Towards the development of a cyclisation-release screening methodology for new C-C bond forming reactions

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Contents

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List of Schemes

Abstract

The objective of this project is primarily to develop a cyclisation-release methodology which could be applied to the investigation of Baylis-Hillman reactions, and to further develop a methodology, enzymatic or chemical, suitable to screen reaction products. The screening process will ultimately be incorporated to identify a potential Baylis-Hillmanase, developed through directed evolution by other members of the Berrisford group. This area of work is based around evolving aldolase enzymes as they are reversibly catalytic in living organisms, and can be of much aid in working towards a Baylis-Hillman catalysing enzyme, thus an ideal starting point for directed evolution.

There is wide-spread enthusiasm in the Baylis-Hillman synthesis and the manufacture of abiotic, asymmetric organic catalysts. There is no general asymmetric catalyst or even a biocatalytic analogue of this reaction.

In a wider context, development of a screen will help validate successful directed evolution, of a totally new C-C bond forming enzyme originating from a class of aldolases.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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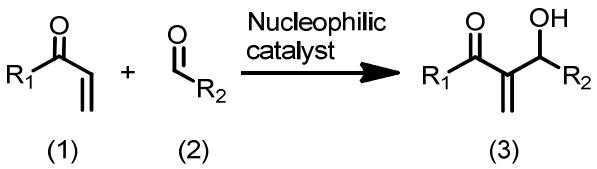
1. Introduction

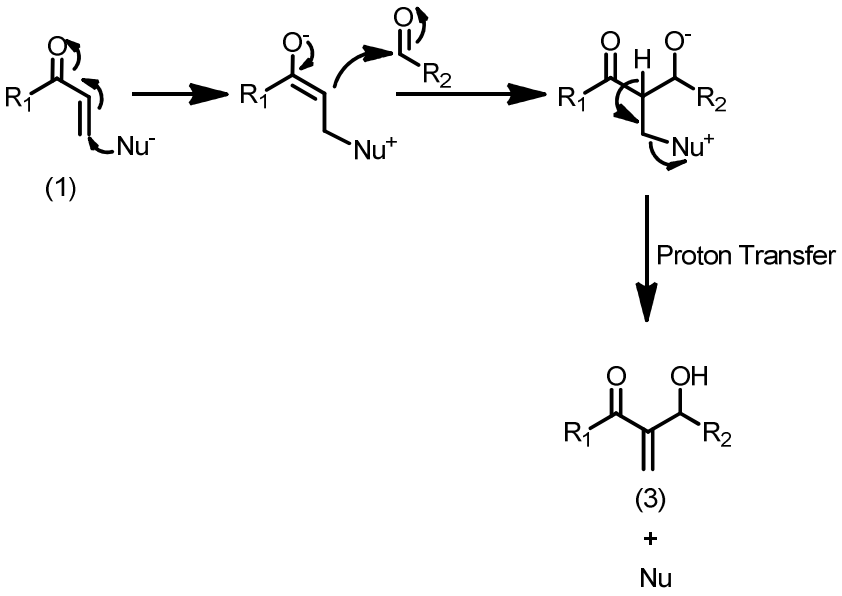
1.1 The Baylis-Hillman reaction

The synthetically important Baylis-Hillman reaction forms a new carbon-carbon bond from an aldehyde and an activated alkene (**Scheme 1.1.1**), typically an α,β-unsaturated compound with an electron-withdrawing group attached. These reactions require nucleophilic catalysts such as tertiary amines and phosphines, with DABCO being the most commonly used.[1]



Scheme 1.1.1 General Baylis-Hillman reaction

The general reaction mechanism can be represented extremely simplistically as follows (**Scheme 1.1.2**), proceeding *via* enolate formation of the α,β-unsaturated ketone through nucleophilic attack.



Scheme 1.1.2 Simplistic Baylis-Hillman mechanism

Morita[2] was the principal founder of this reaction, in 1968 reporting tricyclohexylphosphine catalysed reactions between acrylonitrile/methyl acrylate and various aldehydes. It was the work carried out up by Baylis and Hillman[1], in 1972, utilising tertiary amines and catalysts such as DABCO and quinuclidine which brought much promise to this area. However these reactions were not followed up until the 1980's, after which the area finally blossomed. This is evident in the vast literature currently available on the Baylis-Hillman reaction.

Basavaiah has searched through and brought together much of the work on the Baylis-Hillman reaction.[3] Accounts[4-8] following this have not been as broad in scope. Basavaiah[4] later, in 2007, looked into future prospects of the reactivity. Shi[6] has looked at Aza-Baylis Hillman reactions and Zhu *et al*[7] have reviewed

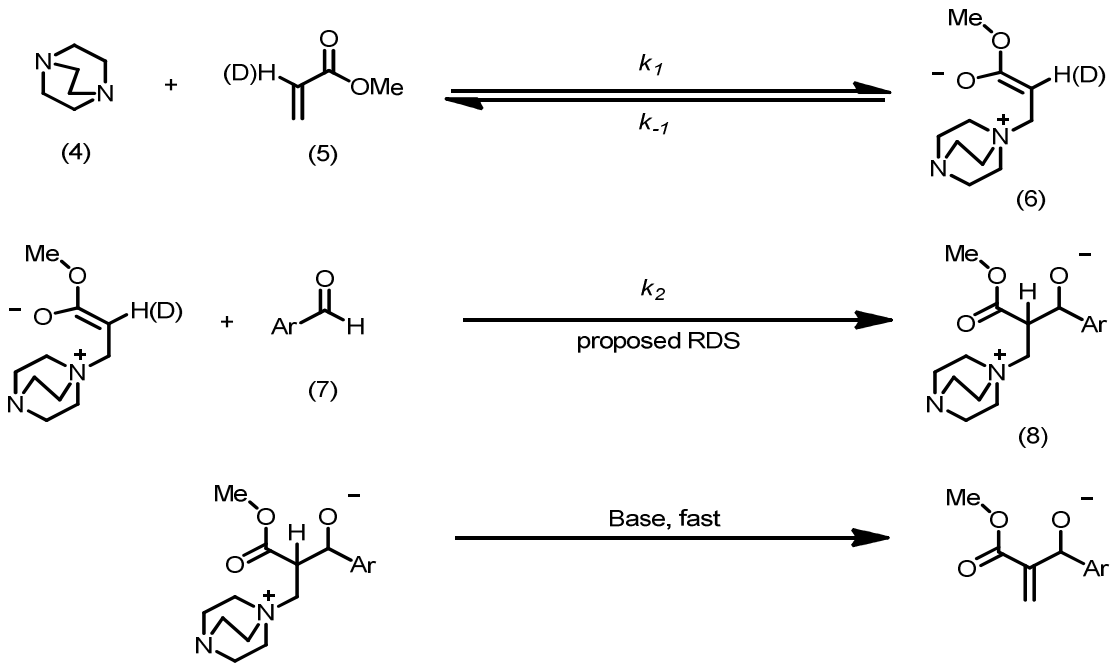
recent advances on enantioselectivity. Recently Batra[8] has highlighted work on cyclic structures.

In this thesis reference to the Baylis-Hillman reaction will focus on recent mechanistic understanding, and the factors which determine the enantioselectivity of these reactions.

1.2 Mechanistic understanding of the Baylis-Hillman reaction

The earliest mechanisms for the Baylis-Hillman reaction were first suggested by Morita[2] and Hoffman,[9] which were universally trusted. Their proposed mechanism was based on fundamental principles of pressure dependence, reaction rates and the kinetic isotope effect.

Taking simple kinetics into account for the reaction between acrylonitrile, acetaldehyde and DABCO; Hill and Isaacs[10] suggested the first detailed mechanism (**Scheme 1.2.1**).



Scheme 1.2.1 Hill and Isaacs mechanism

This is essentially a break-down of the previous mechanism (**scheme 1.1.2**) and shows the rate determining step; the attack of the enolate ion (**6**) on the aldehyde (**7**) following Michael addition of the amine base (**4**) to the alkene (**5**). This is followed by quick elimination of the base. However, with the aid of KIE data, a figure of 1.03 ± 0.1 for the α-proton, they established there is in fact no proton cleavage in the RDS. This proposed mechanism was backed by Bode and Kyle[11] with a rate law, using rate data on acrylates.

$$\text{Rate} = k_1 k_2 [\mathbf{4}][\mathbf{5}][\mathbf{7}]$$

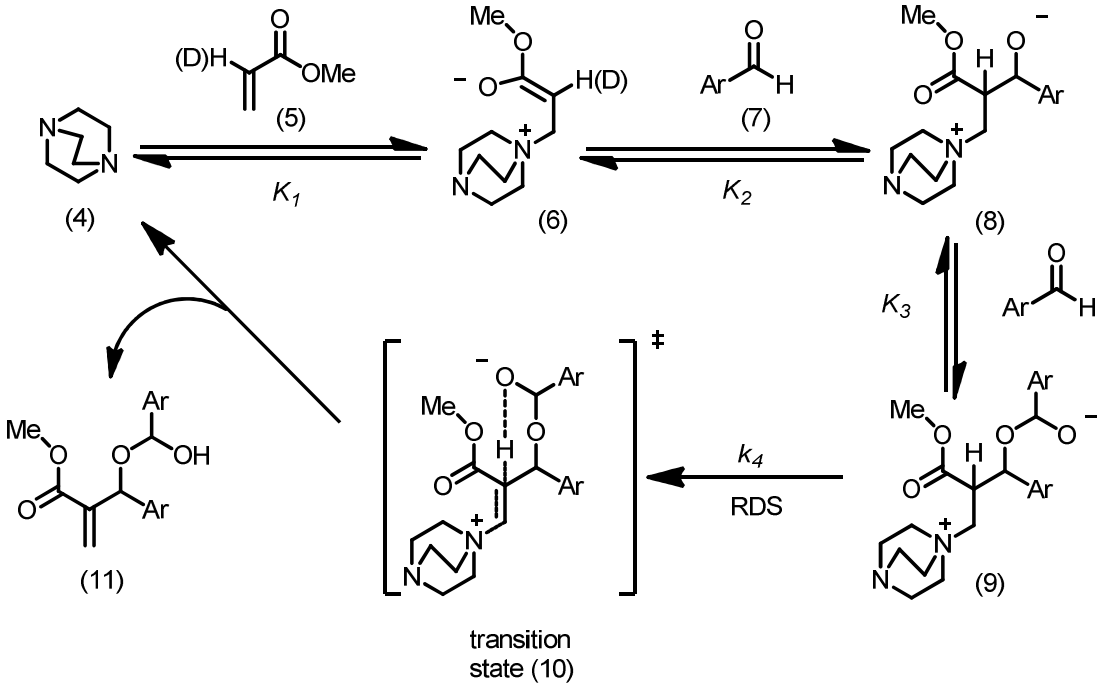
Recent literature[12] suggests occurrences such as slow reaction rates, dioxanone production, tedious control of steroselectivity, autocatalysis and proton aided rate enhancement are all un-accounted for in this proposed mechanism.

McQuade[12,13] studied reaction rates of the Baylis-Hillman reaction on methyl acrylate, *p*-nitrobenzaldehyde and DABCO under aprotic conditions and derived the following rate law:

$$\text{Rate} = k_{obs}[\text{aldehyde}]^2[\text{DABCO}][\text{acrylate}]$$

The rate law suggests the RDS of the Baylis-Hillman mechanism must contain two equivalents of aldehyde, thus the second step of the previous mechanism (**Scheme 1.2.1**) must be invalid as the RDS.

This lead to the postulation of a new mechanism as seen in (**Scheme 1.2.2**). This mechanism agrees with the reaction of enolate (**6**) and aldehyde (**7**) to give (**8**), however this reacts with another equivalent of (**7**) to produce hemiacetal (**9**). It is this species which undergoes rate determining deprotonation to form enone (**11**) *via* a cyclic transition state. The Baylis-Hillman product is then obtained *via* a proceeding set of reactions.

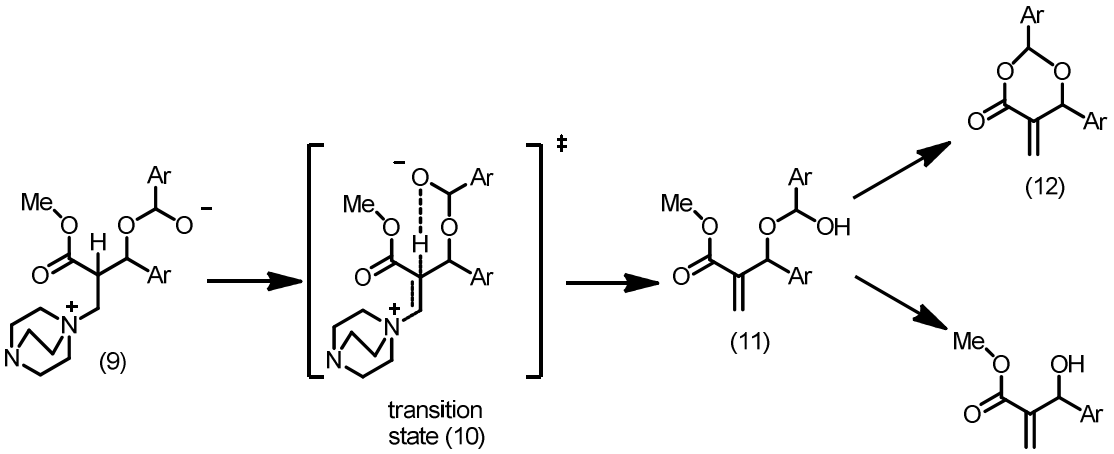


Scheme 1.2.2 McQuade mechanism

The above mechanism was supported with KIE data. One experiment performed using α- ^{2}H acrylate, resulted in primary kinetic isotope effects observed in all of the several solvents used. The effect was most substantial in the more polar solvents, confirming the proposed mechanism and proton deprotonation as its RDS. This was further supported with another experiment, where α-deutereo-*p*-nitrobenzaldehyde showed significant inverse isotope effects in various different solvents.

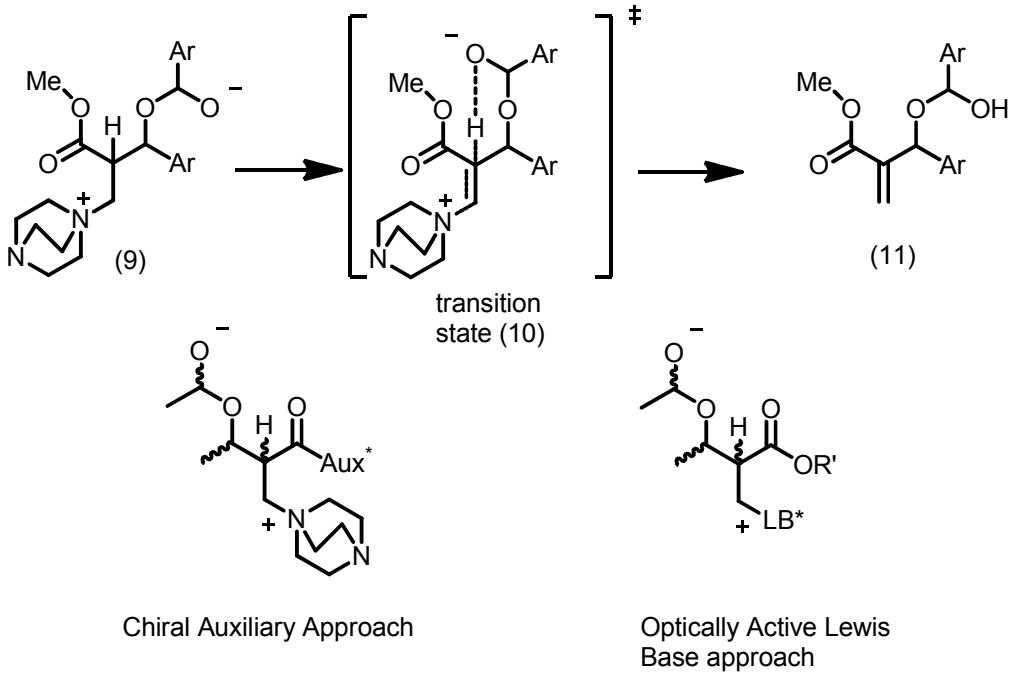
This proposed mechanism by McQuade also accounts for dioxanone formation (**12**), as seen in (**Scheme 1.2.3**) below. It has also been observed that

hemiacetal species such as (**9**) and (**11**) can undergo intramolecular transesterification where the acrylate is an activated ester.



