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Biocatalysts in synthetic organic chemistry

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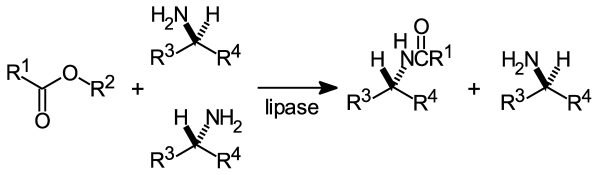
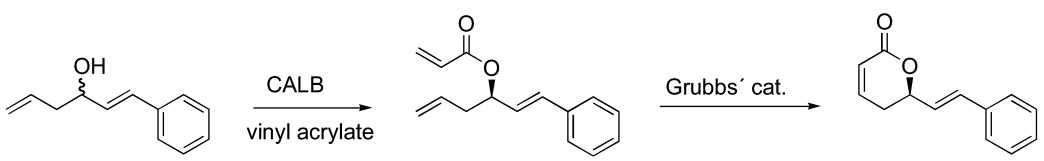
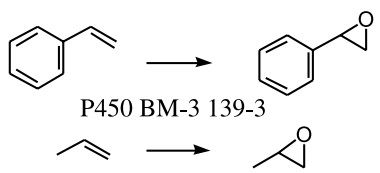
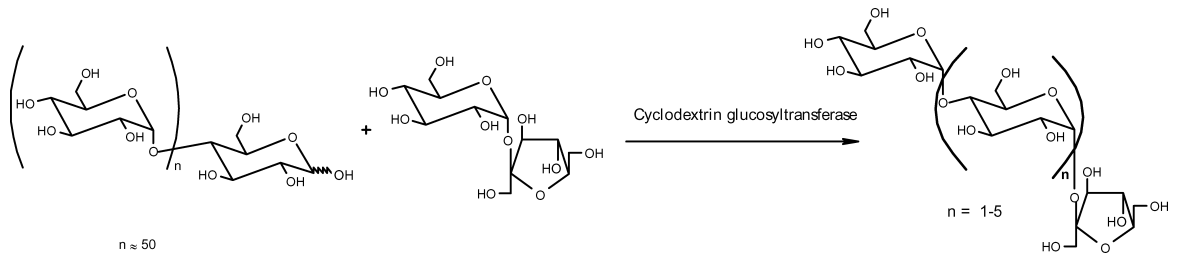
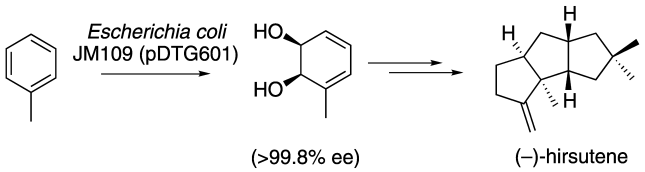


