J* 7.4, (NCH_2CH_2*CH_3*)$_A$), 0.88 (3H, t, *J* 7.4, (NCH_2CH_2*CH_3*)$_A$), 0.77 (3H, d, *J* 6.6, 5-C-CH_3); δ_C (125 MHz, $CDCl_3$); 168 (C=O), 98.5 (2-C), 73.9 (4-C), 65.7 (6-C), 49.1 and 47.9 (NCH_2), 30.0 and 29.4 (2-C-CH_3), 22.6, 20.8 and 18.6 (NCH_2*CH_2* and 5-C), 12.8 (5-C-CH_3), 11.4 and 11.2 (NCH_2CH_2*CH_3*); *m/z* (ES^+) 280.2 (100%, MNa^+); HRMS Found *MNa*$^+$: 280.1872, $C_{14}H_{27}NO_3$ requires *MNa*$^+$ 280.1883.

and the *title compound* **164** (5.5 mg, 28%) as colourless film; R_f: 0.24 (3:7 EtOAc–petrol); v_{max}/cm^{-1} (film) 3965, 1645, 1464 and 1381; δ_H (500 MHz, $CDCl_3$) 4.73 (1H, d, *J* 3.0, 4-H), 4.12 (1H, dd, *J* 11.5 and 3.0, 6-H), 3.63 (1H, dd, *J* 11.5 and 2.2, 6-H), 3.44 (1H, ddd, *J* 15.0, 10.3 and 5.6, (NCH_A)$_A$), 3.31-3.09 (3H, m, (NCH_B)$_A$ and ($NCH_{A,B}$)$_B$), 2.01-1.93 (1H, m, 5-H), 1.68-1.51 (4H, m, NCH_2*CH_2*), 1.46 (6H, s, 2-CH_3), 1.14 (3H, d, *J* 6.8, 5-C-CH_3), 0.90 (3H, t, *J* 7.3, ((NCH_2CH_2*CH_3*)$_A$), 0.89 (3H, t, *J* 7.3, (NCH_2CH_2*CH_3*)$_B$); δ_C (75 MHz, $CDCl_3$); 169.5 (C=O), 99.6 (2-C), 72.8 (4-C), 66.9 (6-C), 49.6 and 49.0 (NCH_2), 31.7 and 29.5 (2-C-CH_3), 23.0, 21.1 and 19.5 (NCH_2*CH_2* and 5-C), 12.4 (5-C-CH_3), 11.9 and 11.5 (NCH_2CH_2*CH_3*); *m/z* (ES^+) 280.2 (100%, MNa^+); HRMS Found *MNa*$^+$: 280.1873, $C_{14}H_{27}NO_3$ requires *MNa*$^+$ 280.1883.

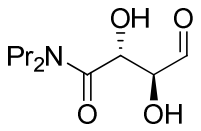
(2*R*, 3*R*)-2,3-Dihydroxy-pent-4-enoic acid dipropylamide[118] 170



Trifluoroacetic acid–water (9:1, 60 mL) was added to the amide **169** (750 mg, 2.93 mmol) and evaporated under reduced pressure. Column chromatography, eluting with 1:1 Petrol–EtOAc, afforded the title compound [118] **170** (365 mg,

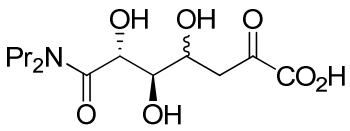
58%) as a off white needles; R_f: 0.16 (6:4, petrol-EtOAc); $[\alpha]_D^{22}$: + 13.8º (c. 0.8, $CHCl_3$) [lit.[118] $[\alpha]_D^{22}$: + 17.1 (c. 0.84, $CHCl_3$)]; ν_{max}/cm^{-1} (solid) 3328, 2967, 1626, 1474, 1431, 1380 and 1317; δ_H (500 MHz, $CDCl_3$) 5.98 (1H, ddd, *J* 16.7, 10.3 and 5.6, 4-H), 5.12 (1H, d, *J* 16.7, 5-H_A), 4.99 (1H, d, *J* 10.3, 5-H_B), 4.20 (1H, d, *J* 4.3, 2-H), 4.11 (2H, br s, 2 × OH), 4.05-4.00 (1H, m, 3-H), 3.37-3.29 (1H, m, $(NCH_A)_A$), 3.25-3.15 (1H, m, $(NCH_A)_B$), 3.01-2.93 (1H, m, $(NCH_B)_A$), 2.90-2.82 (1H, m, $(NCH_B)_B$), 1.47-1.28 (4H, m, 2 × $(NCH_2\mathit{CH_2})$), 0.72 (3H, d, *J* 7.7, $(CH_3)_A$), 0.69 (3H, d, *J* 7.3, $(CH_3)_B$); *m/z* (ES^+) 216.1 (100%, MH^+).

(2*R*,3*S*)-2,3-dihydroxy-4-oxo-*N,N*-dipropylbutanamide[118] 35a



Oxygen (15min, 0.6 psi), ozone (6 min, 0.6 psi), and oxygen (15 min, 0.6 psi,) were bubbled sequentially through a stirred solution of **170** (150 mg, 0.688 mmol) in methanol (10 mL) at –78 ºC. DMS (0.15 mL) was added, and the mixture was allowed to warm to room temperature, once the ozonide was quenched the solvent removed. The title compound was used in the subsequent reaction immediately.

(5*R*, 6*R*)-6-Dipropylcarbamoyl-2-oxo-4,5,6-trhydroxy-hexanoic acid[118] 36a

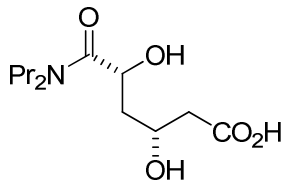


The aldehyde **35a** (150mg, 0.688 mmol)was dissolved in an aqueous potassium phosphate buffer (10 mL, 20 mM, pH 7.4), sodium pyruvate (164 mg, 1.5 mmol) was added, the pH was adjusted to 7.4 with 2M aqueous solution of NH_4OH, and E192N (3 mL, 1.62 mg/mL in 50 mM Tris and 50 mM NaCl) was added. After stirring at room temperature for 20h further E192N (3 mL, 1.62 mg/mL in 50 mM Tris and 50 mM NaCl) was added. After at 24h the pH adjusted to 5.5