Scheme 1.2.3 Proposed method of dioxanone formation

The next mechanism further highlights the effect of stereochemistry and its difficult control (**Scheme 1.2.4**).



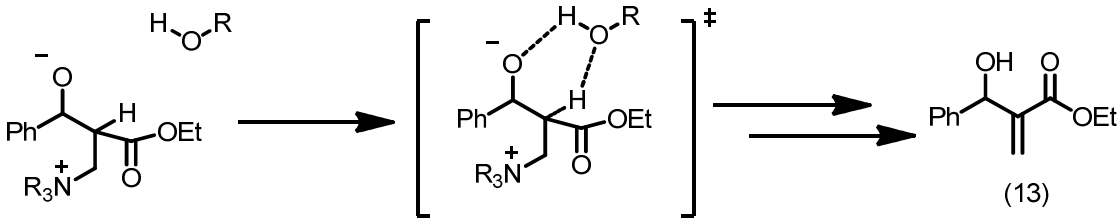
Scheme 1.2.4 Stereochemistry of pathway towards product

Use of a chiral auxilary or a Lewis base would be unsuccessful for the Baylis-Hillman reaction; as three stereogenic centres in the transition state (**10**) could give rise to eight sterioisomers at that point and lead onto four as product, which is far too many for the above approaches.

Aggarwal[14] was eager to fully explore the mechanisms of protic solvent aided acceleration, following Hill and Isaacs finding of autocatalysis of Baylis-Hillman reactions in aprotic solvents.[15] Using KIE data on the quinuclidine catalysed reaction between ethyl acrylate and benzaldehyde under aprotic conditions, it was found that the proton cleavage step is not truly part of the RDS. It is in-fact

only the initial RDS and following 20% turnover of starting materials, the enolate-aldehyde step takes over as the rate-limiting step.

Aggarwal[15] proposed a hydroxyl model (**Scheme 1.2.5**) for autocatalysed proton deprotonation. A simple proton source such as an alcohol or water, can bind to the enolate ion and proceed *via* a six-membered cyclic transition state, to protonate the alkoxide and simultaneously deprotonate the α-methine. Elimination of the amine completes conversion to the Baylis-Hillman adduct.

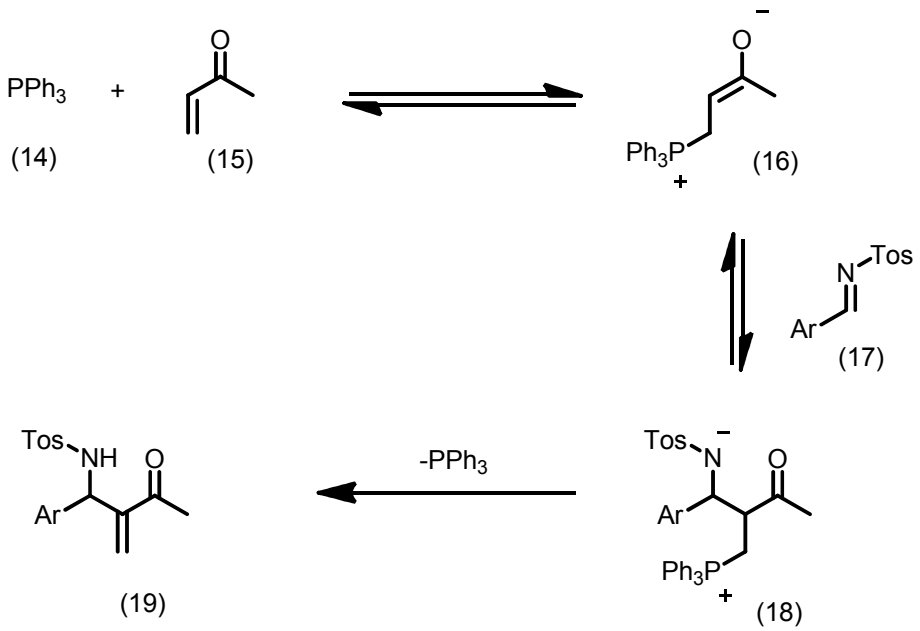


Scheme 1.2.5 Proton transfer mechanism

Aggarwal was able to deduce from the above model, that the stereochemistry of the catalysts is key in asymmetric catalysis of this reaction. He observed that nucleophiles resulting in yields over 80% ee had hydrogen bond donors attached to themselves. It is believed that all four stereoisomers of the transition-state complex are formed, however, only the one with the correctly orientated stereochemistry for hydrogen-bonding successfully converts to product. The other stereoisomers fall back to reactant and reaction subsequently proceeds *via* the fast-elimination pathway. There has been little

success in developing sufficient chiral catalysts, but it is thought this is due to focus on the initial carbon-carbon bond forming step and not on proton elimination. These findings suggest non-protic solvents possibly aid in achieving high enantioselectivity.

Lietner[16] studied the kinetics of the aza-Baylis-Hillman reaction of methyl vinyl ketone (**15**) and 4-methylbenzenesulfonamide (**17**) with catalytic triphenyl phosphine (**14**) in THF at room temperature (**Scheme 1.2.6**). The occurrence of product was observed with ^{19}F NMR and a rate law was formulated from data on initial rates based on concentration.



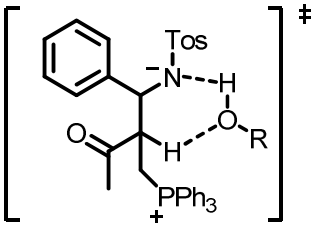
Scheme 1.2.6 aza-Baylis-Hillman mechanism

Autocatalysis was not detected here, and the contribution of imine (**17**) in the rate law suggests that the rate limiting step is linked to proton cleavage.

Upon addition of Bronsted acids such as 3,5-bis(CF_3)phenol, the overall rate increased by a factor of up to fourteen. This effect is reduced though with stronger acids as they can protonate the enolate (**16**). Further study of the reaction kinetics with phenol as an additive lead to the derivation of the following rate law:

$$\text{Rate} = k_{obs}[\mathbf{14}][\mathbf{15}][\mathbf{17}]$$

Here the concentration of imine (**17**) is positive and linearly influential on the initial rate and thus proton cleavage is in-fact enhanced and this lead to a new transition state; essentially a modified version of Aggarwal's (**Scheme 1.2.5**).



Scheme 1.2.7 Transition state for Brønsted acid-aided proton transfer

Leitner's studies confirmed that dual functionality constituting a basic and proton source is a suitable pathway in the design of a catalyst for the asymmetric aza-Baylis-Hillman reaction. It was noted that phosphine catalysts, even in the

absence of protic solvents, are likely to cause racemisation *via* proton exchange at the chiral centre of the adduct. Thus it is essential the geometry of a bifunctional chiral catalyst also avoids the occurrence of racemisation at a latter stage.

Further work was carried out on the aza-Baylis-Hillman reaction by Jacobsen,[17] who researched DABCO accelerated reactions between methyl acrylate and nosylimines in chloroform. A large primary kinetic isotope effect was measured on analysis of the initial rate of methyl acrylate and that of its deuterated analogue, indicating that cleavage of the α-H(D) was part of the rate determining step. Jacobsen also concluded that the imine is not involved in the proton cleavage, unlike McQuade's suggestion, where it is an electrophile in the transformation.

Recently the Baylis-Hillman reaction has been studied by various researchers using computational modelling.

Xu[18] looked into the trimethyl phosphine catalysed reaction between acrylonitrile and ethanol in DCM. With the aid of Density Funtional Theory (DFT) he extrapolated that intramolecular proton transfer is rate-limiting and observed rate kinetics concurrent with previous experimental work.

Roy and Sunjoy[19] utilised ab initio and DFT to study the effects of polar aprotic solvent DMSO, in the DABCO catalysed reaction between benzaldehyde and MVK. They were able to confirm the intramolecular proton transfer step as rate-limiting, as had been stated by Xu. Comparison of the data with initial computation of water as solvent strongly suggested the intramolecular transition state is sufficiently stabilised energetically in a polar-protic medium; confirming the research done by Aggarwal. Water was found to not only lower the activation barrier but also excel the carbon-carbon bond formation.

Aggarwal and Harvey[20] studied the triethylamine catalysed reaction of methyl acrylate and benzaldehyde using DFT, under aprotic, neat conditions and additionally with methanol.

It was calculated that without the aid of protic solvent, proton transfer is the rate determining step and the reaction would proceed *via* a cyclic transition state where intramolecular proton transfer occurs within a hemiacteal alkoxide (**Scheme 1.2.2**). This structure (**10**) is produced from reaction of the intermediate alkoxide with another equivalent of aldehyde. These results and the predicted rate kinetics, second order in aldehyde, all correlate with the experimental findings by McQuade.

Computation of methanol as solvent resulted in a lower activation barrier due to concerted proton transfer, accelerated by methanol or product (**Scheme 1.2.5**).

This phenomena rationalises the wide-spread observance of proton aided rate enhancement of the Baylis-Hilman reaction.

Prediction of a high energy barrier in the absence of protic solvent, possibly justifies the slow rates typically found with this transformation.
There are essentially two reaction trajectories, aldehyde-accelerated and alcohol-accelerated, and this has a profound impact on the stereochemistry of the reaction. The proton transfer transition state has two chiral centres in the alcohol pathway and three in the aldehyde pathway. This means a potential four diastereoisomers for the former and eight for the latter. It should also be noted that these transition states are not only part of the RDS, but also determine the selectivity. A complex transition state would considerably reduce selectivity; diastereoisomers in this case would not easily be differentiated by a chiral catalyst as they are all within a narrow energy range. Under aprotic conditions both mechanisms are in operation, alcohol catalysis takes over from aldehyde catalysis once there is turnover. This switch in mechanism also alters the transition state that would result in enantiomeric enrichment, thus diminishing selectivity. This would explain the little success in achieving high selectivity for this reaction without the aid of protic additives.

Aggarwal and Harvey[20] completed their study by formulating a set of criteria they envisaged essential, in order to obtain good selectivity in an asymmetric Baylis-Hillman reaction:

1) Perform the reaction in the presence of proton donors – ideally as part of a bifunctional catalyst.
2) Control stereoselectivity of the proton transfer step.
3) Control stereoselectivity of the C-C bond forming step.
4) Have no more than one source of proton donor in a reaction, *i.e.* do not use a protic solvent, as this can lead to competitive proton transfer catalysis and product selectivity.

A finding by Leitner[16] is relevant here and could be added to this criteria:

5) The positioning of a proton donor in a bifunctional catalyst should not only help control stereoselectivity, but it must also be able to prevent subsequent racemisation.

To summarise, recent work on the Baylis-Hillman reaction has brought much consolidation to the field. Findings suggest deprotonation is the rate limiting step as oppose to C-C bond formation, for which separate mechanisms exist under aprotic and protic conditions. This is indeed reflected in experimental findings. Issues surrounding the difficulty in achieving high enantioselectivity with asymmetric Baylis-Hillman reactions have been brought forward. Most crucially it should be noted there is no general chiral catalyst available to date, which could produce relatively good selectivity as well as have the potential to convert a wide-range of substrates.

1.3 Directed Evolution

Enzymes provide a broad scope[21,22] in terms of being utilised as catalysts, and there is great interest commercially, especially from a pharmaceutical stand-point, in achieving new stereo-products *via* enzymatic catalysis. The criteria for a good accelerant is; high conversion rate of starting materials to product, high enantioselectivity, broad substrate specificity, stability at elevated temperatures and high tolerance to organic solvents. These points do not wholly fit naturally occurring enzymes, as of course they were synthesised for biological purposes and not for industrial use.

Directed evolution is a process and means which can help achieve the necessary qualities of an industrial catalyst. It is based on Darwinian evolution and works to alter a natural enzyme in such a way, that it ends up matching the attributes and function needed for industrial application. This can in-fact be achieved even without detailed structural information.

There are essentially two parts to the methodology;

1. The mutagenesis of the gene encoding the enzyme of interest and the generation of a library of mutant enzymes.
2. Analysis of the library of mutants on the basis of attribute or function of interest by screening or selection.

Mutant enzymes play a vital role in the process, they are generated iteratively and undergo rounds of mutagenesis through identification of the appropriate genetic information, until they analytically appear to have embedded in them the attributes and function for required purposes.

1.4 Screening versus Selection[23,24]

Directed evolution methods are typically dependant on screening or selection techniques; and require to sieve out the extremely large number of mutants that are created.

Screening and selection methods vary in that the former is applied to single mutants where as the latter is applied to a pool of mutants. The good aspect to screening is that information on single mutants is produced, but screening can be wasteful as every single mutant is analysed, including all non active and wrongly folded mutants (usually accounting for 50-80% library). Selection is not as laborious and has a greater output as groups of mutants can be analysed at the same time. Hence screening library capacity around 10^4 mutants; where as selection library capacity 10^{10} – 10^{13} mutants. Selection strategically works so that desired mutants are created under conditions which they will thrive, and unwanted mutants will not feature. The main downside to selection is that it can easily generate false positives, failing the design and requiring a new selection process.

Common screening methods include UV/Vis spectroscopy,[25] fluorescence spectroscopy,[26] GC and HPLC chromatographies.[27] Selection has been used within a microorganism auxotroph deficient in a chemical needed for growth, this chemical is allowed to be engineered by the desired enzyme; hence useful mutants are distinguished by microorganism growth.