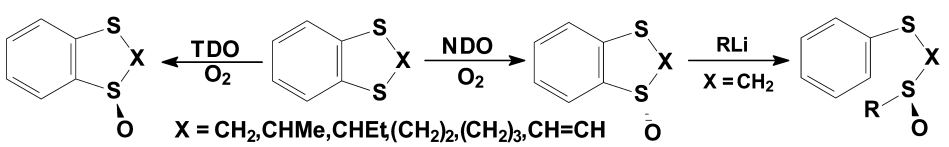


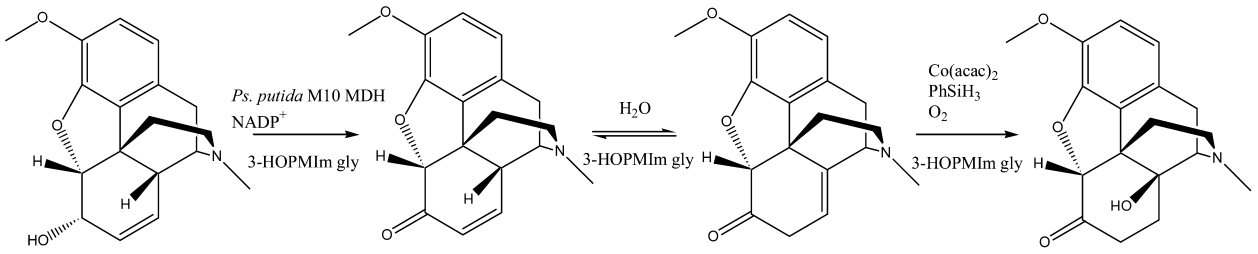
(±)-**2** *R*-**3** (+)-Goniothalamin

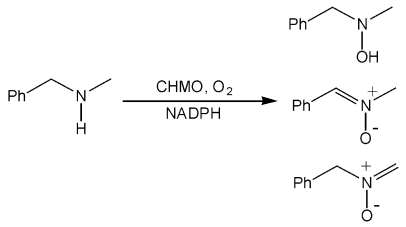




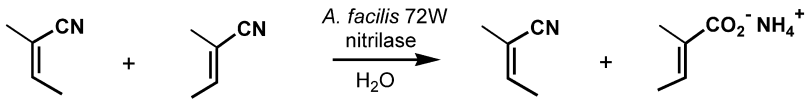


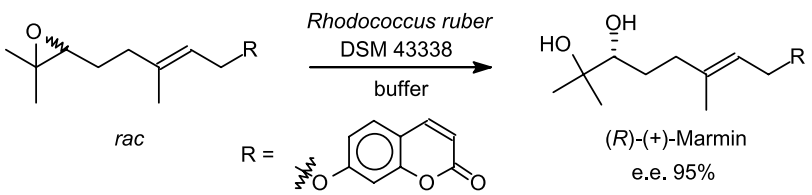




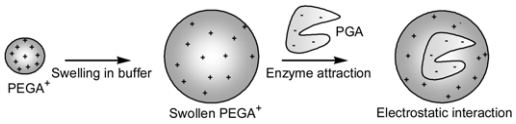


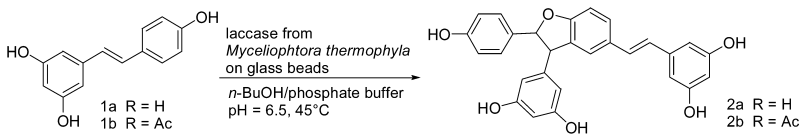
The formation of *N*-oxides, hydroxylamines and nitrones via enzymatic oxidation with cyclohexanone monooxygenase is described.

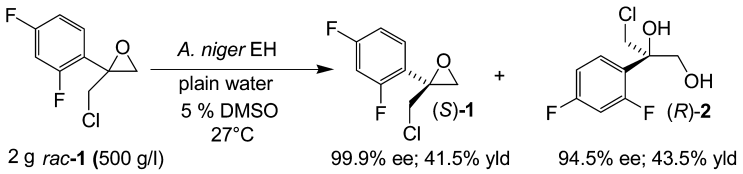


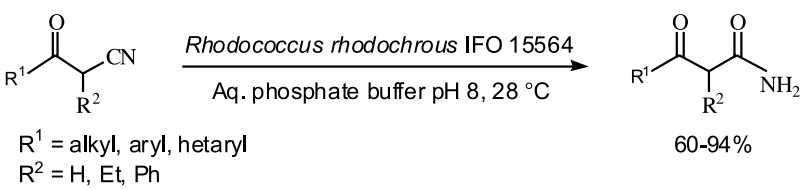


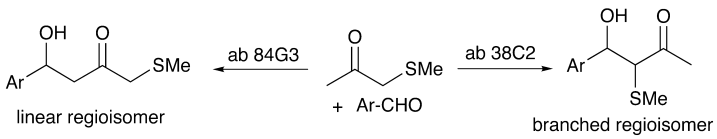
(*R*)-(+)-Marmin was obtained by asymmetric enantioconvergent biohydrolysis of the corresponding oxirane in 95% ee.