with aqueous ammonium (1 M) and pyruvate decarboxylases (3 µL, 14 mg/mL from Bakers yeast) added. After stirring for a further 16 h the reaction was concentrated and ion exchange performed (DOWEX strongly basic X1-2 formate form) eluting from 0 to1.0 M formic acid, the resultant residue was dissolved in an aqueous potassium phosphate buffer (20 mM, pH 7.4) the pH adjusted to 5.5 with aqueous ammonium (1 M) and pyruvate decarboxylases (20 µL, 14 mg/mL from Bakers yeast) added and reaction stirred for 3 days. Dialysis overnight followed by ion exchange (DOWEX strongly basic X1-2 formate form) eluting from 0 to1.0 M formic acid and column chromatography, eluting with 1:3:5 H_2O–IPA–EtOAc, afforded the acid[118] **36a** (42 mg, 20%) as a colourless film; R_f: 0.6 (5:2:2, EtOAc–H_2O–AcOH); v_{max}/cm^{-1} (film) 3372, 2967, 1612 and 1437; δ_H (500 MHz, D_2O) 4.37 (1H, d, *J* 8.5, 6-$H_{fur(maj)}$), 3.74-3.11 (1H, m, 4-$H_{fur(maj)}$), 3.39 (1H, t, *J* 9.4, 5-$H_{fur(maj)}$), 3.31-3.11 (4H, m, NCH_2), 1.91 (1H, dd, *J* 13.3 and 6.0, 3- $H_{Bfur(maj)}$), 1.70 (1H, t, *J* 12, 3- $H_{Afur(maj)}$), 1.40-1.20 (4H, m, NCH_2CH_2), 0.64-0.54 (6H, m, $NCH_2CH_2CH_3$); *m/z* (ES^+) 328.3 (80%, MNa^+), 306.3 (20%, MH^+).

The reaction produces two diastereomers which have four forms each. The major product is the *S* diastereomer in one of its furanose forms. Full interpretation of the seven alternative products not achieved but spectra obtained were identical to authentic samples.[118]

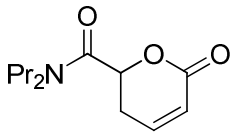
(3*R,5*R**)-6-(dipropylamino)-3,5-dihydroxy-6-oxohexanoic acid 172**



An aqueous hydrogen peroxide solution (24 µL, 0.212 mmol, 30% *w/w* solution) was added to a solution of acid **4a** (24 mg, 0.0830 mmol, 82:12 *cis*:*trans*) in water (500 µL). The pH was adjusted to at pH 2 with a 1M solution of aqueous hydrochloric acid at 0 °C. The reaction was warmed to room temperature and stirred for 2 h. The reaction was neutralised with 2M aqueous sodium

hydroxide solution, cooled to 0 °C and sodium sulphite (133 mg, 1.06 mmol) added stirred for 20 min. The reaction mixture was then tested for peroxide with starch-iodide paper before the reaction was then concentrated and ion exchange performed (DOWEX strongly basic 1X8 200-400 formate form) eluting from 0 to2.0 M formic acid which gave the *title compound* **172** (13.5 mg, 63%, 78:22 *anti*:*syn*) as a colourless film; R_f: 0.41 (5:2:2 EtOAc–H_2O–IPA); ν_{max}/cm^{-1} (solid) 3406, 2966, 1718, 1626, 1401 and 1301; δ_H (500 MHz, D_2O) 4.03-3.94 (1H, m, 5-H_{anti}), 3.88-3.81 (1H, m, 5-H_{syn}), 4.25-4.02 (1H, m, 3-H), 3.45-2.96 (4H, m, NCH_2), 2.58-2.40 (2H, m, 2-H), 1.72-1.62 (2H, m, 4-H), 1.62-1.36 (4H, m, $(NCH_2CH_2)_{A,B}$), 0.80 (3H, t, *J* 7.7, $(CH_3)_A$), 0.77 (3H, t, *J* 7.7, $(CH_3)_B$); δ_C (100 MHz, D_2O) 178.7 (C=O), 177.8 (C=O), 67.8 and 67.3 (3-C and 5-C), 51.8 and 50.7 (NCH_2), 44.9 and 43.7 (4-C and 6-C), 24.4 and 23.0 (NCH_2CH_2), 13.3 and 13.1 (CH_3); *m/z* (ES^+) 262.2 (100%, MH^+); HRMS Found MH^+: 262.1655, $C_{12}H_{23}NO_5$ requires MH^+ 262.1649.

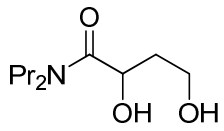
6-oxo-*N,N*-dipropyl-3,6-dihydro-*2H*-pyran-2-carboxamide 171



TFA (5 µL, 28.4 µmol) was added to the acid **172** (13.0 mg, 49.8 µmol) in MeCN (0.50 mL) and stirred at room temperature for 3h. The reaction was heated to 50 °C for 20.5 h and further TFA added (20 µL, 113 µmol). The reaction was stirred for a further 7 h and heated to 80 °C for 18 h. The reaction was cooled and diluted with water (20 mL) and extracted with EtOAc (3 × 20 mL), the combined organics were dried ($MgSO_4$) and concentrated. Column chromatography, eluting with 1:1 Petrol–EtOAc raising to EtOAc afforded the *title compound* **171** (3.07 mg, 28%) as a colourless oil; R_f: 0.26 (EtOAc); ν_{max}/cm^{-1} (film) 3397, 2917, 1732, 1651 and 1384; δ_H (500 MHz, $CDCl_3$) 6.95 (1H, ddd, *J* 10.1, 5.3 and 3.4, 4-H), 6.04 (1H, ddd, *J* 10.1, 2.4 and 1.5, 5-H), 5.17 (1H, dd, *J* 9.5 and 4.8, 2-H), 3.57-3.12 (4H, m, NCH_2), 3.00 (1H, dddd, *J* 18.8, 9.5, 3.4 and 2.4, 3-H_A), 2.52 (1H, ddt, *J* 18.8, 5.3 and 1.5, 3-H_B), 1.81-1.51 (4H, m, NCH_2CH_2), 0.94 (3H, t, *J* 7.5, $(CH_3)_B$), 0.90 (3H, t, *J* 7.5, $(CH_3)_A$); δ_C (75

MHz, $CDCl_3$); 179.3 and 166.2 (6-C and amide), 145.2 (4-C), 120.7 (5-C), 74.1 (2-C), 49.3 and 48.0 (NCH_2), 25.9 (3-C), 22.3 and 20.6 ($NCH_2\mathit{CH_2}$), 11.3 and 11.2 (CH_3); *m/z* (ES^+) 226.1 (100% MH^+); HRMS Found MH^+: 226.1448, $C_{12}H_{19}NO_3$ requires MH^+ 226.1438.