1.5 Towards the evolution of a new enzyme

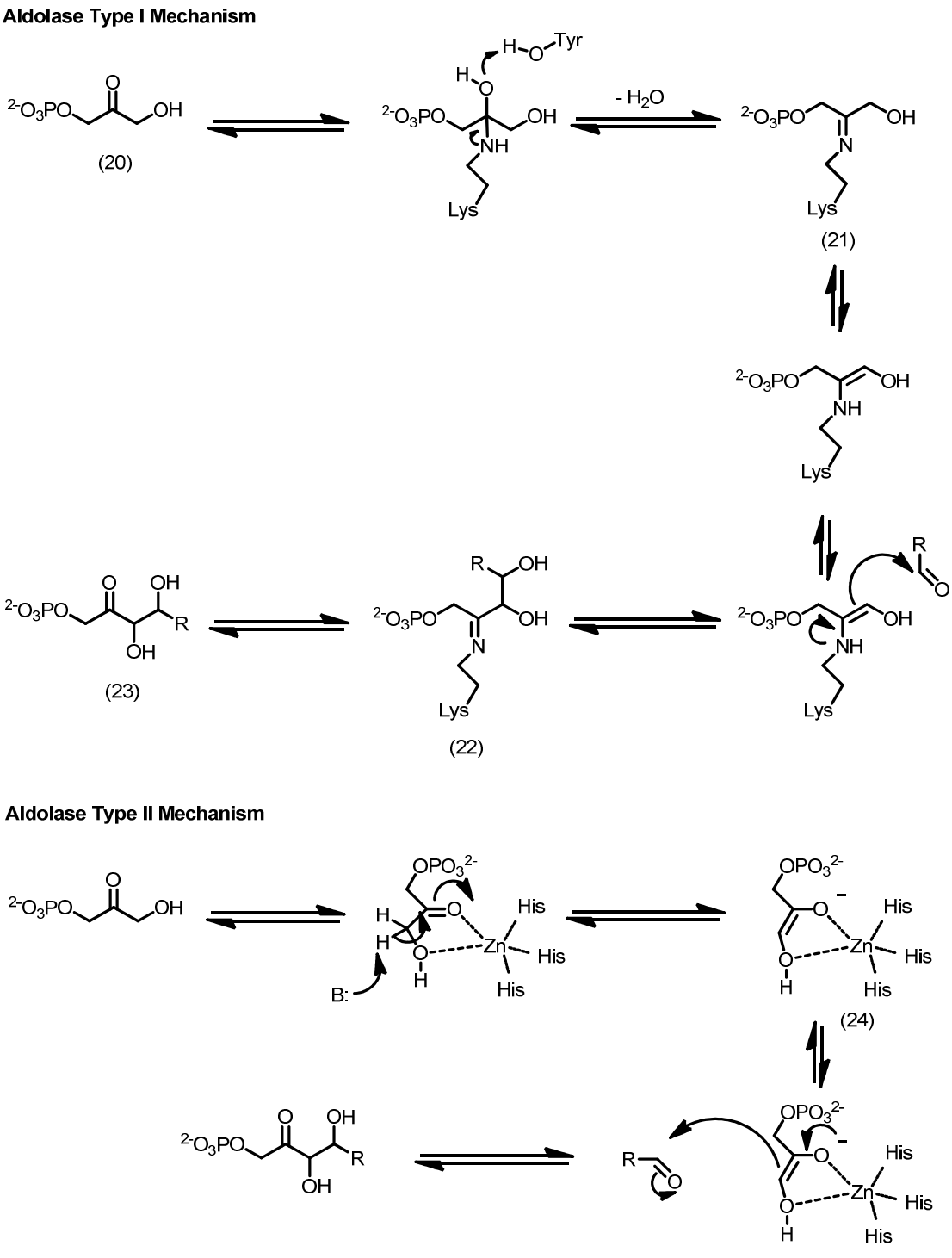
1.5.1 Introduction to aldolases

Aldolases accelerate carbon-carbon bond forming aldol reactions in living organisms.[28-30] Aldolases are involved in the stereoselctive reaction of a keto donor and an aldehyde receiver substrate. Condensation and cleavage transformations are also crucial in nature, in the major sugar metabolic pathways of all organisms. For instance, in glycolysis, fructose-1,6-bisphosphate is reversibly converted into dihydroxyacetone phosphate (DHAP) and glyceraldehye-3-phosphate.

Aldolases typically show high specificity for the nucleophilic donor molecules, and low specificity for the electrophilic counterpart molecules.

They can be generally classed as two main types (**Scheme 1.5.1.1**) depending on reaction mechanism:

Type I aldolases,[31] usually found in animals and higher plants, proceed *via* Schiff base formation (**21**) upon addition of the ketone (**20**), with a retained active site lysine. There is in fact carbinolamine formation preceding the Schiff base stage, which expels the hydroxyl group (as water) with the aid of a tyrosine residue. The Schiff base then tautomerises to an enamine and reacts with the correct face of bound aldehyde, with high selectivity, producing adduct. The product is then freed upon hydrolysis of the imine (**22**).



Scheme 1.5.1.1 Aldolase categorisation by mechanism type

Type II aldolases,[32] mainly present in microorganisms, function with the aid of a metal co-factor held by histidine residues. The metal is typically Zn^{2+} (Co^{2+} and Fe^{2+} can also exist in this role), which is a Lewis acid and activates the attached keto molecule.

1.5.2 Aldolase selection criteria

A small number of aldolases, comprising type I & type II, were chosen for study and experimental reactivity with Baylis-Hillman substrates. Theoretically, Baylis-Hillman substrates of the same size and nature as the natural aldolase substrates, would show some enzyme interaction. These type of enzymes were selected with this in mind, but also as they satisfied the following criteria:

1. **Availability of X-ray data of enzyme structure** – Useful for obtaining data on mutations revealing active-site structure.
2. **Enzyme mechanistic classification** – One enzyme of each type should be tested, in order to analyse varying mechanistics.
3. **Substrate acceptance and scope** – Aldolases readily accept DHAP, should determine other acceptable molecules, natural or synthetic. Phosphate groups tend to make synthesis problematic, hence acceptance of non-phosphorylated molecules would be appropriate. Is there scope to include Baylis-Hillman resembling reactants?
4. **Little or no application in synthesis** – Should the *in vitro* evolution experiments not fully materialise, then there is the possibility of exploring

more basic biochemical, inhibition and syntheses for aldolase reactions not previously covered.

5. **Ease of expression and purification** – It would be useful if procedures already exist for chosen enzymes and/or physical properties observed for enzymes can be utilised, e.g. extreme thermophilic stability.

Based on the above points, these aldolases were chosen for the experiments:

- Fructose-1,6-bisphosphate aldolase from *Thermus aquaticus*.
- Fructose-1,6-bisphosphate aldolase from *Thermoproteus tenax.*
- Fructose-6-phosphate aldolase from *Escherichia coli*.

1.5.3 Attributes of the selected aldolases

Thermus aquaticus fructose-1,6-bisphosphate aldolase (*Taq* FBP aldolase),[33,34] is revealed by X-ray structural analysis (2.3 Å resolution), to be a tetramer of doublet dimers with a single subunit constituting a $(\alpha/\beta)_8$ barrel fold. It is a type II aldolase, with Co^{2+} present as the metal cofactor. The active sites contain loop structures, which are open when the active site is unoccupied and closed when occupied; providing more flexibility than available with type I enzymes. *Taq* FBP aldolase is extremely thermophilic, with optimal activity between 60-95 ℃ and denaturation occurring well above 105 ℃.

Thermoproteus tenax fructose-1,6-bisphosphate aldolase, is revealed by X-ray structural analysis (1.9 Å resolution, and 2.1 Å bound with DHAP),[35] to be a dimer of pentamers with a single subunit constituting a $((\alpha/\beta)_8$ barrel fold.[36,37] X-ray data also exists for attached reactant intermediates to mutants (1.9 Å resolution).[38] *T. tenax* FBP aldolase, classed as type I and of archaic origin, is a thermophile of unknown range.

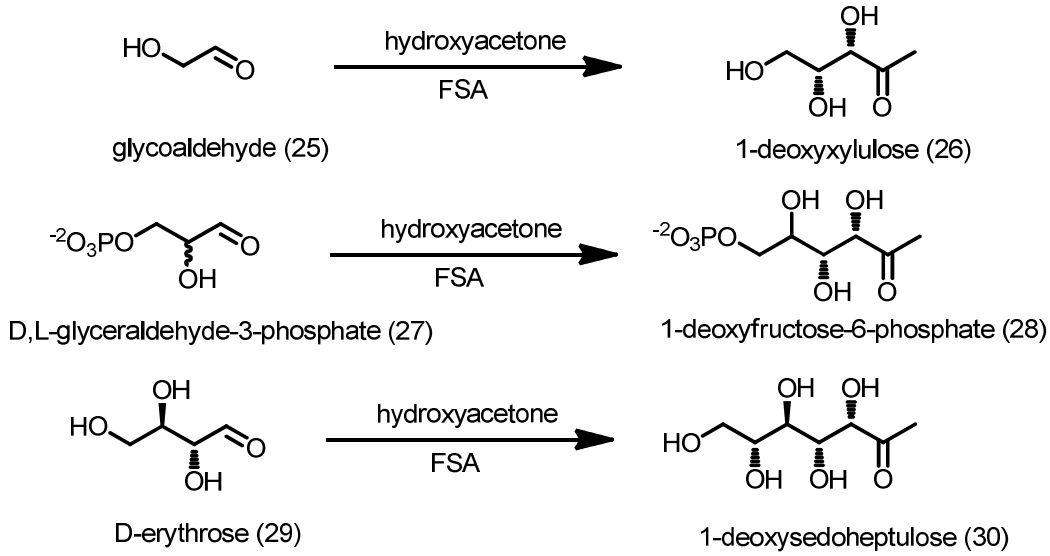
Escherichia coli fructose-6-phosphate aldolase (FSA) is revealed by X-ray structural analysis (1.93 Å resolution, with bound glyceraldehyde),[39] to be a decamer, sandwiching two circular disc-like pentamers, with a subunit constituting a $(\alpha/\beta)_8$ barrel fold. It structurally resembles transalodolases, the class of enzymes it is thought to have evolved from, but is more compact because of considerable secondary structure omissions. Minor changes in the active site have possibly converted the enzyme from transaldolase to aldolase. It is a type I aldolase, but is able to accelerate the breakage and production of fructose-6-phosphate from dihydroxyacetone (DHA) and glyceraldehyede-3-phosphate, without the need for DHAP as a donor molecule. The active site consists of an attached water molecule which propels protonation of product and detachment of the active site lysine (analogous to the Aggarwal mechanism, assisted by protic additives, for Baylis-Hilman reactions).

1.5.4 Substrate scope and synthetic utilisation of enzymes to date

Taq FBP aldolase drives the breakage and production of fructose-1,6-phosphate, but little else is known about alternative substrates and reactivity of this aldolase.

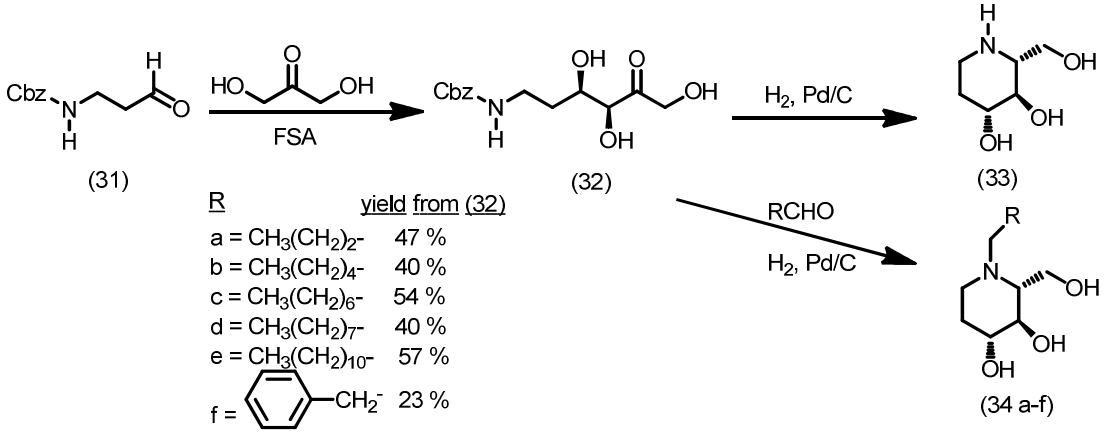
T. tenax FBP aldolase accelerates the reversible production of fructose-1,6-phosphate and additionally fructose-1-phosphate, with fructose-1,6-phosphate believed to be the natural physiological substrate.[35] No reactivity with alternative substrates has been published, but the acquisition of fructose-1-phosphate as a substrate, indicating the enzyme might allow the use of non-phosphorylated substrates.

Escherichia coli fructose-6-phosphate aldolase (FSA) drives the reversible production of fructose-6-phosphate from dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate. Notable that it prefers DHA and not DHAP, FSA also exhibits a strange liking of alternative donor substrates. Sprenger[40] instilled hydroxyacetone (HA) to produce 1-deoxysugars (**Scheme 1.5.4.1**).



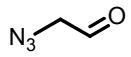
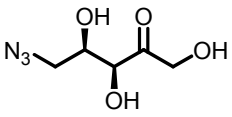
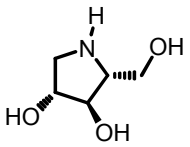
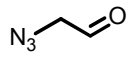
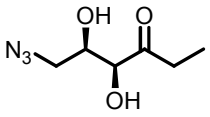
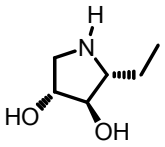
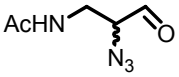
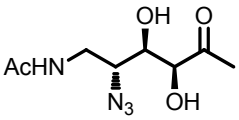
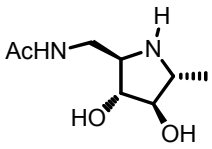
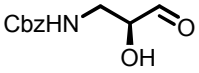
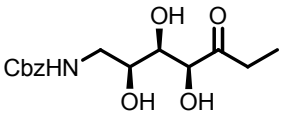
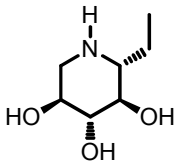
Scheme 1.5.4.1 Reaction of FSA, hydroxyacetone and certain aldoses

Joglar and Clapés[41] made use of the FSA driven reaction of dihydroxyacetone and *N*-Cbz-3-aminopropanal (**31**) in a two step synthesis to afford *D*-fagomine (**33**) in 51 % yield and 99 % de (**Scheme 1.5.4.2**). Various *N*-alkylated species (**34 a-f**) were also produced from compound (**32**) and the counterpart aldehyde *via* a single reductive amination step.



Scheme 1.5.4.2 FSA catalysed production of *D*-fagomine and derivatives

Resembling syntheses on iminocyclitols were conducted by Greenberg and Wong.[42] They ran pilot kinetic tests on FSA with glyceraldehyde-3-phosphate as the acceptor moolecule and hydroxyacetone (HA), dihydroxyacetone (DHA) and 1-hydroxy-2-butanone (HB) as donor molecules. Interestingly all these substrates were taken up with comparable success (k_{cat}/k_M values of 33, 75 and 20 M^{-1} s^{-1}). Single stage transformations were conducted using the three donor substrates HA, DHA and HB and certain azido and Cbz-amino aldehyde acceptors excelled by FSA; aldol and reductive amination steps ran in one single stage producing 5- and 6-membered iminocyclitols in good yield (3-5 % of other diastereomeric products were observed by NMR spectroscopy). Selected examples are shown in **Scheme 1.5.4.3**.

Acceptor	Donor	Aldol product	Iminocyclitol	Yield (%)
	DHA			73
(35)		(36)	(37)	
	HB			83
		(38)	(39)	
	HA			57
(40)		(41)	(42)	
	HB			53
(43)		(44)	(45)	

Scheme 1.5.4.3 FSA catalysed iminocyclitol synthesis

1.6. Screening methodology

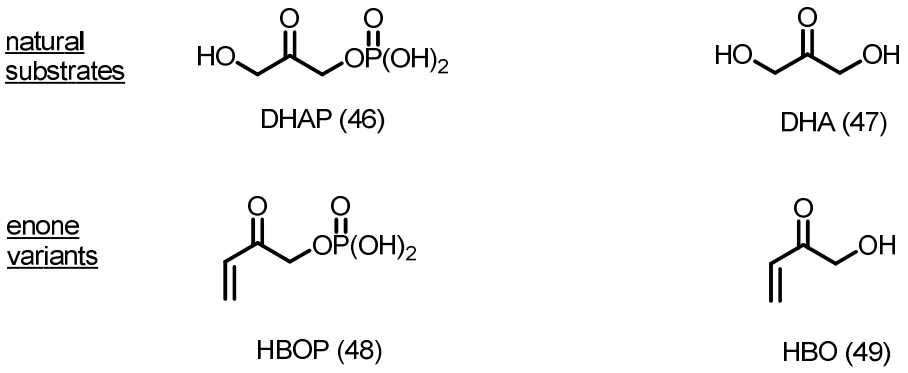
1.6.1 Consideration of natural substrates[43]

There are essentially two points to consider in the creation of the screening process:

1. Structure and synthesis of substrates that are utilised by the selected enzymes and that might participate in a catalytic reaction resulting in the production of Baylis-Hillman products.
2. Designing substrates and reaction conditions, flexible for the identification and analysis of any reaction that takes place.