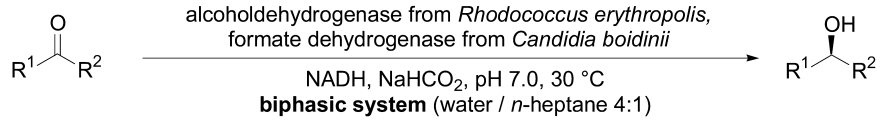




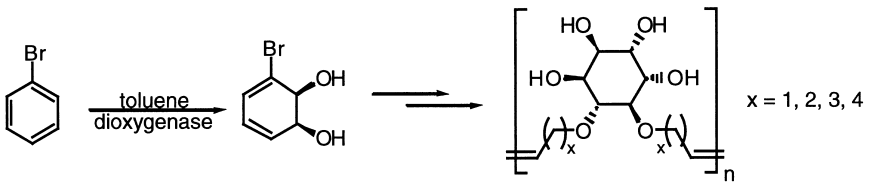


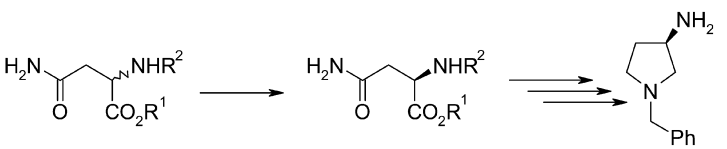


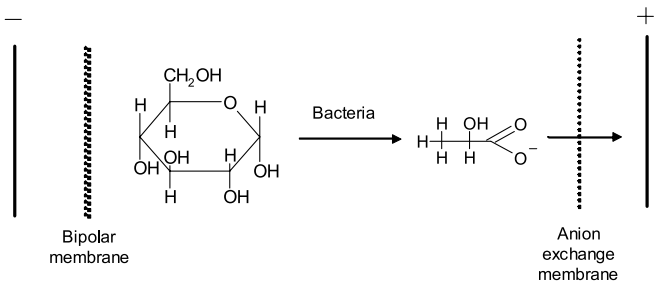


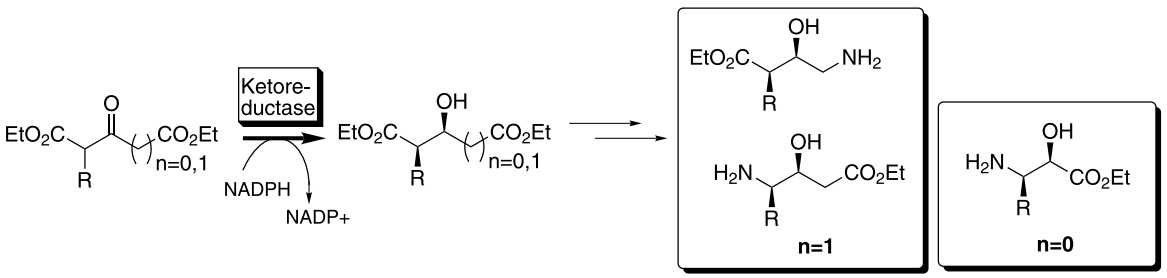


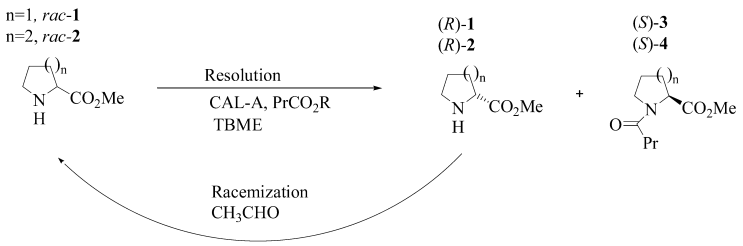
An enzyme-compatible biphasic reaction media for the asymmetric reduction of ketones with in situ-cofactor regeneration has been developed. As enzymes, a novel recombinant (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* and a formate dehydrogenase were used.