2,4-dihydroxy-*N,N*-dipropylbutanamide 173



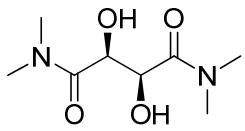
Alkene **123a** (10 mg, 0.0498 mmol) was ozonolysed following *General Procedure* F in MeOH (1.7 mL) quenching with DMS (1.7 mL). The reaction was then concentrated and residue dissolved in dry EtOH (1 mL) and then $NaBH_4$ (19 mg, 0.498 mmol) and stirred at RT for 2.5 h. The reaction was cooled to 0 ℃ and quenched with a 1M aqueous solution of hydrochloric acid. The reaction was then extracted with EtOAc (3 × 10 mL) and the combined organics dried (Mg_2SO_4), and concentrated. Column chromatography, eluting with EtOAc afforded the *title compound* **173** (4 mg, 40%) as as a colourless oil;

The *title compound* can also be prepared as follows:
Acetaldehyde (54 µL, 0.955mmol) was added to a mixture of diamine **69** (3.6 mg, 9.55 µmol) and glyoxylaldehyde **1a** (15 mg, 95.5 µmol) in buffer (307 µL, pH 7.4, 20 mM potassium phosphate) and reaction stirred for 25 h at RT. $NaBH_4$ (37 mg, 0.955 mmol) was added and reaction stirred for 2.5 h at RT then quenched with a 1M aqueous solution of hydrochloric acid. EtOAc (3 × 20 mL) and the combined organics dried (Mg_2SO_4), and concentrated. Column chromatography, eluting with EtOAc afforded the *title compound* **173** (6 mg, 32%) as as a colourless oil;

R_f: 0.18 (9:1 EtOAc–MeOH); v_{max}/cm^{-1} (film) 3392, 2965, 1633, 1465 and 1402; δ_H (500 MHz, $CDCl_3$) 4.57-4.50 (1H, br s, 2-H), 3.95-3.92 (3H, br s, 4-H and OH), 3.59 (1H, ddd, *J* 14.0, 9.0 and 6.6, $(NCH_A)_A$), 3.24 (1H, dt, *J* 14.2 and 8.1, $(NCH_A)_B$), 3.12-3.03 (2H, m, $(NCH_B)_A$ and $(NCH_B)_B$), 2.34 (1H, br s, OH), 1.92-

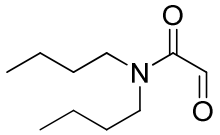
1.83 (1H, m, 3-H_A), 1.73-1.50 (5H, m, 3-H_B), 0.93 (3H, t, *J* 7.3, $(CH_3)_A$), 0.90 (3H, t, *J* 7.5, $(CH_3)_B$); δ_C (75 MHz, $CDCl_3$); 172 (1-C), 64.6 (2-C), 57.8 (4-C), 46.3 and 45.4 ($(NCH_2)_{A,B}$), 35.3 (3-C), 19.9 and 18.6 ($(NCH_2CH_2)_{A,B}$), 9.3 and 9.1 ($(CH_3)_{A,B}$); *m/z* (ES^+) 204.2 (60%, MH^+), 226.1 (40%, MNa^+); HRMS Found *MH*$^+$: 204.1590, $C_{10}H_{21}NO_3$ requires *MH*$^+$ 204.1594.

(2*R,3*R**)-dihydroxy-*N*^{1},*N*^{1},*N*^{4},*N*^{4}-tetramethylsuccinamide[147] 156h**



The amide **127h** (800 mg, 4.71 mmol) was reacted following *General Procedure E* with NMO in water (606 mg, 5.17 mmol in 1 mL), citric acid (1.82 g, 9.48 mmol), $K_2OsO_2(OH)_4$ (88 mg, 0.188 mmol), in 1:1 ^{t}BuOH–water (10 mL) for 3 days. Column chromatography eluting with 9:1 EtOAc–MeOH gave the title compound [147]**156h** (93 mg, 10%) as a colourless film; R_f : 0.31 (8:2 EtOAc–MeOH);□$_{max}$/cm^{-1} (film) 3369, 2931, 1634 and 1504; δ_H(300 MHz, CD_3OD) 4.53 (2H, s, CH), 3.08 (6H, s, $(NCH_3)_A$), 2.82 (6H, s, $(NCH_3)_B$); *m/z* (ES^+) 227.1 (100%, MNa^+); HRMS Found *MNa*$^+$: 227.1006. $C_8H_{12}N_2O_4$ requires, *MNa*$^+$ 227.1002.

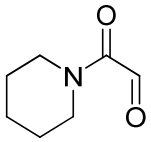
***N,N*-Dibutyl-2-oxoacetamide 1b**



The amide **127b** (1.03 g, 3.03 mmol) was ozonolysed following *General Procedure F* in DCM (50 mL) quenching with DMS (30 mL). The reaction was allowed to warm to room temperature and after stirring overnight the reaction was concentrated to give the crude product. Column chromatography eluting with 3:7 to 1:1 EtOAc–petrol afforded the *title compound* **1b** (881 mg, 78%) as a colourless oil.

The title compound **1b** exists as 42% hydrate and 8% aldehyde in $CDCl_3$ with a mixture of other oligomers; R_f: 0.42 (1:1 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3364, 2959, 1724, 1651, 1466 and 1413; δ_H (300 MHz, $CDCl_3$) 9.49 (0.08 H, s, aldehyde), 5.65 (0.42 H, s, hydrate), 3.62-3.02 (4H, m, $(NCH_2)_{A,B}$), 1.74-1.42 (4H, m, $(NCH_2\textit{CH}_2)_{A,B}$), 1.42-1.22 (4H, m, $(NCH_2CH_2\textit{CH}_2)_{A,B}$), 0.98-0.87 (6H, m, $(CH_3)_{A,B}$); δ_C (75 MHz, $CDCl_3$, hydrate only) 167.8 (amide), 86.1 (hydrate), 47.2 and 46.6 ($(NCH_2)_{A,B}$), 31.2 and 29.7 ($(NCH_2\textit{CH}_2)_{A,B}$), 20.5 and 20.3 ($(NCH_2CH_2\textit{CH}_2)_{A,B}$), 14.2 and 14.1 ($(CH_3)_{A,B}$); *m/z* ($ES^+$) 204.2 (100%, MH^+); HRMS Found MH^+: 204.1598, $C_{10}H_{21}NO_3$ requires MH^+ 204.1594.

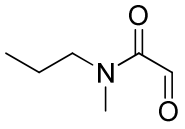
2-oxo-2-(piperidin-1-yl)acetaldehyde[150] 1c



The amide **127c** (1.45 g, 6.42 mmol) was ozonolysed following *General Procedure F* in DCM (30 mL) quenching with DMS (30 mL). The reaction was allowed to warm to room temperature and after overnight stirring concentrated to give the crude product. Column chromatography eluting with 7:3 to 9:1 EtOAc–petrol afforded the title compound[150] **1c** (448 mg, 69%) as a colourless oil.

The title compound **1c** exists as 40% hydrate and 10% aldehyde in $CDCl_3$ with a mixture of other oligomers; R_f: 0.25 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3369, 2938, 1735, 1649, 1447 and 1252; δ_H (300 MHz, $CDCl_3$) 9.51 (0.10 H, s, aldehyde), 5.65 (0.40 H, s, hydrate), 3.69-3.30 (4H, m, $(NCH_2)_{A,B}$), 1.77-1.45 (6H, m, $(NCH_2\textit{CH}_2)_{A,B}$ and $(NCH_2CH_2\textit{CH}_2)_{A,B}$); δ_C (75 MHz, $CDCl_3$, hydrate only) 166.2 (amide), 86.3 (hydrate), 46.6 and 44.5 ($(NCH_2)_{A,B}$), 26.2, 25.9 and 24.7 ($(NCH_2\textit{CH}_2)_{A,B}$ and $(NCH_2CH_2\textit{CH}_2)_{A,B}$); *m/z* ($ES^+$) 160.1 (60%, MH^+), 182.1 (20%, MNa^+); HRMS Found MH^+: 160.0974, $C_7H_{13}NO_3$ requires MH^+ 160.0968.