Type I FBP aldolases typically show high specificity with respect to the donor substrate they utilise; in this case, dihydroxyacetone phosphate (DHAP). FSA shows, as already illustrated, a reasonably lenient specificity for donor substrates; its normal physiological donor substrate is dihydroxyacetone (DHA). A starting point for possible donor candidates for aldolase enzymes, to be prepared by other members of the Berrisford group, are 1-hydroxy-3-buten-2-one phosphate (HBOP) for FBP type I aldolases and 1-hydroxy-3-buten-2-one (HBO) for FSA (**Scheme 1.6.1.1**). The target structures, HBOP and HBO, deliberately resemble their respective natural donor substrates, with the introduction of an enone group to provide the scope for a Baylis-Hillman reaction. The phosphate group in HBOP is absolutely essential for utilisation by

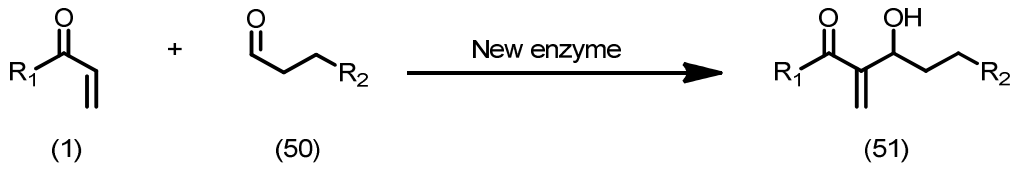
Type I FBP aldolases. Depending on the outcome of utilisation with these new substrates, more structurally diverse molecules will be studied.



Scheme 1.6.1.1 Initial synthetic target donor molecules

Specificity of acceptor aldehydes is much more flexible than for donor substrates. The selection of aldehyde to be utilised in a screen is crucially dependant on the type of screening technique, which is the focus of this project.

The Baylis Hillman reaction between an enone and an aldehyde affords a product constituting three functionalities; an enone, a secondary hydroxyl and an allylic alcohol (**Scheme 1.6.1.2**).

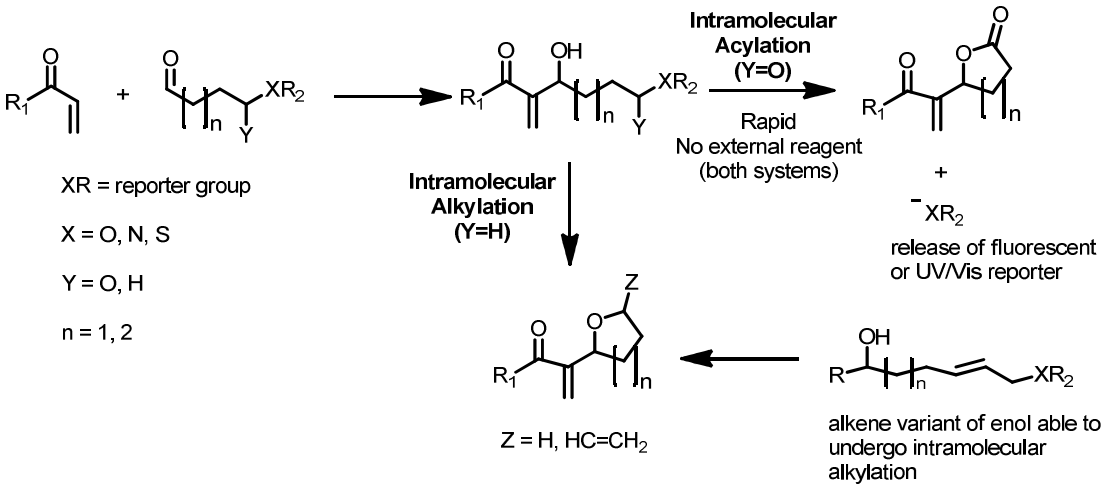


Scheme 1.6.1.2 The Baylis-Hillman reaction between enone and aldehyde

The new functionalities consist of a secondary alcohol and an allylic alcohol, a distinctive product from the enone starting material, and hence it would be rational to explore screening of these groups.

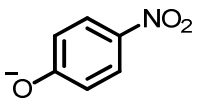
1.6.2 The unique functionality in the secondary alcohol

The alcohol could be considered as a nucleophile, and beneficially, an intramolecular cyclisation with a slightly electrophilic group would allow for the expulsion of a reporter group, XR or HXR, where X = oxygen/nitrogen. It is the intramolecular feature of this reaction which provides scope to develop a system capable of instant cyclisation without external reagent or catalyst interference; the product is predisposed and able to expel the reporter group. There are potentially two routes with this chemistry, involving internal acylation, or alkylation as shown in **Scheme 1.6.2.1** below. The acylation chemistry being more intrinsically reactive, would favour it over the alkylation option. However, with either route it must be ensured measures are in place to account for the background release of the XR (HXR) reporter; which can occur *via* direct hydrolysis at that pH (and buffer) or by aldehyde hydration leading onto cyclisation.

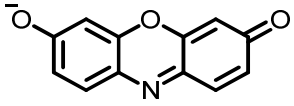


Scheme 1.6.2.1 Cyclisation-release strategy

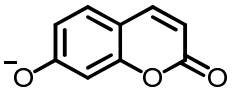
There is flexibility with the structure of the molecule bearing the reporter group, and this can include an ester (X = *O*), amide or hydrazine (X = *N*) functionality. Most fundamental is that the reporter group, XR (XHR), must be readily detectable. Common reporter groups in the literature include nitrophenolate (UV/Vis), resorufin (UV/Vis) and the coumarin umbelliferone (fluorescence). It is evident in the literature that the latter, is only liberated after evolved proteins are screened with chemical or secondary enzymatic reactions on a microtitre plate. A single step process to release the fluorophore would be greatly beneficial. We ideally need to compare UV/Vis and fluorescence detection, with the latter likely to be less sensitive to detection. A good starting point for UV active alcohols would be nitrophenolate, but this might be less sensitive than resorufin, which posses an extensive π-system.



Nitrophenolate (52)
UV/Vis 405nm



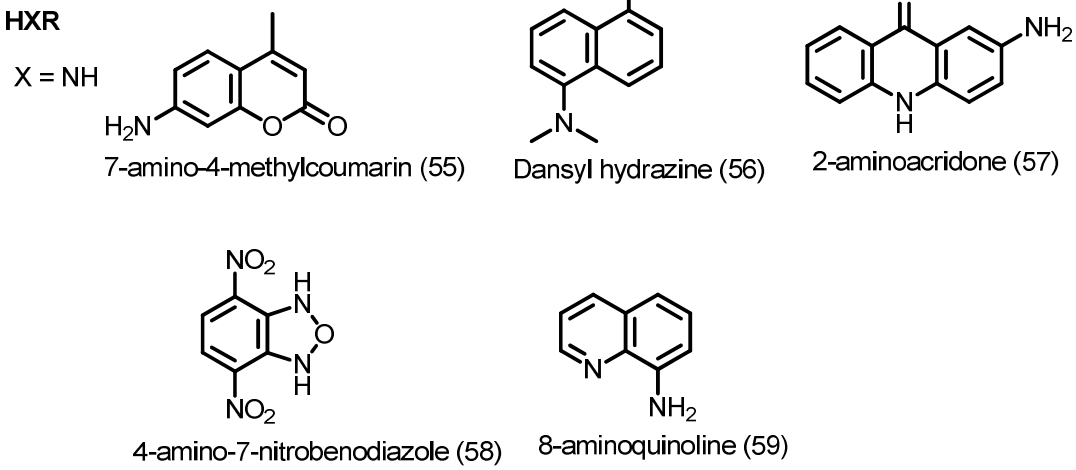
Resorufin (53)
UV/Vis 570nm



Umbelliferone (54)
Fluorescent

Scheme 1.6.2.2 Potential reporter groups

It is generally believed systems with X = *O* would work better, possessing greater reactivity towards cyclisation, than systems with X = *N*. A selection of possible reporter groups for X = *N* is shown below (**Scheme 1.6.2.3**), where a switch in chromophore from amide to amine (protonated form) results in UV activity. All those shown have been used as fluorescent probes/labels and produce reasonable Stokes' shifts.



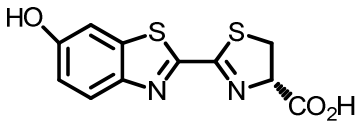
Scheme 1.6.2.3 Potential amine groups

One problem with using an amide is that the cromophore detected post-cyclisation would be amplified in the presence of neutral amine as the major component, *i.e.* from the electron withdrawing amide pre-cyclisation, with the *N*-lone pair in conjugation with the carbonyl, to an electron rich system with the *N*-lone pair in conjugation with the extended π-system (protonation likely to minimise this). Hence the coumarin is a better option to start off with.

An alternative strategy for a reporter group is the example of Luciferin. It is catalytically oxidised by Luciferase with the addition of ATP. This results in luminescence and is commonly used for bioassays. We could exploit this characteristic by using a Luciferin ester as substrate, by either utilising with Luciferase, or if not able to do so then have release of the molecule detected.

Luciferin + ATP → luciferyl adenylate + PPi

Luciferyl adenylate + O_2 → oxyluciferin + AMP + light



Luciferin (60)

Scheme 1.6.2.4 Reaction of Luciferin to produce light

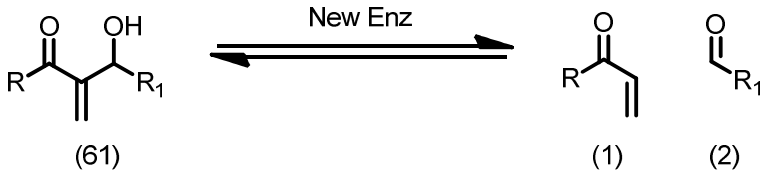
1.6.3 The allylic alcohol functionality as a probe

The alcohol could specifically be oxidised by a number of available reagents and catalysts. However, this would mean introducing a reagent-substrate interaction, concentration factor and a work-up step to a relatively simple intramolecular reaction. Simpler than this would be an enzymatic oxidation, based on methods common in the literature.[44] This could be possible by allowing the oxidising agent to be reduced to the UV active specie, for instance a stiochiometric transition metal changing chromophore upon reduction. Or perhaps even simpler would be enzymatic dehydrogenase oxidation, producing reduced cofactor, say NAD(P)H from $NADP^+$, for detection at 340 nm or use as a reporter in a subsequent enzyme reaction. In theory, the direct cofactor detection method could be utilised to assay the enantiomeric excess *via* a particular dehydrogenase. Certain dehydrogenases believe to exist which would be able to discriminate between R and S enantiomers of substrate, hence providing potential for a more advanced screen after success with a simpler model.

Scheme 1.6.3.1 Enzyme oxidation reaction affords detectable reduced cofactor

1.6.4 Screening for the reverse reaction

Recently aldolases have been evolved to reversibly catalyse aldol reactions, simultaneously releasing pyruvate.[45] This could potentially be exploited, to screen for aldehyde release from Baylis-Hillman adducts. The aldehyde functionality is unique in this system, analogous to the secondary alcohol in the reverse direction, hence a suitable candidate for screening.

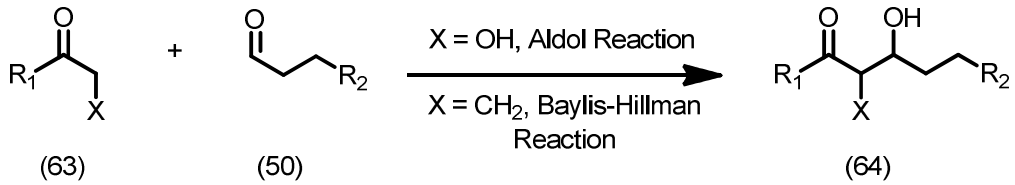


Scheme 1.6.4.1 Reverse screening

1.7 Overall proposal of project

The objective of this project is primarily to develop a cyclisation-release methodology which could be applied to the investigation of Baylis-Hillman reactions, and to further develop a methodology, enzymatic or chemical, suitable to screen reaction products. The screening process will ultimately be incorporated to identify a potential enzyme accelerated Baylis-Hillman reaction, developed through directed evolution by other members of the Berrisford group. This area of work is based around evolving aldolase enzymes as they are

reversibly catalytic in living organisms and bear much resemblance to the Baylis-Hillman reaction, thus an ideal starting point for directed evolution.



(63) (50) (64)

Scheme 1.7.1 Similarity between Aldol and Baylis-Hillman Reactions

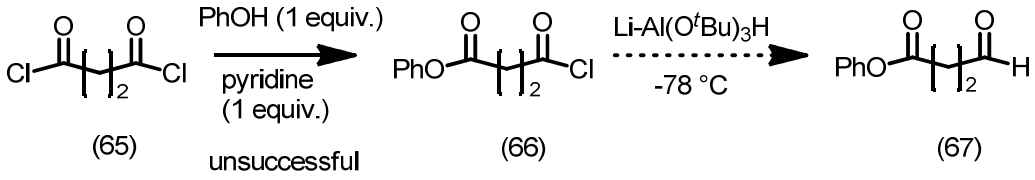
There is wide-spread enthusiasm in the Baylis-Hillman synthesis and the manufacture of abiotic, asymmetric organic catalysts. There is no general asymmetric catalyst or even a biocatalytic analogue of this reaction.

In a wider context, development of a screen will help validate successful directed evolution, of a totally new C-C bond forming enzyme originating from a class of aldolases.

2. Results & Discussion

2.1 First attempt towards cyclisable aldehyde systems; bis-acid chlorides

The main focus of the project lay in creating 1,5 or 1,4-ester/amide-aldehydes, bearing an appropriate reporter group. The reporter group could be detected upon a cyclisation-release mechanism following conversion of the aldehyde to alcohol. Initially, the shortest path to the envisaged cyclisable aldehydes, was investigated with use of bis-acid chlorides (**Scheme 2.1.1**). The four or five carbon bis-acid chlorides, glutaryl & succinyl chloride respectively, could potentially provide us with the desired aldehydes in two steps; firstly mono-esterification of bis-acid chloride and secondly chemoselective transformation of remaining acid chloride group to aldehyde.[46] It should be noted that we were most interested in creating aryl esters.