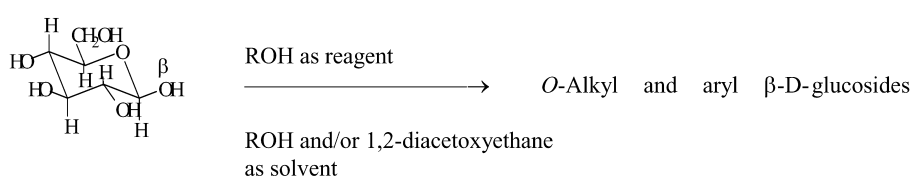


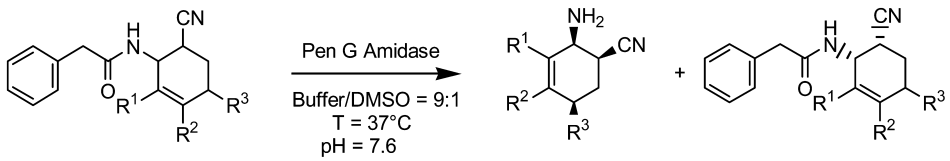


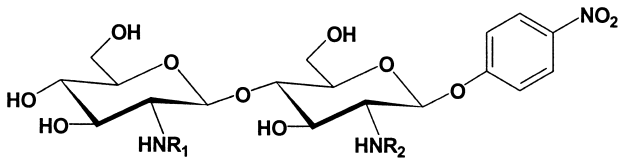


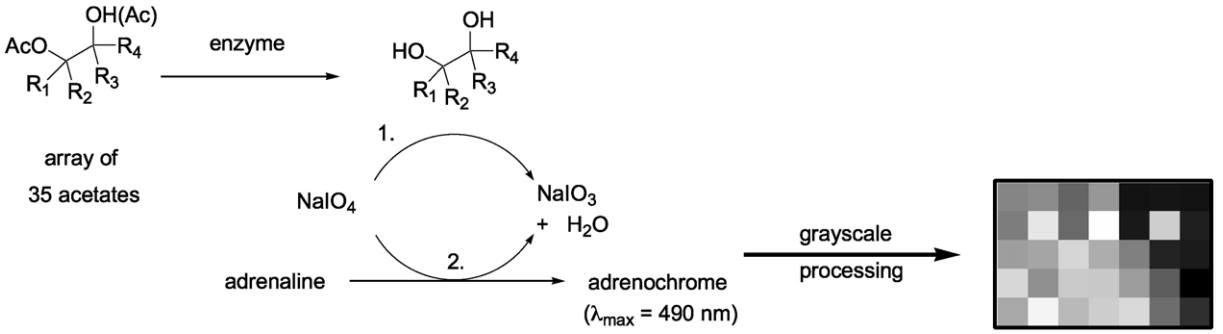




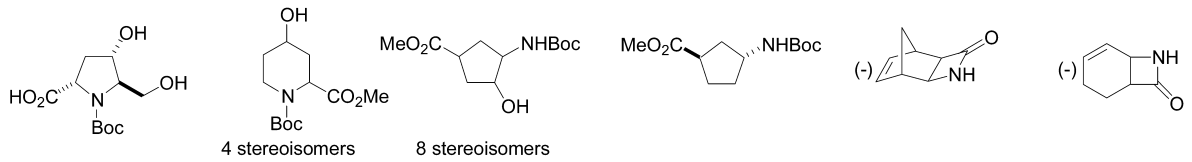


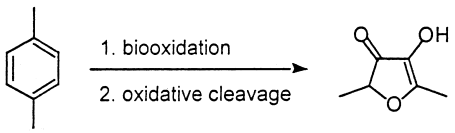


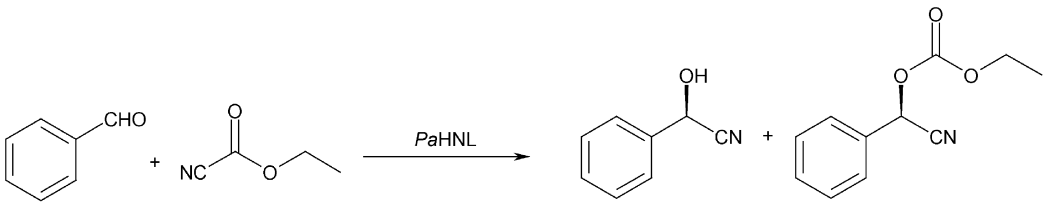


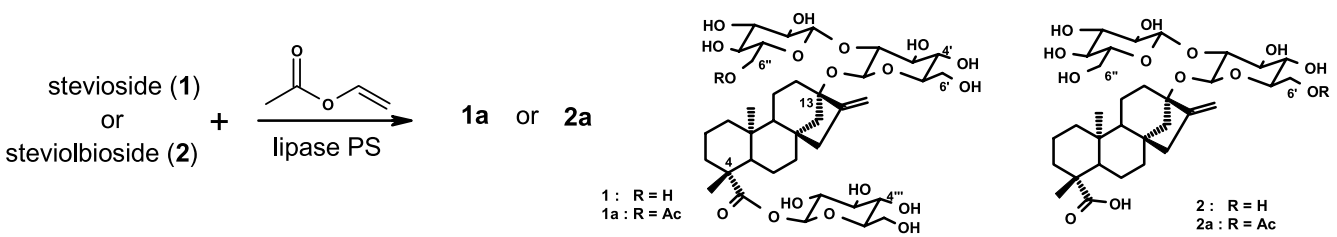


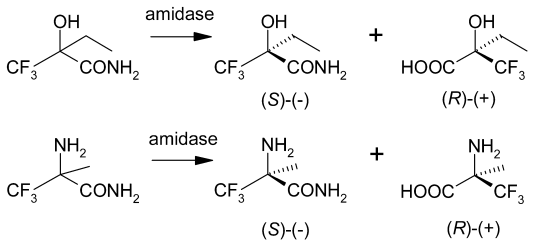
An enzyme that selectively cleaves the (+)-enantiomer from a racemic mix of γ-lactam has been isolated from the thermophilic archaeon *Sulfolobus solfataricus* MT4. The temperature optimum for the cleavage reaction of the γ-lactam substrate was 85 °C in phosphate buffer at pH 7.0. The enzyme also exhibits general amidase activity by cleaving linear and branched aliphatic and aromatic amides. It can catalyse the synthesis of benzoic hydrazide from benzamide preferentially to benzamide cleavage in the presence of excess hydrazine. It has potential for use in industrial biotransformations for the production of both carbocyclic nucleosides and hydrazides.