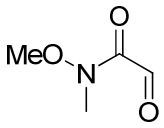
_N_-methyl-2-oxo-_N_-propylacetamide 1e



The amide **127e** (928 mg, 4.10 mmol) was ozonolysed following *General Procedure F* in DCM (50 mL) quenching with DMS (30 mL). The reaction was allowed to warm to room temperature and after 2 h concentrated to give the crude product. Column chromatography eluting with 7:3 EtOAc–petrol afforded the *title compound* **1e** (839 mg, 79%) as a colourless oil.

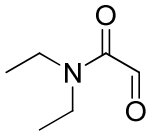
The title compound **1e** exists as 60% hydrate in D_2O with a mixture of other oligomers; R_f: 0.29 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3363, 2963, 1732, 1648 and 1459; δ_H (500 MHz, D_2O, *ca*. 1:1 mixture of rotomers) 5.45 (0.30 H, s, hydrate^{rotA}), 5.43 (0.30 H, s, hydrate^{rotB}), 3.21 (1H, t, *J* 7.3, NCH_A^{rotA}), 3.18-3.10 (1H, m, NCH_A^{rotB} and NH_B), 2.92, 2.85, 2.76 and 2.73 (3H, s, NCH_3), 1.56-1.32 (2H, m, $NCH_2\mathit{CH_2}$), 0.80-0.63 (3H, m, $NCH_2CH_2\mathit{CH_3}$); δ_C (75 MHz, D_2O, hydrate only) 170.7 and 170.5 (amide), 84.5 and 84.1 (hydrate), 51.1 and 50.4 (NCH_2), 34.8 and 33.8 (NCH_3), 20.9 and 19.9 ($NCH_2\mathit{CH_2}$), 10.7 and 10.4 ($NCH_2CH_2\mathit{CH_3}$); *m/z* (ES^+) 170 (80%, MNa^+), 148.1 (20%, MH^+); HRMS Found MH^+: 148.0968, $C_6H_{14}NO_3$ requires MH^+ 148.0968.

_N_-methoxy-_N_-methyl-2-oxoacetamide[149] 1f



The title compound **1f** was prepared following *General Procedure F* with amide **127f** (255 mg, 1.08 mmol) in DCM (5 mL) quenching with DMS (5 mL). Reaction was allowed to warm to room temperature and after 2 h concentrated to give the crude product[149] which was used directly in subsequent reactions.

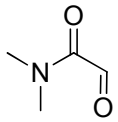
_N,N_-diethyl-2-oxoacetamide 1g



The amide **127g** (577 mg, 2.31 mmol) was ozonolysed following *General Procedure F* in DCM (50 mL) quenching with DMS (30 mL). The reaction was allowed to warm to room temperature and after 2 h concentrated to give the crude product. Column chromatography eluting with 7:3 EtOAc–petrol afforded the *title compound* **1g** (1.03 g, 62%) as a colourless oil.

The *title compound* **1g** exists as 50% hydrate in D_2O with a mixture of other oligomers. The data presented is for the hydrate only; R_f: 0.33 (7:3 EtOAc–petrol); ν_{max}/cm^{-1} (film) 3368, 2979, 1738, 1645, 1462 and 1215; δ_H (300 MHz, D_2O) 5.58 (0.50 H, s, hydrate), 3.64-3.32 (4H, m, $(NCH_2)_{A,B}$), 1.70-1.45 (6H, m, $(CH_3)_{A,B}$); δ_C (75 MHz, D_2O, hydrate only) 168.7 (amide), 84.3 (hydrate), 46.9 and 44.4 ($(NCH_2)_{A,B}$), 25.6 and 24.1 ((CH_3))$_{A,B}$; *m/z* (ES^+) 148.1 (70%, MH^+), 170.1 (30%, MNa^+); HRMS Found MH^+: 148.0971, $C_6H_{13}NO_3$ requires MH^+ 148.0968.

_N,N_-dimethyl-2-oxoacetamide[148] 1h



Water (0.4 mL) was added dropwise to a vigorously stirred suspension of sodium periodate (178 mg, 0.833 mmol), diol **156h** (85 mg, 0.42 mmol) and DCM (1.6 mL). The reaction was stirred at room temperature for 6 h, diluted with DCM (40 mL) and Na_2SO_4 (10g) added. The reaction was the filtered and evaporated under reduced pressure to give to give the crude product as a colourless oil. Column chromatography, eluting with 9:1 EtOAc–petrol, afforded the title compound[148] **1h** (22 mg, 26%) as colourless oil.

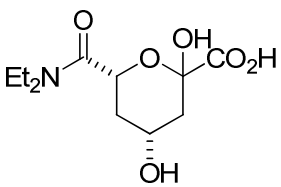
The title compound **1h** can also be prepared following *General Procedure F* with amide **127h** (300 mg, 1.75 mmol) in MeOH (20 mL) quenching with DMS (20 mL). Reaction was allowed to warm to room temperature and after 2 h concentrated to give the crude product. Column chromatography eluting with EtOAc afforded the title compound **1h** (272 mg, 76%) as a colourless oil.

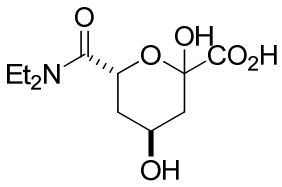
the title compound **1h** was fully characterised was as the hydrate; R_f: 0.30 (EtOAc); v_{max}/cm^{-1} (film); 3273, 2470, 1659, 1509 and 1459; δ_H (500 MHz, D_2O) 5.55 (1H, s, 1-H), 3.04 (3H, s, $(NCH_3)_A$), 2.89 (3H, s, $(NCH_3)_B$); δ_C (75 MHz, D_2O) 170.8 (1-C), 84.5 (2-C), 36.9 and 36.17 ($(NCH_3)_{A,B}$); m/z (EI^+) 101.0 (M^+, 100%); HRMS Found M^+: 101.0478, $C_4H_7NO_2$ requires M^+101.0477.

One-Pot Reactions

Please note that acids the **4a**, **4c**, and **4e** prepared using the one-pot reaction are described earlier alongside the procedure describing their preparation from the homoallylic **123**.