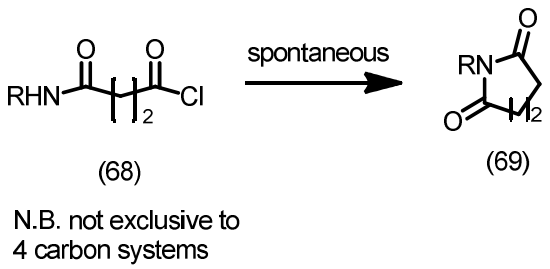
N.B. only 4 carbon acid attempted-
5 carbon acid also possible

Scheme 2.1.1 Attempted mono-esterification of bis-acid chloride and proposed aldehyde synthesis

There was some limited precedence in the literature for mono-esterification of bis-acid chlorides. A literature search revealing only one case,[46] this involved the selective mono-esterification of glutaryl chloride with a phthalimide derivative, using pyridine at room temperature. This encouraged us to test this chemistry, and so we attempted a mono esterification of succinyl chloride (**Scheme 2.1.1**), using controlled amounts of phenol, and pyridine at various temperatures (0 ℃ → RT) in DCM.

Several attempts were made at 0 ℃; with variation of concentration (approx. 0.1 → 0.01 M) and alteration of order of addition (the mixture of phenol & base into acid chloride seemed most likely to succeed). However, all attempts were unsuccessful and this reaction just seemed too aggressive. In any case, the bis-substituted product was not observed either. The nmr data on the solution separated from the precipitate indicated significant presence of free phenol (OH proton at 7.7 ppm), suggesting the base was reacting with the bis-acid chloride to give a pyridinium salt. On this evidence, it was concluded that bis-acid chlorides were generally too reactive for mono-substitutions, even with the use of mild base (although we did not look into sterically hindered bases). Hence there would need to be some variation between the acid-chloride groups to give an unsymmetrical system, in order to introduce selectivity and enhance scope for mono-substitution reactions.

Amines were being considered as alternatives to phenols for the above work; however there was already a problem applying this reaction to amines, as their presence within an acid chloride could result in nucleophilic substitution, even without the assistance of base. In this case we would likely observe premature cyclisation (**Scheme 2.1.2**).



(68)

N.B. not exclusive to 4 carbon systems

(69)

Scheme 2.1.2 *N*-cyclisation of amide acid chloride

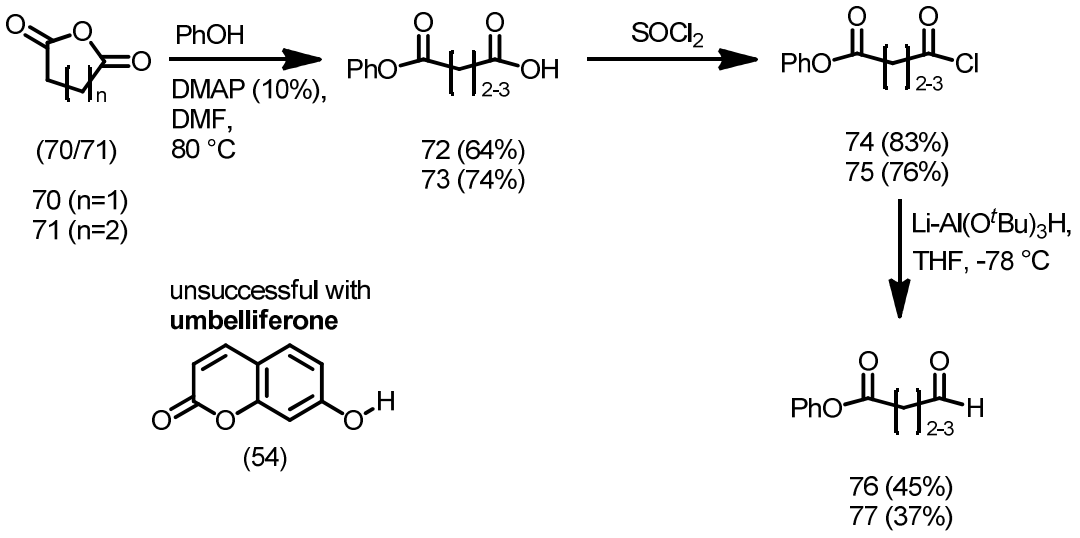
In this type of cyclisation, as the reaction is intramolecular, the acid-chloride would not be isolable and thus we would be unable to override this process with an intermolecular reaction.

It should be noted that premature cyclisation can also occur *via* hydration at the following aldehyde stage, applicable to both esters and amides, however this shall be addressed later.

2.2 Another direct route towards aldehyde systems; cyclic anhydrides

After the unsuccessful bis-acid chloride chemistry, the next direct route adapted to get to the aldehyde systems was *via* ring opening of cyclic anhydrides. Commercially available five and six membered cyclic anhydrides, succinic and glutaric anhyride respectively, would be able to provide us with aldehyde in essentially three steps (**Scheme 2.2.1**):

1) Alcohol/phenol nucleophilic attack to ring open anhydride, producing 1,5 or 1,4 ester-acids,
2) Formation of the acid chloride,
3) Chemoselective conversion of the acid chloride to aldehyde in presence of ester.



Scheme 2.2.1 Synthesis of ester aldehyde *via* anhydride

This is actually a modification of the bis-acid chloride approach, in that with an additional step we are able to get to ester acid chloride, and this would then be treated with hydride reagent in same manner to get to aldehyde.

The synthesis was attempted primarily with phenol and then later with umbelliferone. With the former, successful reactions were observed for both five and six membered anhydrides.

The first step involved reaction of phenol (1.1 equiv.) with anhydride and catalytic DMAP (10%) in DMF, heating at 80 ℃ overn ight. The reaction was worked up with dilution and aqueous wash, followed by back-extraction from DMF in the aqueous layer. NMR signal complexity (2.71 – 1.84 ppm range) was observed for the CH_2 region, indicating a characteristic lack of symmetry. Yields of 74% and 64% were obtained for products of glutaric and succinic anhydride (**72** & **73**) respectively.

The second step involved the simple conversion to acid chloride with the use of neat thionyl chloride. Reaction was completed within two hours and the thionyl chloride taken off with rotary evaporation, aided by small aliquots of pentane. The reaction was monitored by TLC, spot moving from R_f of 0.1 (SM) to 0.3 (product). In both cases the acid chloride was isolated as a yellow oil, distinct from the colourless starting material which was also an oil. The crude material

was taken directly onto the next stage. Yields of 83% and 76% were obtained for products of glutaric and succinyl esters (**74** & **75**) respectively.

The final stage involved the conversion of acid chloride to aldehyde with the use of lithium tri-*tert*-butoxyaluminium hydride (1.3 equiv.). This is a chemoselctive reagent that reacts with acid chloride and not the ester. This reaction was carried out at -78 ℃ in THF, with careful addition of reagent. The mixture was quenched with EtOAc and washed with phosphate buffer. This aided in removing the mixture of reagent and its by-products. The conversion was confirmed by presence of an aldehyde proton on nmr spectrum (compound **76** 9.88 ppm (br), compound **77** 9.73 ppm (br)). Crude products (**76** & **77**) were purified by column chromatography to give yields of 45% & 37% respectively.

Under the same conditions as above, esterification with umbelliferone was not achieved. In fact no reaction was observed. We believe the variation in reactivity is possibly due to a difference in acidity between phenol and umbelliferone (pK_a values of 10 and 7.7 respectively).

Alternative amino reporter groups (*e.g.* aniline) were being considered in addition to the phenol/umbelliferone; however amines were not suitable due to the earlier issue of amide acid chloride instability leading to premature cyclisation (**Scheme 2.1.2**).

It should be noted that premature cyclisation extends to aldehyde ester/amide systems (**Scheme 2.2.2**), and this occurs through a distinct mechanism to amide acid chloride cyclisation

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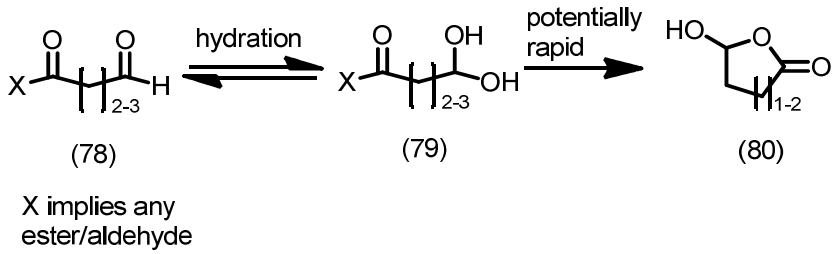


Scheme 2.2.2 Premature cyclisation *via* aldehyde hydration

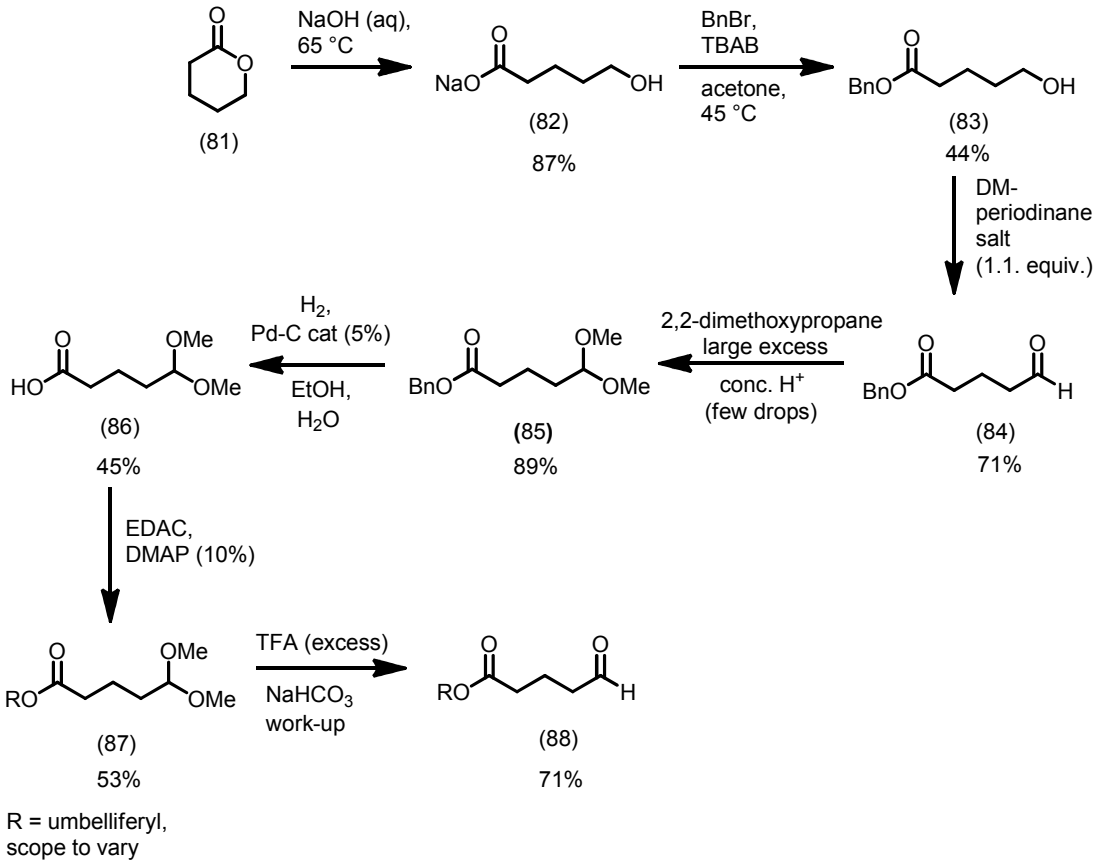
This feature can only just be minimized, its effect along with simple hydrolysis means these aldehydes are generally not stable enough to store for any longer than a few days. Aldehydes (**76** & **77**) were not stable for this amount of time at room temperature (degradation indicated by change of colour), particularly as they are liquids; however they are stable when stored at -18 ℃.

2.3 Alternative method to incorporate umbelliferone; δ-valerolactone

Although the phenol ester aldehyde systems made in the previous section were useful, we essentially required a system that could help us screen efficiently. Umbelliferone (**54**) (strong absorption at 300, 305 & 325 nm, with logarithmic molar absorption co-efficients of 3.9, 3.95 and 4.15 respectively) is a more suitable choice for screening as it is a fluorescent molecule and thus can be

used to detect reaction instantly. The aim now was to develop a methodology that could provide us with umbelliferone ester-aldehyde.

Looking at the previous failed attempt it was decided that the umbelliferone ester, was perhaps not as stable as the simple phenyl ester, hence the umbelliferone moiety should be incorporated at the latest stage possible. In such a case, we would require an aldehyde acid system of some sort, though the stability of the aforementioned molecule might limit its shelf life. Taking a retro-synthetic approach and using a protected aldehyde acid system as the target molecule, we designed a synthesis originating from δ-valerolactone (**Scheme 2.3.1**).



Scheme 2.3.1 Aldehyde synthesis *via* valerolactone

This is in essence, is a variant of the anhydride model, but with valerolactone proving more useful in this case as it provides easy access to aldehyde at one end of the open chain *via* the primary alcohol. In many ways, the most difficult stage is initial ring opening, as once done the molecule is susceptible to cyclisation *via* attack of the free alcohol at other end. In this case, our first product is more unstable than its ester-acid equivalent (**72**/**73**). To avoid premature cyclisation at the first step, we decided to ring open with aqueous NaOH,[47] in order to have the open chain as a stable salt. This would then allow easier esterification of the carboxyl group by substitution. It would not be

possible to esterify directly using a phenol or umbelliferone. Benzyl esters are of moderate stability, so benzyl bromide was chosen[47] to make the ester, and have in place whilst the other functional group in the molecule would be converted to aldehyde and subsequently protected. At the benzyl ester-acetal stage, there is a role reversal in regards to protection, and we can cleave the benzyl ester to give free acid with the acetal intact. This takes us to the last two steps where the umbelliferone would be coupled with the acid to give ester, and finally the acetal cleaved to reveal the aldehyde product.

The first step as mentioned above, is the valerolactone ring opening using aqueous sodium hydroxide solution (1 M) and heating to 65 ℃ overnight. This reaction is relatively simple and gives good conversion. However, there is difficulty in drying the salt as when isolated the material is ‘paste’ like and uneasy to handle. The material was firstly ground up thoroughly and then dried under high vacuum for several weeks.

The second step involves benzyl esterification using benzyl bromide and TBAB in dry acetone at 45 ℃ for 24 hours. It requires a high concentration (minimal acetone) and two equivalents of benzyl bromide to accelerate the reaction. Attempts were made using reduced quantities of benzyl bromide (1.1 equiv.), but this only slowed the reaction to the order of several days. Initially, the salt is a slurry in the acetone but gradually goes into solution, with heating and aid of the phase transfer catalyst. The salt and any by-products are washed out by

working up with water, aqueous $NaHCO_3$ and subsequently brine solution to wash out the base. Care has to be taken with the basic wash, because if not promptly removed the benzyl ester can cleave due to instability outside neutral pH. After the work-up there is still benzyl bromide impurity, the removal of which requires column chromatography. This does have to be fairly quick and the purified product promptly taken onto next stage before the onset of cyclisation. Yields of around 44% have been obtained.

The third step entails the simple oxidation of a primary alcohol to an aldehyde using a mild reagent, Dess-Martin periodinane in our case. This reaction is complete within two hours, notable change by TLC (R_f 0.3 to 0.5), and being fairly clean enables filtration and straight column chromatography. NMR analysis confirmed the presence of the aldehyde peak (broad singlet around 9.72 ppm).

The fourth step involves aldehyde protection to the acetal using 2,2-dimethoxy-propane and a few drops of concentrated HCl. The reaction is performed with an excess of reagent, without the need for solvent and this excess is easily taken off by rotary evaporation. Again the reaction progress was confirmed by TLC (0.5 to 0.6 R_f), and nmr (acetal singlet peak around 3.30 ppm).