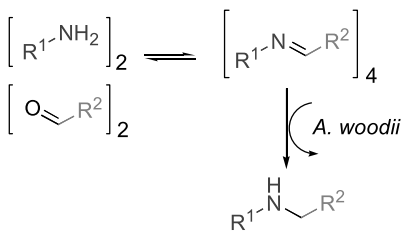




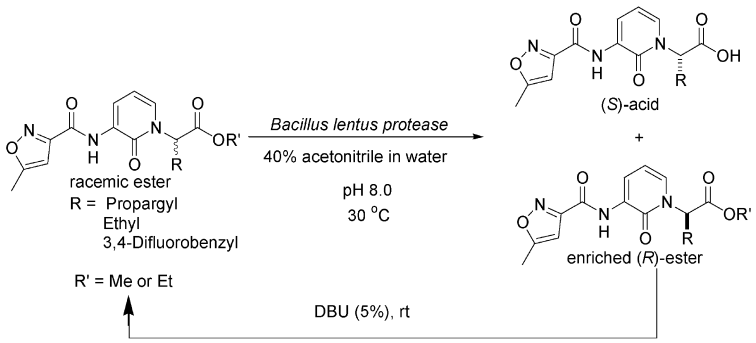


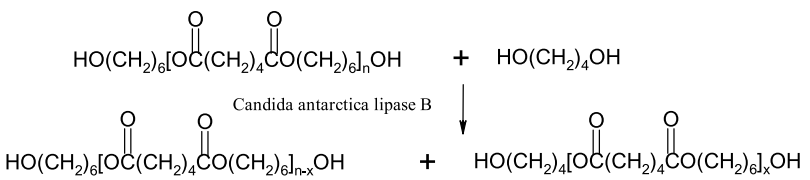


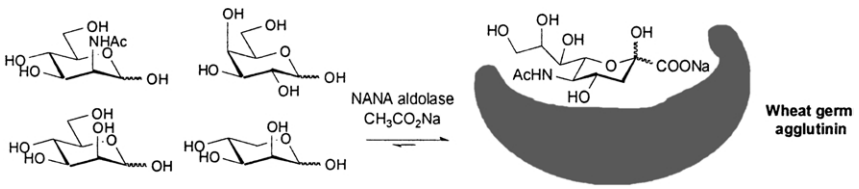


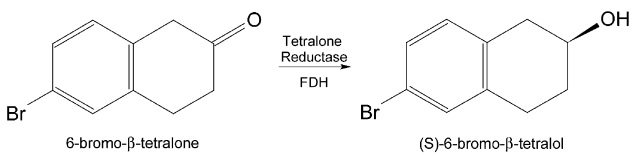


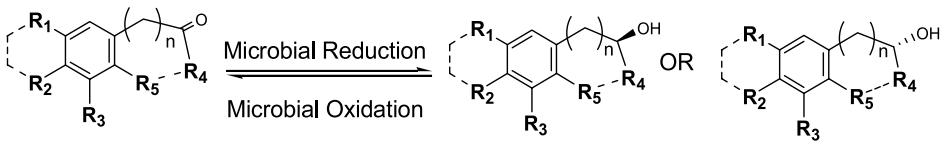
A new screen has been developed for novel biocatalytic imine reduction using virtual dynamic combinatorial libraries. Imine reductase activity has been detected in the strict anaerobe, *Acetobacterium woodii*.

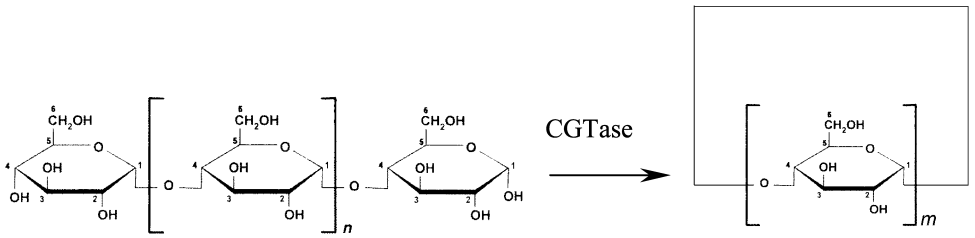












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*Corresponding author
ⓘ+ Supplementary data available via ScienceDirect

COVER

This picture shows the active site of the *Aureobacterium* (-)γ-lactamase enzyme (PDB code 1HK7). The catalytic triad made up of Ser98, His259 and Asp230, is shown as ball and stick and the secondary structure elements are shown as ribbons. A tetrahedral intermediate of the cyclic carbonate, (3aR,7aS)-3a,4,7,7a-tetrahedro-benzo [1,3] dioxol-2-one, is shown in pale blue covalently bound to the catalytic serine residue. (*J. Mol. Biol.* **2004**, submitted)
Authors: Kirsty Line, Michail N. Isupov and Jennifer A. Littlechild

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Preface

Biocatalysts in synthetic organic chemistry

The employment of enzymes and whole cells has been important in many industries for centuries. The most obvious usages have been in the food and drink businesses where the production of wine, beer, cheese etc. is dependent on the effects of microorganisms. For years fermentation technology provides a variety of amino acids, citric acid and other feed additives. Nowadays, the enzyme transglutaminase is widely used to give meat a fresh appearance, while an isomerase is used to produce fructose on a very large scale.