(4*R,6*R**)-6-(Diethylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid and (4*S**,6*R**)-6-(diethylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid 4g**

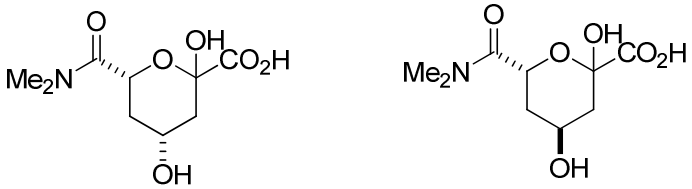




Glyoxylaldehyde **1g** (13.5 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (54µL, 0.955 mmol) diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 0-30% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **4g** (13.2 mg, 51%, 61:39 *cis*:*trans*) as a

colourless film; ν_{max}/cm^{-1} (film) 3369, 2979, 1761, 1627, 1462 and 1202; δ_H (500 MHz, D_2O) 5.13 (0.20H, dd, *J* 9.2 and 3.4, 6-$H_{trans(maj)}$), 5.07 (0.20H, dd, *J* 6.4 and 4.2, 6-$H_{trans(min)}$), 4.84 (0.53H, dd, *J* 11.7 and 1.6, 6-$H_{cis(maj)}$), 4.56-4.51 (0.28H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.26 (0.20H, br s, 4-$H_{trans\ (maj)}$),4.14-4.04 (0.53H, m, 4-$H_{cis(maj)}$), 3.94-3.84 (0.08H, m, 4-$H_{cis(min)}$) 3.40-3.06 (2H, m, NCH_2), 2.15-1.48 (2H, m, H-5 and H-3), 1.12-1.02 (3H, m, CH_3), 0.96 (3H, t, *J* 6.8, CH_3); δ_C (100 MHz, D_2O, *cis(maj)* only) 163.2 (amide or 1-C), 67.2 and 62.7 (6-C and 4-C), 42.5, 14.2, 39.1 and 35.3 (5-C, 3-C and NCH_2), 13.4 and 11.7 (CH_3); *m/z* (ES^-) 260.1 (100%, $[M–H]^-$); HRMS Found *$[M–H]^-$*: 260.1152, $C_{11}H_{20}NO_6$ requires *$[M–H]^-$* 260.1140. Correlation of the ^1H NMR spectrum with that of the acid **4a** allowed the identification of the anomers of the *cis* and *trans* diastereoisomers. The ratio of the species were determined by the integration of the signals at: 5.13 ppm (*trans(maj)*), 5.07 ppm (t*rans(min)*), 4.14-4.04 ppm (*cis(maj)*), 3.94-3.84 ppm (*cis(min)*).)). Analysis by 500 MHz ^1H NMR revealed that a 61:39 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer existed as an 87:13 mixture of anomers and the *trans* isomer existed as a 50:50 mixture of anomers.

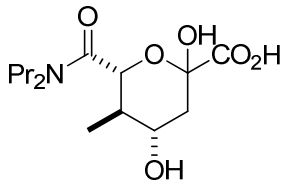
(4*R,6*R**)-6-(dimethylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid and (4*S**,6*R**)-6-(dimethylcarbamoyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid 4h**



Glyoxylaldehyde **1h** (38.6 mg, 0.382 mmol l) in buffer (1.23 mL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with acetaldehyde (216µL, 3.82 mmol) diamine **69** (7.26 mg, 19.1 µmol), diluted with sodium pyruvate (420 mg, 3.82 mmol) in buffer (460 µL, pH 7.4, 20 mM potassium phosphate) and E192N (1.66 mL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 0-20% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The

reaction gave the *title compound* **4h** (40.3 mg, 45%, 71:29 *cis*:*trans*) as a colourless film; ν_{max}/cm^{-1} (film) 3293, 2916, 1678 and 1595; δ_H (500 MHz, D_2O) 5.27-5.17 (0.29H, m, 6-$H_{trans(maj)}$ and 6-$H_{trans(min)}$), 4.97 (0.57H, d, *J* 11.5, 6-$H_{cis(maj)}$), 4.70-4.63 (0.27H, m, 6-$H_{cis(min)}$ and 4-$H_{trans(min)}$), 4.48-4.38 (0.16H, m, 4-$H_{trans\ (maj)}$), 4.26-4.17 (0.57H, m, 4-$H_{cis(maj)}$), 4.09-3.99 (0.14H, m, 4-$H_{cis(min)}$), 3.07 (3H, s, NCH_3), 2.94 (3H, s, NCH_3) 2.25-2.11 (2H, m, 5-H_A and 3-H_A),1.70-1.47 (2H, m, 5-H_A and 3-H_A); δ_C (125 MHz, D_2O, *cis(maj)* only) 172.0, 163.6 and 163.2 (amide and 1-C), 68.2 (6-C), 63.2 (4-C), 39.5, 37.2, 36.2 and 35.1 (5-C, 3-C, $(NCH_3)_A$ and $(NCH_3)_B$); *m/z* (ES^-) 232.1 (100%, $[M–H]^-$); HRMS Found *[M–H]*$^-$: 232.0824, $C_9H_{15}NO_6$ requires *[M–H]*$^-$ 232.0827. Correlation of the ^1H NMR spectrum with that of the acid **4a** allowed the identification of the anomers of the *cis* and *trans* diastereoisomers. The ratio of the species were determined by the integration of the signals at 5.27-5.17 ppm (*trans(maj) and* (t*rans(min)*), 4.70-4.63 ppm (t*rans(min) and*(*cis(min)),* 4.26-4.17 ppm (*cis(maj)*), 4.09-3.99 ppm (*cis(min)*). Analysis by 500 MHz ^1H NMR revealed that a 71:29 mixture of *cis* and *trans* diastereomers was present and that the *cis* isomer exists as a 80:20 mixture of anomeric forms; the *trans* isomer exists as a 56:44 mixture of anomeric forms.

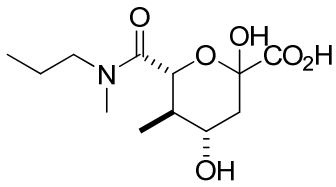
(4*S,5*R**,6*R**)-6-(dipropylcarbamoyl)-2,4-dihydroxy-5-methyltetrahydro-2*H*-pyran-2-carboxylic acid 174a**



Glyoxylaldehyde **1a**(15.0 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with propionaldehyde (69µL, 0.955 mmol) diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 15-46% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The