The fifth step involves hydrogenolysis in order to cleave the benzyl ester, this is done on Pd-carbon catalyst in ethanol, with water (5%). The reaction goes to

completion within two hours and is filtered through a fine filter with a small layer of silica. This ensures removal of the reagent. The progress of the reaction was checked by crude nmr; confirming presence of acid proton peak (singlet around 11.12 ppm), and loss of the benzylic protons (singlet around 5.10 ppm).

The sixth step is the coupling reaction with water soluble reagent- EDAC (1.2 equiv.). This is used with catalytic DMAP (10%). Originally a different carbodiimide, DCC, was tried and it proved to be very difficult to remove from the reaction (only separable by chromatography). Where as EDAC is easily washed out in the aqueous phase. This reaction gave an overall yield of 53%.

The last step is the acetal cleavage using TFA in DCM, reaction being complete within one to two hours and the reagent washed out using bicarbonate.

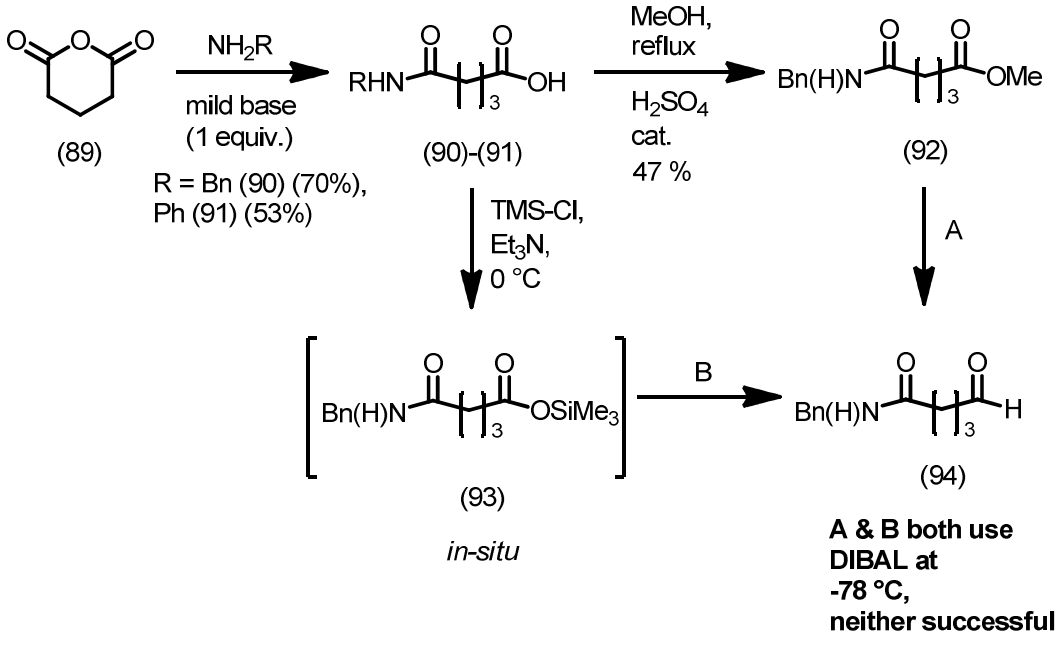
This overall synthesis, albeit slightly tedious, finally provided us with the umbelliferone ester aldehyde needed for screening.

2.4 Methodology for amides

Although theoretically the above methodology could be applied to amides; unpublished work relating to this chemistry by colleague M. Swiatyj, suggests amides are prone to cyclisation (**Scheme 2.1.2**) during the acetal cleavage. To overcome this problem, cleavage of these acetals by an external nucleophilic

attack at the methyl group is required. Of course, successful cleavage would only be possible if the reaction was to proceed *via* an intermediate oxonium ion.

There was still the possibility of exploiting earlier anhydride chemistry, in order to provide us with a short route to amide aldehydes. Previously, the attempted formation of an acid chloride would result in premature cyclisation. We needed an alternative intermediate in place of the acid chloride to get us to the aldehyde. There was precedence in the literature[48] for the reduction of an ester to an aldehyde with the selective reagent DIBAL. This would selectively reduce the ester without any reactivity with the amide. This was the key step in this chemistry (**Scheme 2.4.1**).



Scheme 2.4.1 Amide aldehyde access *via* anhydrides

The first step is analogous to earlier ring opening with esters. The product is the amide acid (**90**/**91)**), produced from coupling using triethylamine (1 equiv.).

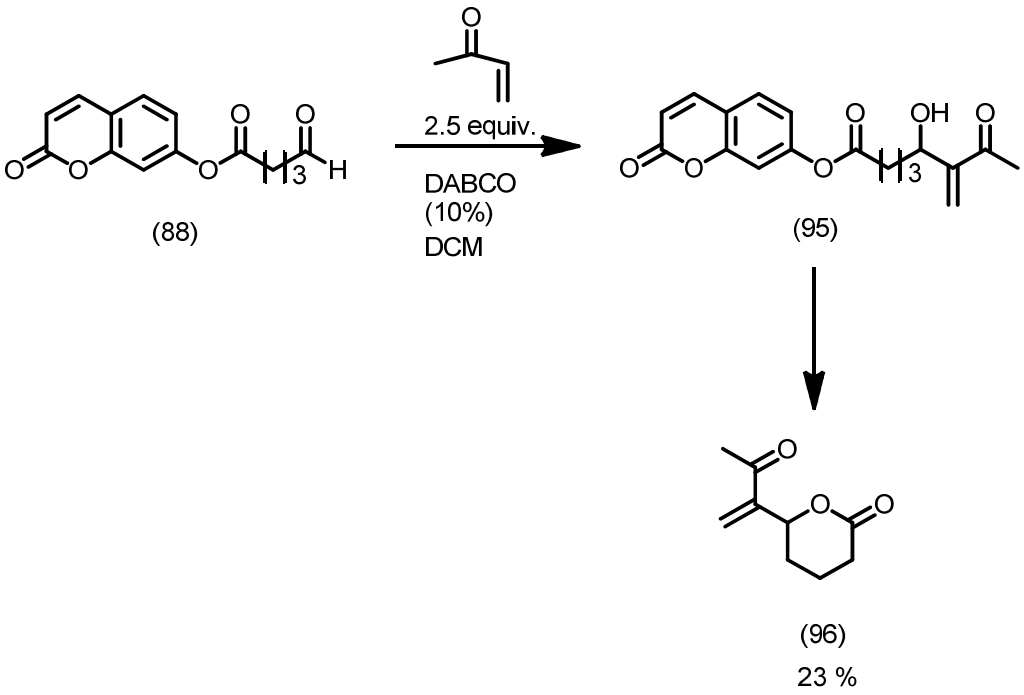
The second step, is the relatively simple Fischer esterification in an excess of methanol, aided of course with a few drops of concentrated H_2SO_4. We can also switch the esterification to the first step, to get ester acid and then make amide *via* coupling to the acid chloride. The advantage of that methodology is that the coupling is both easier (base independent) and performed at a latter stage. However, prior ring-opening of the anhydride with an alcohol is more difficult than with an amine. Anhydride ring opening with methanol was attempted, and we observed rapid reversibility of the reaction, apparent within a few hours of recovery of the product. Hence, only a low yield (30%) could be obtained.

It is the last stage, generating the aldehyde (**94**) from the ester amide (**92**/**93**), which did not come to fruition. The addition of **92**/**93** was carefully controlled and reactions were conducted at -78 ℃. Reactions were given up to several hours with both 1 equivalent and an excess of DIBAL (1.5 → 2.5 equiv.), but on neither occasion was any product observed. Reaction progress was difficult to monitor *via* TLC as was observing several spots around the reactant spot. The reaction was quenched with a few drops of methanol and worked-up with aqueous wash. NMR data of recovered material showed starting material alone (81% of reactant mass).

There was an alternative to the methyl ester, S. Chandrasekhar[49] had reduced a silyl ester (formed *in-situ*) to aldehyde, using trimethylsilyl chloride. The silyl ester formation is suppose to be instantaneous at 0 ℃, and is assisted by triethylamine. The reduction was attempted using the conditions in the literature, using DIBAL (1 equiv.) at -78 ℃, and left for their stated time of half an hour and even longer, but no apparent reaction was observed (recovered free acid (**90**)).

2.5 Application of cyclisation concept

With the umbelliferone ester aldehyde (**88**) to hand, we were in a position to demonstrate the principle of cyclisation. In order to do this it was decided a simple method would be to react the aldehyde with a nucleophile, and then observe reaction. The nucleophile selected would be a Baylis-Hillman reactant (enone system), a choice that also confirms the chemical Baylis-Hillman reactivity of this system.



Scheme 2.5.1 Chemical Baylis-Hillman reaction triggering cyclisation

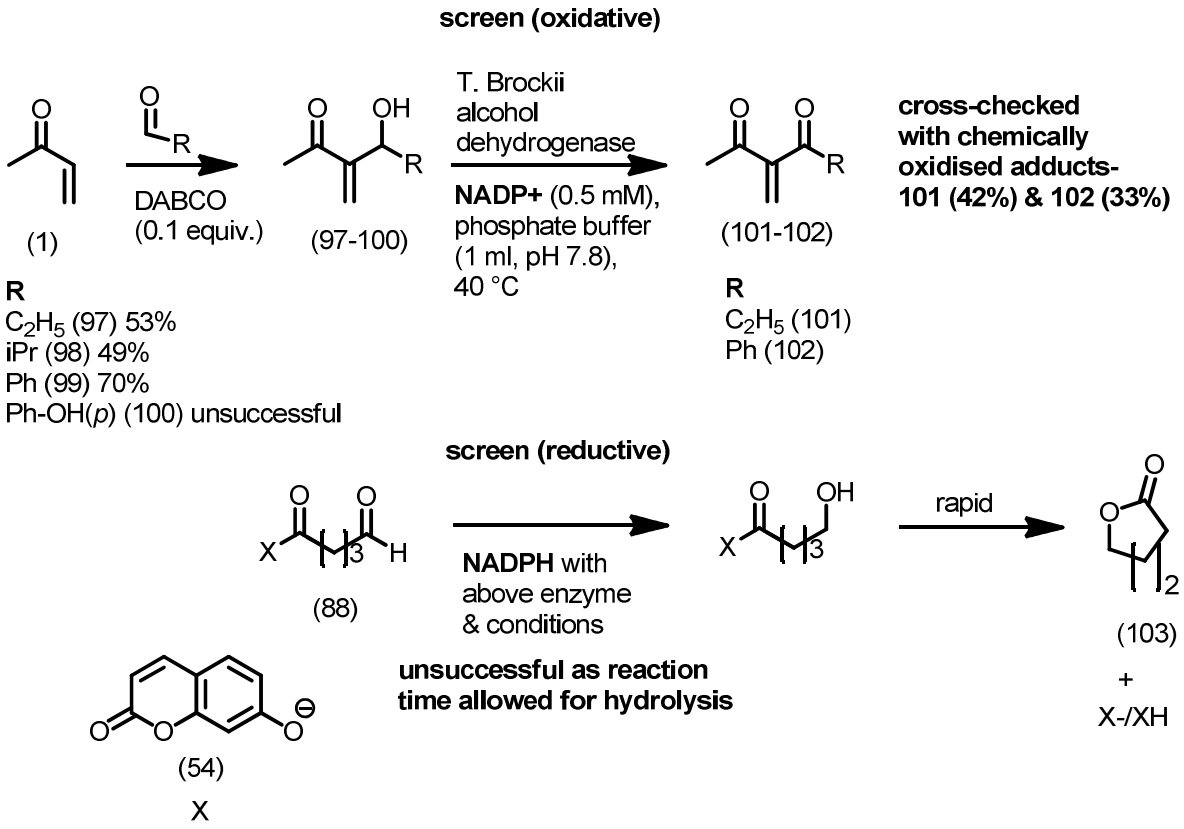
The general methodology for the Baylis-Hillman reaction was adapted from the literature.[46] The reactant, MVK (excess to accelerate reaction), was treated with the aldehyde in DCM with DABCO, and monitored over the course of six hours to decent conversion, with monitoring of fine CH_2 structure that exists for the glutaryl moiety (2.71 – 1.84 ppm range) with use of proton nmr. Successful reaction of the aldehyde to give the cyclic adduct was observed, with only the starting materials remaining in the reaction mixture. In the first instance, there was already free umbelliferone present (slight insolubility present in solvent), presumably due to competing hydrolysis. With this experiment it is difficult to quantify level of background umbelliferone release from hydrolysis, however the final product (**96**) is only viable through Baylis-Hillman reaction. Intermediate

(**95**) was not detected over the course of the reaction, suggesting it has a relatively short life-time, confirming our hypothesis on cyclisation-release with the appropriate leaving group.

2.6 Utilisation of Baylis-Hillman adducts and umbelliferone ester aldehyde in a screen

As an alternative screening methodology we decided to perform a simple enzymatic screening experiment, using selected Baylis-Hillman adducts (**97** & **99**) and the umbellifeone system (**88**) prepared previously (**Scheme 2.3.1**), as substrates. The purpose was to compare substrate suitability and reactivity of these compounds with *Thermoanaerobium brockii* alcohol dehydrogenase (**Scheme 2.6.1**). These were designed as simple screening experiments, based on the use of TLC and mass spectrometric detection of products. We originally indented to use UV Spectroscopy, but felt it would not work effectively for phenolic compounds, due to little discrimination between chromophores of phenol ester aldehyde (**76**/**77**/**88**) and unbound phenol. With regards to the reaction itself, for the umbelliferone compound we would be looking for enzymatic reduction (NADPH co-factor) of the aldhyde to give the secondary alcohol, resulting in cyclisation and the release of umbelliferone. With the Baylis-Hillman adducts we would be looking for the reverse reaction, enzymatic oxidation ($NADP^+$) of secondary alcohol to ketone. The experiments would also

confirm the suitability of simple Baylis-Hillman adducts, and the umbelliferone ester-aldehyde as enzyme substrates. The preparation of the Baylis-Hillman adducts (**97**-**100**) and the enzymatic screening reactions were as follows:



Scheme 2.6.1 Enzymatic screening experiments for detection using TLC

The basic Baylis-Hillman methodology was the same as previously used. We applied the reaction of MVK (1.2 equiv.) in the presence of DABCO, to four different aldehydes of which three were successful, but with varying reaction time-scales (1-3 days). 4-Hydroxybenzaldehyde showed no reactivity under

these conditions and a longer reaction time in excess of 72 hours. The adducts from successful reactions however, were purified, and the propanal (**97**) and benzaldehyde (**99**) compounds taken further onto dehydrogenase experiments. Additionally, they were oxidised chemically using Dess-Martin periodinane to 1,3-diketones (loss of secondary alcohol at around 6.5 ppm), in order to obtain data for cross-examination. Details of the enzyme experiments were as follows:

The substrate (approx. 5 mg) was added to phosphate buffer (1 ml, pH 7.8) containing *Thermoanaerobium brokii* alcohol dehydrogenase (0.5 µg, 5-15 units/mg protein) and $NADP^+$/NADPH (0.5 mM). This gave an approximate substrate concentration of 150 mM. In the case of the benzaldehyde adduct (**99**) solubility was aided with DMSO (1 drop). These enzymatic oxidation reactions[50] were stirred at 40 ℃ and monitored by TLC hourly; with R_F data on previously attempted chemical oxidation of these adducts used for cross-checking product formation (0.4 & 0.5 for benzaldehyde and propanal adducts respectively). Reaction was only apparent though after 48 hours (both BH adducts).