reaction gave the *title compound* **174a** (29.1 mg, 70%, 55:45 *maj*:*min*) as a colourless film; δ_H (500 MHz, D_2O) 4.91-4.80 (0.45H, m, 6-H_{min}), 4.53 (0.50H, d, *J* 10.3, 6-$H_{maj(maj\ anomer)}$), 4.22 (0.05H, d, *J* 10.3 Hz, 6-$H_{maj(min\ anomer)}$), 4.06-4.00 (0.45H, m, 4-H_{min}), 3.72 (0.50H, td, *J* 11.5 and 4.7, 4-$H_{maj(maj\ anomer)}$), 3.61-3.51 (0.05H, m, 4-$H_{maj(min\ anomer)}$), 3.49-2.85 (4H, m, $(NCH_{A,B})_{A,B}$), 2.18-2.10 (1H, m, 3-H_A), 1.82-1.65 (2H, m, 3-H_B and 5-H), 1.65-1.41 (4H, m, $(NCH_2CH_2)_{A,B}$), 0.86 (1H, d, *J* 6.5, 5-C-CH_3), 0.83-0.72 (6H, m, $(NCH_2CH_2CH_3)_{A,B}$); δ_C (125 MHz, D_2O, *major diastereomer (maj anomer)* only) 70.9 (6-C), 67.8(4-C), 49.7 and 48.5 (NCH_2), 39.7 and 39.0 (3-Cand 5-C), 21.7 and 19.8 (NCH_2CH_2) 10.9, 10.2 and 10.0 ($NCH_2\,CH_2CH_3$ and 5-CH_3),; *m/z* (ES^+) 302.2 (100%, MH^+); Found MH^+: 302.1620, $C_{14}H_{24}NO_6$ requires MH^+ 302.1609. *Maj* refers to the major (4*S**,5*R**,6*R**)-**174a** product as depicted above and *min* refers to all other diastereomers. TOCSY and NOESY analysis allowed the identification the *maj* and *min* anomers of the *4S* isomer. The ratio of the species were determined by the integration of the signals at:4.91-4.80 ppm (*minor diatereomers*), 3.72 ppm (*major diastereomer(major anomer)*), 3.61-3.51 ppm (*major diastereomer (minor anomer)*). Analysis by 500 MHz ^{1}H NMR revealed that a 55:45 mixture of maj and min diastereomers was present and that the major (4*S**,5*R**,6*R**)-**174a** product exists as a 90:10 mixture anomers.

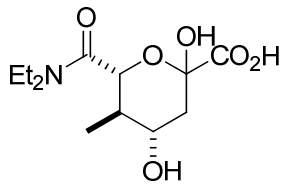
(4*S,5*R**,6*R**)-2,4-dihydroxy-5-methyl-6-(methyl(propyl)carbamoyl)tetrahydro-2*H*-pyran-2-carboxylic acid 174e**



Glyoxylaldehyde **1e** (13.5 mg, 95.5 µmmol) in buffer (308 µL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with propionaldehyde (69µL, 0.955 mmol) diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC

eluting with 0-100% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **174e** (12.3 mg, 47%, 56:44 *maj: min*) as a colourless film; ν_{max}/cm^{-1} (film)3249, 2098 and 1644; δ_H (500 MHz, D_2O) 5.09-4.58 (0.44H, m, 6-H_{min}), 4.71 (0.36H, d, *J* 10.4 Hz, 6-$H_{maj(maj\ anomer)}$),, 4.45 (0.10 H, d, *J* 10.4, 6-$H_{maj(min\ anomer)}^{rotA}$),4.44 (0.10H, d, *J* 10.4, 6-$H_{maj(min\ anomer)}^{rotB}$) 4.23-4.15(0.44H, m, 4-$H_{min}$), 3.92-3.82 (0.36H, m, 4-$H_{maj(maj\ anomer)}$), 3.78-3.68 (0.20 H, m, 4-$H_{maj(min\ anomer)}$), 3.40-3.05 (2H, m, NCH_2), 3.07-2.93 (3H, m, NCH_3^{rotA}), 2.88-2.72 (3H, m, NCH_3^{rotB}), 2.10-2.59 (3H, m, 5-H and 3-H), 1.58-1.32 (2H, m, NCH_2CH_2), 0.85-0.65 (6H, m, $NCH_2CH_2CH_3$ and 5-C-CH_3); δ_C (125 MHz, D_2O, *major diastereomer (maj anomer)* only); 173.4 and 166.0 (amide and 1-C), 101.4 (2-C), 74.3, 74.1 and 71.2 (6-C and 4-C), 54.7 and 53.6 (NCH_2), 42.9 and 42.7 (3-C), 42.3 and 42.2 (5-C), 38.5 and 37.1 (NCH_3), 24.5 and 22.5 (NCH_2CH_2), 14.3, 14.2, 13.3 and 13.0 (5-C-CH_3 and $NCH_2CH_2CH_3$); *m/z* (ES^-) 274.1 (100%, $[M–H]^-$); HRMS Found *[M–H]*$^-$: 274.1287, $C_{12}H_{21}NO_6$ requires *[M–H]*$^-$ 274.1296. *Maj* refers to the major (4*S**,5*R**,6*R**)-**174e** product as depicted above and *min* refers to all other diastereomers. Correlation with the acid **174a** allowed the identification the *maj* and *min* anomers of the *4S* isomer. The ratio of the species were determined by the integration of the signals at 5.09-4.58 (*minor diatereomers*), 4.71ppm (*major diastereomer(major anomer)*), 4.45 ppm (*major diastereomer (minor anomer) rotomer A*) 4.44ppm (*major diastereomer (minor anomer) rotomer B*). Analysis by 500 MHz ^1H NMR revealed that a 56:44 mixture of *maj* and *min* diastereomers was present that the major (4*S**,5*R**,6*R**)-**174e** product exists as a 65:35 mixture of anomers and as a 1:1 mixture of rotomers.

(4*S,5*R**,6*R**)-6-(diethylcarbamoyl)-2,4-dihydroxy-5-methyltetrahydro-2*H*-pyran-2-carboxylic acid 174g**



Glyoxylaldehyde **1g** (13.5 mg, 95.5 µmmol) in buffer (0.308 mL, pH 7.4, 20 mM potassium phosphate) was reacted following *General Procedure J* with propionaldehyde (69µL, 0.955 mmol) diamine **69** (1.81mg, 4.76 µmol), diluted with sodium pyruvate (105 mg, 0.955 mmol) in buffer (115 µL, pH 7.4, 20 mM potassium phosphate) and E192N (416 µL, 6.98 mg/mL in pH 7.4, 20 mM potassium phosphate). The product was purified using preparative HPLC eluting with 0-100% (1% TFA in H_2O–1% TFA in MeCN) over 30 minutes. The reaction gave the *title compound* **174g** (18.8 mg, 69%, 75:25 *maj*:*min*) as a colourless film; ν_{max}/cm^{-1} (film) 3398, 1679, 1629, 1364 and 1206; δ_H (500 MHz, D_2O) 4.81-4.70 (0.25H, m, 6-H_{min}), 4.44 (0.62H, d, *J* 10.5, 6-$H_{maj(maj\ anomer)}$), 4.14 (0.13H, d, *J* 10.5 Hz, 6-$H_{maj(min\ anomer)}$), 3.97-3.92 (0.25H, m, 4-H_{min}), 3.65 (0.62H, td, *J* 11.3 and 4.7, 4-$H_{maj(maj\ anomer)}$), 3.59-3.52 (0.13H, m, 4-$H_{maj(min\ anomer)}$), 3.43-3.02 (4H, m, NCH_2), 2.10-1.98 (1H, m, 3-H_A), 1.75-1.55(2H, m, 3-H_B and 5-H), 1.02 (3H, t, *J* 7.2 Hz, $(NCH_2\mathit{CH_3})_A$), 0.98 (3H, t, *J* 7.3 Hz, $NCH_2\mathit{CH_3})_B$),0.80-0.69 (3H, m, 5-C-CH_3); δ_C (125 MHz, D_2O, *major diastereomer (maj anomer)* only) 170, 163.5 and 163.2 (amide and 1-C), 71.6 (6-C), 68.5 (4-C), 43.4 and 42.1 (NCH_2), 40.4 (5-C), 39.6 (3-C), 14.2, 12.2 and 11.7 (5-C-CH_3 and $NCH_2\mathit{CH_3}$); *m/z* (ES^-) 274.1 (100%, $[M–H]^-$); HRMS Found *[M–H]*$^-$: 274.1282, $C_{12}H_{21}NO_6$ requires *[M–H]*$^-$ 274.1282. *Maj* refers to the major (4*S**,5*R**,6*R**)-**174g** product as depicted above and *min* refers to all other diastereomers. Correlation with the acid **174a** allowed the identification the *maj* and *min* anomers of the *4S* isomer. The ratio of the species were determined by the integration of the signals at:4.81-4.70 (*minor diatereomers*), 3.65ppm (*major diastereomer(major anomer)*), 3.59-3.52 ppm (*major diastereomer (minor anomer)*). Analysis by 500 MHz ^{1}H NMR revealed that a 75:25 mixture of *maj* and *min* diastereomers was present and that the major (4*S**,5*R**,6*R**)-**174g** product exists as a 82:18 mixture of anomers.

Chapter 6

6 Material and Methods

6.1 Bacterial strains, plasmids and media

Proteins were overexpressed using E. coli strains: EP-Max10B F' cells (Bio-rad). The plasmid used was pKK233-3 and was from Amersham Biosciences. The construct pK*nan*A-His6 was previously described[47] and was provided by Amanda Bolt, University of Leeds. Bacterial cultures were grown in 2 × YT rich media consisting of 16 g/L of tryptone, 10 g/L of yeast extract and 5g/L of NaCl. Glycerol stocks were prepared by adding 0.5 mL of an overnight culture to 0.5 mL sterile glycerol and was stored at –20 °C.

6.2 Chemicals and Enzymes

Nicotamide adenine dinucleotide (reduced form) [NADH] sodium salt, sodium pyruvate, agarose, imidazole and TEMED were from Sigma Chemicals Ltd. (Poole, U.K.). Sodium chloride and ampicillin sodium salt was supplied by Fisher Scientific (Loughborough, U.K.). Sodium dodecyl sulphate and acrylamide were from BDH Chemicals Ltd. (Essex, U.K.). L-Lactate dehydrogenase was supplied by Roche. Trytone, yeast extract, tris(hydroxymethyl)aminomethane (Tris) were supplied by Melfor Laboratories (Ipswich, U.K.). Ni^{2+} resin for protein purification "Chelating Sepharose® Fast Flow" was supplied by Pharmacia.

6.3 Over-expression and purification of His-tagged proteins form *E.coli*

E. coli cells harbouring the plasmid pKK233-3 were grown overnight at 37 °C in 5 mL of 2 × YT supplemented with 50 µg/mL ampicillin. The overnight culture was used to inoculate 3 L of 2 × YT supplemented with 50 µg/mL ampicillin and 0.1 mM IPTG, which was grown overnight at 37 °C. The cells were harvested by

centrifugation at 4000g and 4 °C and the pellet suspended in 50 mL of loading buffer (containing 16.2 mM potassium phosphate dibasic and 3.8 mM potassium phosphate monobasic (pH 7.4), and 300 mM NaCl and 10 mM imidazole). The cells were lysed using a Constant Systems cell disrupter (20 kpsi) and the cell debris was collected by centrifugation at 10 000 g for 50 min at 4 °C. The supernatant was aliquoted into 2 portions, each of which were loaded onto 15 mL of Ni^{2+} charged resin that had been pre-equilibrated with loading buffer. The resin was centrifugated at 3000 rpm at 4 °C and the removed and fresh loading buffer added. Contaminanting proteins were removed by washing resin with wash buffer a total of 5 times. The aldolase enzyme was eluted from the column using elution buffer (containing 20 mM potassium phosphate buffer (pH 8.0), 200 mM NaCl and 200 mM imidazole). Fractions were analysed by SDS-PAGE.

6.4 Dialysis

Protein samples were dialysed with dialysis tubing (molecular weight cut-off of 12-14 kDa) against 50-100 times the volume overnight at 4 °C.

6.5 SDS-PAGE

Proteins were separated by SDS-polyacrylamine gel electrophoresis using a 20% resolving gel with 5% stacking gel of the following composition:

Gel component	**Volume required for resolving gel (µL)**	**Volume required for stacking gel (µL)**
30% (*w/v*) acrylamide/ 0.8% (*w/v*) bisacrylamide solution	7500	625
1.5 M Tris/HCl pH 8.8	3750	625
10% (*w/v*) SDS	150	50
25% (*w/v*) ammonium persulfate	50	200
TEMED	5	5
H_2O	3500	3650

Buffers utilised in SDS-PAGE were composed as follow:

2 × SDS Sample Buffer

Buffer component	Quantity
Dithiotheritol	154.0 mg
10% (*w/v*) SDS	2.0 mL
Glycerol	1.0 mL
1M Tris/HCl pH 6.8	170 mL
H_2O	1.63 mL
0.2% (*w/v*) bromophenol blue	200 µL

SDS Running Buffer

Buffer component	Quantity
Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g
β-mercaptoethanol	0.14 mL
H_2O	to 1.0 L

Before loading onto gel, protein samples were mixed in a 1:1 ratio with sample buffer and boiled for 5 minutes. SDS-PAGE was performed at 60 mA for 2-3 h and stained with a 5:1:1 (*v/v/v*) methanol: acetic acid: water solution containing 0.1% (*w/v*) Coomasine Brilliant Blue R-250 for 1 h then destained in the same solution not containing Coomasie Brilliant Blue R-250.

6.6 Determination of Protein Concentration

Protein concentrations were determined by measurement of optical density of sample (10 µL in 1 mL) at 280 nm with a Jasco V-560 UV/VIS spectrophotometer assuming that a 1 mg/mL protein concentration equates to an optical density of 1.

6.7 Robotic liquid handling

Robotic liquid handling was performed using a Hamilton Microlab Star robot with Hamilton pipette tips and Vector software.

6.8 Thiobarbituric Acid Assay

The effect of various potential one-pot reaction conditions on the E192N aldolase variant were analysed by using an adapted literature method: thiobarbituric acid (TBA) assay.