Conversion was expected to take several days for the umbelliferone system (**88**), but we were unable to detect reaction due to complete hydrolysis under less than 24 hours. This was indeed confirmed in a separate control experiment where the umbelliferone ester aldehyde (**88**), was run as a blank in phosphate buffer at pH 7.8. This showed signs of complete ester cleavage over 1 hour (free acid aldehyde spot detected very close to TLC baseline). Mass

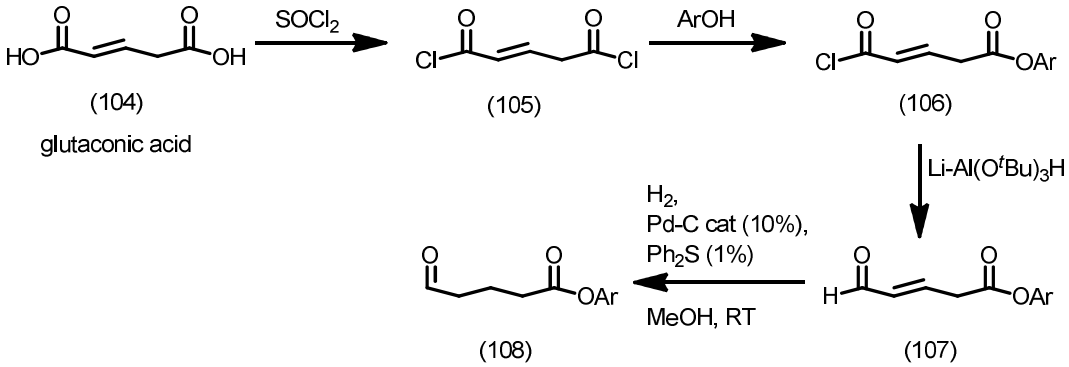
spectrometry data was obtained for the Baylis-Hillman adducts which was in agreement with the data obtained from the corresponding chemical syntheses. It should be noted due to scale of reaction only able to characterise using TLC and MS, and not NMR.

The specification of the *T.Brockii* alcohol dehydrogenase states conversion of 1 µmol of substrate (simple secondary alcohol like propan-2-ol) per unit of enzyme (15/mg) per minute, under pH 7.8 at 40 ℃. This sug gests, with respect to concentrations used, complete conversion of the benzaldehyde adduct (**99**) within 63.1 hrs, and propanal adduct (**97**) conversion within 86.8 hrs. Such details were of course difficult to determine with this scale of experiment, both starting material and product present on TLC at end. The calculation
Is though only a simplistic view, and a significant factor such as enzyme affinity for the substrate would needed to be accounted. Data from the literature[50] suggests propan-2-ol (V_{max} 78 µmol/min per mg of protein) is the most favourable substrate and secondary alcohols with 3-6 carbon chain lengths have an apparent V_{max} ten times greater than their primary hydroxyl analogues. Substrates of the nature of these adducts have not been reported, but judging on size would be expected to have a V_{max} less than 5. It should also be noted that reaction in the reductive direction is considerably slower (V_{max} 7.8 µmol/min per mg of protein for ethanal) and hence more difficult to incorporate experimentally.

2.7 Conclusions and future work

The main target of devising a sound route to 1,5 ester-aldehyde systems with a reporter group moiety has been achieved. This is viable using cyclic anhydrides but for more labile phenols, the longer route using valerolactone is a better option, leaving the coupling of the reporter up to last stage possible. Amide analogues were not successfully prepared with the anhydride chemistry, due to failure of ester reduction to the aldehyde using DIBAL. They can potentially be prepared *via* the valerolactone route, with the final products expected to be more stable than those with an ester. This would also be useful as range of UV active/fluorescent amines available.

The following is a revised synthesis (**Scheme 2.7.1**) for failed mono-substitution of bis-acid chlorides, which could potentially prove to be a better route than the valerolactone chemistry. The starting material is commercially available glutaconic acid. It would be expected that with the un-conjugated acid-chloride being more reactive, esterification would likely occur there only with one equivalent of alcohol/phenol. Furthermore, use of the *trans* alkene would prohibit cyclisation.



Scheme 2.7.1 Proposed aldehyde synthesis from conjugated bis-acid

There is evidence in the literature to support general allylic acid conversion to acid chloride[51], and other evidence to support selective hydrogenation of general alkenes[52]. This would be the key step in the synthesis, using diphenyl sulphide as a catalyst poison to allow selective alkene reduction in the presence of ester (benzyl in literature). Phenol esters could be tested on this evidence. Of course the aldehyde might need protecting, as previously shown to be the case (**Scheme 2.3.1**).

These aldehyde systems can be utilised to undergo cyclisation and release detectable reporter group, under any reaction which would convert the aldehyde to alcohol. They were initially devised to screen for new C-C bond forming reactions, and this has been put to the test with a chemical Baylis-Hillman reaction. Successful reaction has been confirmed with the presence of cyclised product.

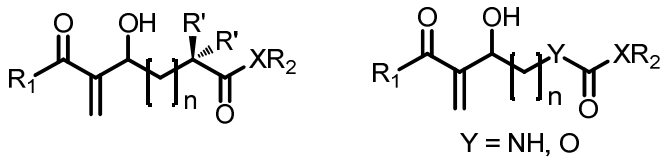
Screening needs to be worked on however, whilst attempts to monitor dehydrogenase activity with various aldehydes (**97**/**99**) has been successful, this has not been the case with the umbelliferone aldehyde (**88**), as it has shown to be unstable outside neutral pH (due to a combined effect of hydration-cyclisation and direct hydrolysis). Such a system requires a selective screen, and so this could possibly be attempted under the following scenarios;

1) Use of a neutral pH solution, allowing the ester to react under stable conditions.
2) Use of amides (prepared *via* valerolactone), or other less reactive leaving groups than esters, allowing reaction under original conditions (pH 7.8).
3) Increase in the aldehyde chain length, subsequently decreasing the rate of cyclisation.
4) Exploration of non aqueous conditions (umbelliferone aldehyde (**88**) already shown to be stable in DCM at room temperature), enabling this type of methodology to be applied with catalytic transition metal chemistry. For example looking to demonstrate reactions of the aldehyde, to allow for easier detection.

Regarding the attempted screening experiments (**Scheme 2.6.1**), we were also unable to generate detailed and quantified data. This could be possible with nmr on a larger scale experiment (integration change of the significant functionality over time), or perhaps even better fluorometry experiments with active reporter groups, *e.g*. umbelliferone. We suspected simple UV spectroscopy would not

work unless free reporter molecule absorption uniquely distinct from its bound state.

In addition to the type of aldehyde systems already in place, certain aspects can be modified to increase the rate of cyclisation (**Scheme 2.7.2**). Although cyclisation rate is not the main issue regarding our present stand-point; it is a feature we would like more control over once the main aldolase screen is in place. For example in the case where R'= Me, this would produce a gem-dimethyl moiety (can actually be on any position between cyclisable components), resulting in enhanced cyclisation due to the Thorpe-Ingold effect. Alternatively, change in ring size (n) from six-membered to five-membered, or constraints such as unsaturation introduction in the linker (*cis* alkenes or aromatic); all help with the cyclisation rate. The rate of cyclisation is expected to be greater with a five-membered[53] compound, where n=1. Further variation by the introduction of a heteroatom in between the dicarbonyl system would also alter the rate of cyclisation.



Scheme 2.7.2 Structural variations to alter cyclisation rate and general hydrolytic stability

3. Experimental

3.1 General Experimental

^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 300/400 MHz spectrometers. Chemical shifts are given in ppm and are referenced to tetramethylsilane and residual protonated solvent shifts. Standard abbreviations are used throughout (s = singlet; br = broad singlet; d = doublet: t = triplet; q = quartet; m = multiplet).

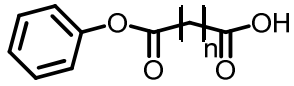
Infrared spectra were recorded as evaporated films or liquid films on potassium bromide discs using a Mattson Genesis Series FTIR spectrometer.

Low resolution mass spectra were recorded on a Micromass Trio 2000 spectrometer for EI/CI spectra, and a Micromass Platform II spectrometer for electrospray spectra. All high resolution mass spectrometry was performed using a Thermo Finnigan MAT95XP spectrometer. All mass spectrometry results are reported in the form *m*/*z*.

Thin layer chromatography was performed using 0.25 mm pre-coated aluminium-backed silica gel 60 F_{254} plates and column chromatography with silica gel (particle size 40-63 μm), both supplied by Merck.

3.2 Individual Procedures

Expts. 1-2 Preparation of Phenyl succinate & glutarate[54] (**72-73**)



n = 2-3

Phenol (2.02 g, 21.5 mmol, 1.1 equiv), glutaric anhydride (2.2g, 19.3 mmol)/succinic anhydride (1.93 g, 19.3 mmol) and DMAP (0.236 g, 1.93 mmol, 0.1 equiv) were heated to 80 ℃ overnight in dry DMF (2 ml). The reaction mixture was diluted with EtOAc (50 ml) and extracted with water (1 x 50 ml). The aqueous extract washed with EtOAc (2 x 50 ml). These were combined and dried over magnesium sulphate, before being concentrated for purification by column chromatography on silica gel using an EtOAc:hexane (1:6 → 1:4 v:v) solvent system as eluent.

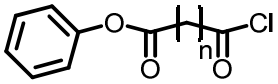
2.28 g (11.8 mmol) of purified phenyl succinate (**72**) was obtained as a colourless liquid (yield 64%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.49 – 7.31 (5H, m, -Ar-H), 2.71 (2H, s, -CH_2), 2.55 (2H, s, -CH_2). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 178.9, 173.4, 154.3, 131.0, 124.8, 122.3, 34.4, 31.6. **m/z** (ESI +ve) 195.8 $[M+H]^+$ (20%) (Calculated 194.1840 for $C_{10}H_{10}O_4$). **R_F** (EtOAc:hexane 1:1) 0.1.

2.97 g (14.3 mmol) of purified phenyl glutarate (**73**) was obtained as a colourless liquid (yield 74%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.40 – 7.29 (5H, m, -Ar-H), 2.73 (2H, t, *J* = 7.4 Hz, -C$\underline{H_2}$-COOH), 2.51 (2H, t, *J* = 6.9 Hz, -C$\underline{H_2}$-COOPh), 1.84 (2H, *J* = 6.9 Hz, quintet, -C$\underline{H_2}$-CH_2-COOH). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 177.4, 174.7, 152.7, 130.0, 126.5, 121.3, 34.4, 31.2, 19.0. **m/z** (ESI +ve) 231.7 $[M+Na]^+$ (40%) (Calculated 208.2106 for $C_{11}H_{12}O_4$). **R_F** (EtOAc:hexane 1:1) 0.1.

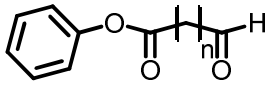
Expts. 3-4 Preparation of Phenyl ester 4-chloro-4-oxo-butanoic acid[55] & Phenyl ester 5-chloro-5-oxo-pentanoic acid[56] (**74-75**)



n = 2-3

Phenyl succinate **72** (0.374 g, 1.93 mmol)/ phenyl glutarate **73** (0.401 g, 1.93 mmol) in thionyl chloride was left to stir for 2 hrs at RT. Reaction confirmed by TLC (EtOAc, hexane 1:1), both products gave R_f values of 0.3. Excess thionyl chloride was removed by vacuum, with addition and removal of pentane (2 x 8 ml). The crude material obtained was carried straight through to the next step. 0.311 g (1.47 mmol) of product (**74**, n=2) was obtained as a yellow oil (yield 76%). 0.362 g (1.60 mmol) of product (**75**, n=3) was also obtained as a yellow oil (yield 83%).

Expts 5-6 Preparation of Phenyl ester 4-oxo-butanoic acid & Phenyl ester 5-oxo-pentanoic acid[57] (**76-77**)



n = 2-3

The acid chloride produced (**74**, 0.311 g (1.47 mmol)/ **75**, 0.332 g (1.47 mmol)) was dissolved in dried THF (4 ml) and cooled to -78 ℃. Lithium tri-*tert*-butoxyaluminium hydride (0.491g, 1.93 mmol, 1.3 equiv.) was dissolved in dried THF (6 ml) and slowly added with the reaction left to stir for 30 mins. This was followed by dilution with EtOAc (70 ml) and extraction with phosphate buffer (50 ml, pH 7.4). The aqueous extract was separated and washed with EtOAc (3 x 50 ml). The washings were combined and dried over magnesium sulfate, followed by concentration and purification by silica gel chromatography using EtOAc:hexane (1:6 → 1:5 v:v) as eluent.

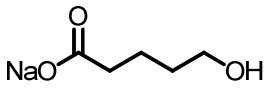
0.118 g (0.66 mmol) of product (**76**) obtained as colurless oil (yield 45%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.73 (1H, br, -COH), 7.44 – 7.30 (5H, m, -Ar-H), 2.64 (2H, t, *J* = 7.1 Hz, -CH_2), 2.48 (2H, t, *J* = 6.8 Hz, -CH_2). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 177.9, 173.7, 154.7, 131.7, 124.1, 120.3, 33.0, 31.1. **m/z** (ESI +ve) 177.1 $[M-H]^+$ (20%) (Calculated 178.1846 for $C_{10}H_{10}O_3$). **R_F** (EtOAc:Hexane 1:1) 0.4.

0.104 g (0.54 mmol) of product (**77**) obtained as a colourless oil (yield 37%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.88 (1H, br, -COH), 7.39 – 7.30 (5H, m, -Ar-H), 2.66 (2H, t, *J* = 7.3 Hz, -CH_2), 2.50 (2H, t, *J* = 6.8 Hz, -CH_2), 1.91-1.85 (2H, m, -CH_2). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 200.9, 173.3, 152.7, 131.0, 124.5, 122.4, 39.6, 31.9, 17.5. **m/z** (ESI +ve) 193.5 $[M+H]^+$ (25%) (Calculated 192.2112 for $C_{11}H_{12}O_3$). **R_F** (EtOAc:hexane 1:1) 0.4.

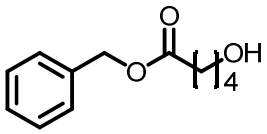
Expt. 7 Preparation of Sodium hydroxybutyrate[47] (**82**)



δ-valerolactone (4.6 ml, 49.6 mmol) was added to a solution of NaOH (50 ml, 1 M) and the reaction was left to heat overnight at 60 ℃. The mixture was concentrated and dried to give product. 5.98 g (42.7 mmol) of product (**82**) was obtained, as a white solid (yield 87%).

^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.28 (2H, t, *J* = 6.0 Hz, -CH_2), 1.94 (2H, t, J = 6.6 Hz, -CH_2), 1.52 (2H, t, *J* = 6.7 Hz, -CH_2), 1.43-1.38 (2H, m, -CH_2). **^{13}C NMR** (DMSO-d_6, 300 MHz) δ 175.1, 66.2, 35.6, 31.9, 23.1. **m/z** (ESI +ve) 139.2 $[M-H]^+$ (50%) (Calculated 140.1129 for $C_5H_9NaO_3$). **IR** 1550 cm^{-1}.