The assay was performed in Varian ^{TM} glass 1.0 mL conical vials in a 96 well plate into which the diamine **69** (10% *w/v* in ethanol) was pipetted using a liquid handling robot into appropriate wells and solvent removed *in vacuo*. The robot pipetted as appropriate the acid **36a** (20 mM in 100 mM, pH 7.4 potassium phosphate buffer), the aldehyde **35a** (20 mM in 100 mM, pH 7.4 potassium phosphate buffer), the glyoxylaldehyde **1a** (50 mM in 100 mM, pH 7.4 potassium phosphate), sodium pyruvate (100 mM in 100 mM, pH 7.4 potassium phosphate buffer) and buffer (100 mM, pH 7.4 potassium phosphate buffer). To the appropriate wells propionaldehyde was added pipetting manually followed by E192N (6.2 mg/mL, in 100 mM, pH 7.4 potassium phosphate buffer) manually using a multi-channel pipette. The plates were shaken, sealed with microplate film and plate left overnight at room temperature. Water was added to the plate (300 µL) followed by $CHCl_3$ (100 µL) and each well mixed with pipette to aid extraction. A portion of the aqueous layer (150 µL) was aliquoted from each well and transferred into a plastic shallow-well plate and solvent removed using a GeneVac^{TM} centrifugal evaporator.

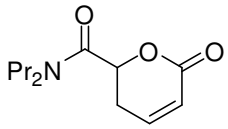
To each well water (20 µL) and sodium periodate (11 µL, 0.2 M in 9 M H_3PO_4) was added and allowed to stand at room temperature. After 20 minutes sodium arsenite (45 µL, 10% (*w/w*) in 0.5 M Na_2SO_4 containing 0.05 M H_2SO_4) was added and plates shaken for 2 minutes to assure the complete discharge of yellow-brown colour, TBA added (135 µL, 0.6% (*w/w*) aqueous solution) was added and plates sealed and heated to 100 °C in an oven for 30 minutes.

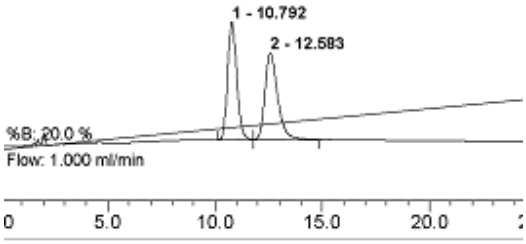
6.9 Coupled enzyme assay for determination of the steady state kinetic parameters k_{cat} and K_M

A coupled NADH and aldolase enzyme assay was used to determine aldolase activity as developed within the group.[49] The assay was performed in a 1 mL cuvette containing 50 mM Tris/HCl buffer pH 7.5, 1 mM NADH, 2 μL coupling enzyme (lactate dehydrogenase, 550 U/mg), a suitable aliquot of purified E192N and varying concentrations of substrate. All substrate were dissolved in 50 mM Tris/HCl buffer pH 7.5, and the pH adjusted to pH 7.5 as required using a saturated aqueous solution of NaOH. The initial rate of decrease of A_{340} was recorded as the measure of enzyme activity on an Ubikon 930 spectrophotometer. One unit of aldolase activity is defined as the amount of enzyme which catalyses the oxidation of 1 μmol NADH/min in this system, using the molar extinction coefficient of NADH as 6220 $M^{-1}cm^{-1}$. The rates of change of A_{340} with different substrate concentrations and a single aldolase concentration were used to determine the steady state kinetic parameters k_{cat} and K_M, using Michaelis–Menton kinetics

Appendix i

Chiral Reverse Phase Chromatography of Racemate Lactone

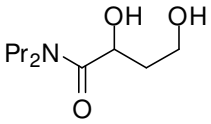


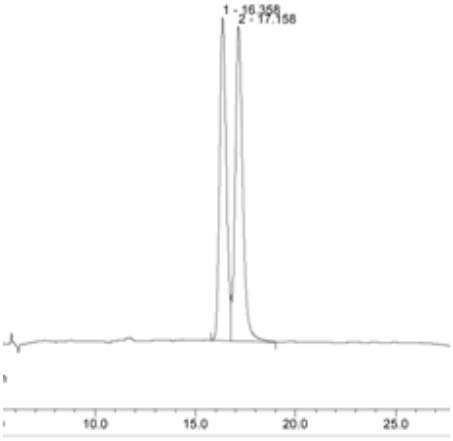


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	10.79	n.a.	220.411	109.529	49.82	n.a.	BMb
2	12.58	n.a.	161.842	110.311	50.18	n.a.	bMB
Total:			382.252	219.840	100.00	0.000	

Appendix ii

Chiral Normal Phase Chromatography of Racemate Diol

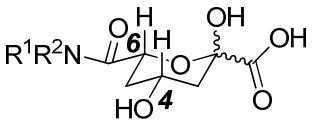




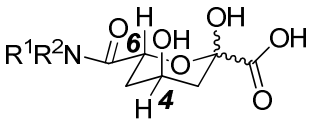
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16.36	n.a.	240.085	92.958	47.95	n.a.	BM
2	17.16	n.a.	233.597	100.893	52.05	n.a.	MB
Total:			473.682	193.851	100.00	0.000	

Appendix iii

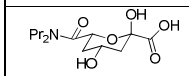
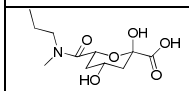
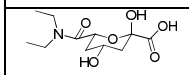
cis diastereomer ***trans diastereomer***

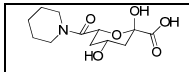
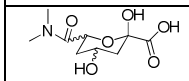


cis-**4**



trans-**4**

Compound	***Cis maj H6eq H4-eq***			***Cis Min H6eq H4-eq***			***Trans maj H6eq H4-ax***			***Trans Min H6eq H4-ax***		
	6-H* δ *ppm	***4-H* δ *ppm***	***J Hz H6-H5***	***6-H* δ *ppm***	***4-H* δ *ppm***	***J Hz H6-H5***	***6-H* δ *ppm***	***4-H* δ *ppm***	***J Hz H6-H5***	***6-H* δ *ppm***	***4-H* δ *ppm***	***J Hz H6-H5***
	4.83	4.17-4.08	10.5	4.69-4.63	4.01-3.93	m	5.18	4.30	9.5, 3.3	5.10	4.69-4.63	6.7, 4.9
	4.85	4.17-4.08	12.0	4.62-4.53	4.00-3.90	m	5.23-2.02	4.33-4.26	m	5.23-5.02	4.62-4.53	m
	4.84	4.14-4.04	11.7 and 1.6	4.56-4.51	3.94-3.84	m	5.13	4.26	9.2 and 3.4	5.07	4.56-4.51	6.4 and 4.2

	4.84	4.14-4.04	11.7 and 1.6	4.56-4.51	3.94-3.84	m	5.13	4.48-4.38	9.2 and 3.4	5.07	4.56-4.51	6.4 and 4.2
	4.97	4.26-4.17	11.5	4.70-4.63	4.09-3.99	m	5.27-5.17	4.48-4.38	m	5.27-5.17	4.70-4.63	m

Summary and comparison of H-4 and H-6 chemical shifts and coupling constants of the acids **4** for both *cis* and *trans* diastereomers and their respective major (*maj*) and minor (*min*) anomeric species; ^{m} multiplet

Appendix iv

0 **+** **++** **+++**

The level of E192N activity was assessed by the amount of pink observed in each well. The above diagram shows the level of "pinkness" which corresponds to each level of E192N activity.

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