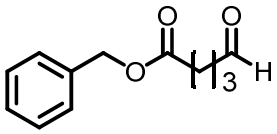
Expt. 8 Preparation of Benzyl 4-hydroxybutyrate[58] (**83**)



To a mixture of sodium 5-hydroxypentaoate (**82**) (569 mg, 4.06 mmol) in acetone (3 ml), was added benzyl bromide (0.97 ml, 8.11 mmol, 2.0 equiv) and tetrabutylammonium bromide (65 mg, 0.203 mmol, 0.05 equiv) and the reaction was left to heat at 45 ℃ for 24 hrs. The mixture w as cooled, concentrated and filtered, with the residue dissolved in EtOAc (200 ml) and subsequently washed with saturated aqueous sodium bicarbonate (75 ml) and brine (75 ml). The extract was dried and concentrated before being purified by column chromatography on silica gel using an EtOAc:hexane solvent system (1:3 → 1:1 v:v) as eluent. 0.372 g (0.179 mmol) of **83** was obtained as a colourless oil (yield 44%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.47 – 7.27 (5H, m, -ArH), 5.10 (2H, s, $-CH_2$), 3.34 (2H, t, *J* = 6.2 Hz, $-CH_2$), 2.52 (2H, t, *J* = 7.5 Hz, $-CH_2$), 1.83-1.75 (2H, m, $-CH_2$), 1.59-1.48 (2H, m $-CH_2$). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 211.4, 139.6, 135.5,130.7, 126.1, 66.3, 51.1, 33.6, 29.9, 23.6. **m/z** (ESI +ve) 206.5 $[M-H]^+$ (20%) (Calculated 208.2536 for $C_{12}H_{16}O_3$). **R_F** (EtOAc:hexane 1:2) 0.3.

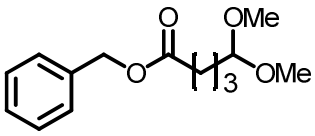
Expt. 9 Preparation of Benzyl 4-oxobutanoate[59] (**84**)



Alcohol (**83**) (300 mg, 1.88 mmol) in DCM (15 ml) was oxidised using Dess-Martin periodinane salt (0.91 g, 1.1 equiv.). The reaction was stirred for 2 hours, following this the mixture was filtered and concentrated; and then purified by column chromatography on silica gel using an EtOAc:hexane solvent system (1:3 → 1:1 v:v) as eluent. 211 mg (1.34 mmol) of purified product was obtained as a yellow oil (yield 71%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.72 (1H, s, -COH), 7.49 – 7.27 (5H, m, -ArH), 5.10 (2H, s, $-CH_2$), 3.20 (2H, t, *J* = 7.2 Hz, $-CH_2$), 2.47 (2H, t, *J* = 7.1 Hz, $-CH_2$), 1.77-1.71 (2H, m, $-CH_2$). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 212.7, 138.0, 134.5, 130.7, 126.6, 76.3, 61.1, 36.6, 28.7, 22.2. **m/z** (ESI +ve) 245.1 $[M+K]^+$ (50 %) (Calculated 206.2378 for $C_{12}H_{14}O_3$). **R_F** (EtOAc:hexane 1:2) 0.5.

Expt. 10 Preparation of acetal (**85**)

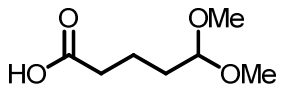


Aldehyde **84** (400 mg, 1.94 mmol), was treated in neat 2,2-dimethoxypropane (2.4 ml, 10.0 equiv.) with 2 drops of concentrated HCl. The mixture was left to stir for 12 hours. Upon completion by TLC the reagent was removed by rotary evaporation to afford product **85** (with little starting material contamination),

which was purified by column chromatography on silica gel using an EtOAc:hexane solvent system (1:4 → 1:1v:v) as eluent. 0.435 g (1.73 mmol) of purified acetal (**85**) was obtained as a colourless oil (yield 89%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.44 – 7.25 (5H, m, -ArH), 5.12 (2H, s, $-CH_2$), 4.33 (1H, t, *J* = 5.2 Hz), 3.30 (6H, s), 3.27 (2H, t, *J* = 6.8 Hz, $-CH_2$), 2.48 (2H, t, *J* = 7.0 Hz, $-CH_2$), 1.77-1.71 (2H, m, $-CH_2$). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 209.4, 137.4, 132.6, 128.7, 126.3, 102.3, 79.1, 54.7, 36.6, 31.8, 21.4. **m/z** (ESI +ve) 274.9 $[M+ Na]^+$ (15 %) (Calculated 252.3062 for $C_{14}H_{20}O_4$). **R_F** (EtOAc:hexane 1:2) 0.6.

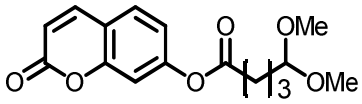
Expt. 11 Preparation of 5,5-Dimethoxypentanoic acid[60] (**86**)



Acetal (**85**) (400 mg, 1.59 mmol) was added to a solution of ethanol (10 ml, 5% water) and Pd/C catalyst (50 mg), under a hydrogen atmosphere (balloon). The reaction was stirred at room temperature for two hours. Following completion the mixture was filtered through a fine filter with a layer of silica; the product was washed through with EtOAc (20 ml) (taking care not to let the silica dry). The filtrate, free from catalyst, was concentrated and purified by column chromatography on silica gel using an EtOAc:hexane solvent system (3:1 → 2:1 v:v). 0.116 g of acid (**86**) (0.712 mmol) was obtained, as a colourless oil (yield 45%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.37 (1H, t, *J* = 5.1 Hz), 3.30 (6H, s), 3.21 (2H, t, *J* = 6.6 Hz, -CH_2), 2.40 (2H, t, *J* = 6.7 Hz, -CH_2), 1.72-1.68 (2H, m, -CH_2). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 182.8, 102.5, 59.9, 33.1, 30.1, 20.4. **m/z** (ESI +ve) 161.7 $[M - H]^+$ (15 %) (Calculated 162.1837 for $C_7H_{14}O_4$). **R_F** (EtOAc:hexane 1:2) 0.2.

Expt. 12 Preparation of acetal (**87**)



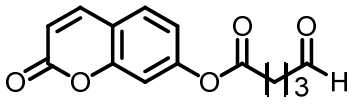
To a stirred solution of umbelliferone (199.3 mg, 1.23 mmol), in DCM (10 ml) were added EDAC (229.1 mg, 1.2 equiv.), DMAP (15 mg, 0.1 equiv.) and acid (**86**) (298.9 mg, 1.5 equiv.). The mixture was stirred at room temperature for 1 hour, after which the reaction was diluted with EtOAc (25 ml) and worked up with aqueous brine (35 ml). The aqueous was further extracted with EtOAc (2 x 20 ml), and the combined organic layers dried (over $MgSO_4$) and concentrated. This was followed with purification by chromatography on silica gel using an EtOAc:hexane solvent system (4:1). 199.5 mg (0.65 mmol) of pure umbelliferone ester acetal (**87**) was obtained, as a white solid (yield 53%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.84 (1 H, d, *J* = 7.4 Hz), 7.44 (1 H, d, *J* = 8.4 Hz), 7.20-7.00 (2H, m), 6.34 (1H, d, *J* = 7.2 Hz), 3.30 (6H, s), 2.67-2.61 (2H, m), 2.19 (2H, t, *J* = 5.4 Hz), 1.67-1.63 (2H, m, -CH_2). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 171.0, 158.1, 153.4, 150.1, 140.0, 127.4, 119.7, 116.2, 113.6, 101.4, 49.9.

m/z (ESI +ve) 317.5 $[M + K]^+$ (20 %) (Calculated 278.2574 for $C_{14}H_{14}O_6$).

R_F (EtOAc:hexane 1:2) 0.6.

Expt. 13 Preparation of aldehyde (**88**)



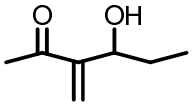
Acetal (**87**) (100 mg, 0.33 mmol), was treated with neat TFA (1 ml) for approximately 1 hour. Following de-acetalation the reaction was diluted with DCM (15 ml) and neutralised with careful addition of saturated aqueous $NaHCO_3$ (15 ml). The organic layer was decanted and the aqueous layer washed several times with more DCM (3 x 15 ml). Following this all organic layers were collected and dried over $MgSO_4$, prior to rotary evaporation and purification by column chromatography on silica gel, using an EtOAc:hexane solvent system (4:1 → 2:1). 54.4 mg (0.23 mmol) of aldehyde (**88**) was obtained as a white solid (yield 71%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.75 (1H, br), 7.84 (1 H, d, *J* = 7.4 Hz), 7.44 (1 H, d, *J* = 8.4 Hz), 7.20-7.00 (2H, m), 6.34 (1H, d, J = 7.2 Hz), 2.63-2.59 (2H, m), 2.18 (2H, t, *J* = 5.4 Hz), 1.55 (2H, s). **^{13}C NMR** ($CDCl_3$, 300 MHz) δ 201.0, 170.9, 160.2, 154.8, 153.0, 142.8, 128.4, 118.4, 116.0, 110.2, 42.5, 33.0, 17.3. **m/z** (ESI +ve) 283.0 $[M + Na]^+$ (40 %), **HRMS** 283.0578 (Calculated 283.0600 for $C_{14}H_{12}O_5Na$). **R_F** (EtOAc:hexane 1:2) 0.4.

Expts. (**14-16**) Preparation of Baylis-Hillman adducts (**97**-**99**)

To a mixture of aldehyde (1 mmol) and methyl vinyl ketone (1.2 mmol, 1.2 equiv.) in DCM (5 ml), was added DABCO (0.1 mmol, 0.1 equiv.) and the reaction left to stir at room temperature for 24-72 hours. Following completion the reaction was diluted with DCM (10 ml), and then washed with saturated aqueous bicarbonate (2 x 10 ml) and brine (10 ml). The crude material was purified by column chromatography on silica gel using an EtOAc:hexane (1:6 – 1:3) solvent system as eluent.

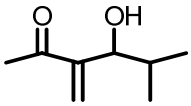
4-hydroxy-3-methylene-2-Hexanone[61] (**97**)



67.9 mg (0.530 mmol) was obtained, as a yellow oil (yield 53%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.49 (1H, s, -OH), 5.92 (1H, s, -C=CH_2), 4.52-4.48 (1H, m, -C=CH_2), 2.73 (1H, br, -HCOH), 2.11 (3H, s, -$COCH_3$), 1.61-1.55 (2H, m, -CH_2), 0.94 (3H, t, *J* = 6.9 Hz, -CH_2CH_3). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 198.7, 146.1, 126.8, 69.7, 28.0, 27.3, 7.9. **m/z** (ESI +ve) 151.0 $[M+Na]^+$ (50%) (Calculated 128.0837 for $C_7H_{12}O_2$). **R_F** (EtOAc:Hexane 1:2) 0.3.

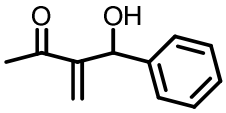
4-hydrxoy-5-methyl-3-methylene-hexan-2-one[62] (**98**)



69.7 mg (0.490 mmol) was obtained as a colourless oil (yield 49%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.22 (1H, s, -OH), 5.84 (1H, d, *J* = 1.2 Hz, -C=CH_2), 4.07 (1H, d, *J* = 6.0 Hz, -C=CH_2), 2.68 (1H, br, -HCOH), 2.31 (3H, s, -$COCH_3$), 1.89 (1H, m, -COHCH), 0.91 (3H, d, *J* = 7.1 Hz, -CHCH_3), 0.84 (3H, d, *J* = 6.9 Hz, -CHCH_3). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 201.7, 148.6, 126.6, 78.1, 33.1, 26.6, 19.7, 17.5. **m/z** (ESI +ve) 182.0 $[M+K]^+$ (55%) (Calculated 142.0837 for $C_8H_{14}O$). **R_F** (EtOAc:Hexane 1:2) 0.3.

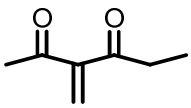
4-hydroxy-3-methylene-5-phenyl-2-pentanone[63] (**99**)



123.2 mg (0.7 mmol) was obtained, as a brown wax (yield 70%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.44 – 7.29 (5H, m, -Ar-H), 6.11 (1H, s, -C=CH_2), 5.94 (1H, s, -C=CH_2), 5.61 (1H, s, -HCOH), 2.34 (3H, s, -CH_3). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 200.36, 150.1, 147.7, 141.6, 136.5, 134.5, 128.4, 77.1, 29.7. **m/z** (ESI +ve) 177.12 $[M+H]^+$ (35%) (Calculated 176.0837 for $C_{11}H_{12}O_2$). **R_F** (EtOAc:Hexane 1:2) 0.2.

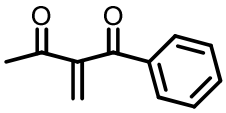
Expt. 17 Preparation of 3-methylene-2,4-dihexanon (**101**)



To adduct **97** (40 mg, 0.31 mmol) in DCM (5 ml), was added dess-martin periodinane salt (0.15 g, 1.1 equiv.) and the mixture left to stir for 2 hours. Following this the reaction was filtered and concentrated, and then purified by column chromatography on silica gel using an EtOAc:hexane (1:4) solvent system as eluent. 17 mg (0.13 mmol) of purified product was obtained, as colourless oil (yield 42%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.88 (1H, s, -C=CH_2), 4.51 (1H, m, -C=CH_2), 2.76 (1H, s, -HCOH), 2.07 (3H, s, -$COCH_3$), 1.61 (2H, m, -COHCH_2), 0.93 (3H, t, *J* = 7.7 Hz, CH_2CH_3). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 199.9, 145.1, 126.2, 72.4, 25.6, 28.9, 11.4. **m/z** (ESI +ve) 149.5 $[M+Na]^+$ (20%) (Calculated 126.1531 for $C_7H_{10}O_2$). **R_F** (EtOAc:Hexane 1:2) 0.5.

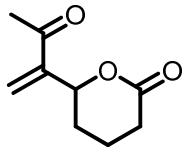
Expt. 18 Preparation of 2-methylene-1-phenyl-1,3-Butanedione (**102**)



Adduct **99** (50 mg, 0.28 mmol) was oxidised in the same manner as stated in experimental **17**. 16.3 mg (93.8 µmol) of purified product was obtained, as a brown solid (yield 33%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.47 – 7.31 (5H, m, -Ar-H), 6.06 (1H, s, -C=CH_2), 5.97 (1H, s, -C=CH_2), 5.53 (1H, s, -HCOH), 2.32 (3H, s, -CH_3). **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 201.2, 148.1, 147.2, 140.6, 135.5, 134.7, 127.9, 75.3, 30.2. **m/z** (ESI +ve) 175.1 $[M+H]^+$ (15%) (Calculated 174.1959 for $C_{11}H_{10}O_2$). **R_F** (EtOAc:Hexane 1:2) 0.4.

Expt 19. Preparation of adduct (**96**)



This adduct was prepared for detection only during the Baylis-Hillman screening reaction. To a mixture of aldehyde (**88**) (278 mg, 1 mmol) and methyl vinyl ketone (0.21 ml, 2.5 mmol, 2.5 equiv.) in DCM (10 ml), was added DABCO (11.3 mg, 0.1 mmol, 0.1 equiv.) and the reaction monitored over six hours, after which nmr analysis on crude material revealed presence of adduct (**96**), with an approximate yield of 23%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.92 (1H, d, *J* = 1.3 Hz), 4.11 (1H, d, *J* = 7.0 Hz), 2.71 (2H, t, *J* = 7.5 Hz, -CH_2), 2.68 (1H, Br), 2.56 (2H, t, *J* = 7.2 Hz, -CH_2), 2.33 (3H, s), 1.94-1.84 (2H, m, -CH_2).

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