Other outlets for enzyme-controlled reactions are in the tanning industry, the household/cosmetic industries (for example biological washing powders), bioremediation and the production of natural flavours such as vanillin.

However, it is only in the last 30 years that the use of enzymes for the synthesis of high-value fine chemicals has enjoyed increasing popularity. Personally, in the 1970s I found lectures by first Bryan Jones then George Whitesides and papers by Charles Sih, highly influential and like many other organic chemists, I was attracted to the field by these brilliant pioneers and their peers.[1] Many breakthroughs have been recorded since these early days; high on the list must be the popularisation of the use of hydrolases in organic solvents for acylation and esterification processes. More recently, the application of error-prone polymerase chain reactions for the construction of 'designer' enzymes and the alteration of metabolic pathways to produce 'cell factories' are two techniques which have only just begun to be exploited for fine chemical manufacture.

For three years (1996–8) I reviewed the field of biotransformations for *J.C.S. Perkin Transactions*; for these reviews I summarised the biotransformations that appeared in the 'Top Twenty' Organic Chemistry Journals: the various sub-divisions of the topic attracted different volumes of work (Table 1). The use of esterases and lipases for the catalysis of hydrolysis and esterification reactions was most popular, not least because many of the enzymes were (and are) available commercially. The potential of epoxide hydrolases, nitrilases and nitrile hydratases was beginning to be explored. Reduction of carbonyl compounds, usually involving readily available microorganisms, such as bakers' yeast, represented about 10% of the activity, on average.

Not surprisingly, various oxidation reactions involving enzymes (e.g., monooxygenases, dioxygenases and oxidoreductases) tended to be undertaken in more specialist laboratories since the transformations often utilised microorganisms which needed to be grown and harvested before use.

The field of carbohydrate chemistry had warmly embraced this aspect of biotechnology, not least because the regioselectivity of the enzyme-controlled processes often circumvented long-winded protection/deprotection sequences. Moreover the mild conditions that could be employed kept the carbohydrate core structure intact.

Interestingly, virtually the same proportions of work in the different areas are seen in this Symposium-in-Print (Table 1).

Esterases and lipases are used almost routinely these days to provide optically active building blocks for the construction of imaginative new routes to chiral target molecules. The increased interest in epoxide hydrolases and the hydrolysis of nitriles is reflected in the submitted papers. While somewhat surprisingly enzyme-catalysed amide formation is not featured to a large extent in this Symposium-in-Print, readers will find an excellent review on enzymic acylation of chiral amines, in the next pages of this issue.[2]

Reduction reactions feature prominently, not only asymmetric reductions of carbonyl compounds but also of imines (for dynamic compound-library formation). Oxidation reactions that are featured herein include the exploitation

Table 1. Popularity of enzyme-catalysed transformations 1996–1998 and topics featured in this issue

Transformation type	Percentage of total biotransformations in the years 1996–1998 and in this Symposium-in-Print			
	1996	1997	1998	S-i-P
Hydrolysis	20	25	22	42
Esterification	18	23	23	
Amide formation	3	5	6	3
Reduction	9	13	9	14
Oxidation	21	12	18	19
C–C Bond formation	8	5	6	3
Carbohydrate modfn.	10	10	6	10
Others	11	6	11	9
	100	100	100	100

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of dioxygenases for the preparation of chiral cyclohexadienediol derivatives and the stereoselective oxidation of sulfides.

Carbon–carbon bond forming reactions are represented by the enzyme-controlled formation of cyanohydrins and the modern-day construction of compound libraries using aldolases. There is a splendid survey of some of the modern work involving carbohydrate modification, while in the 'other' category (equally important, in my view, to all the other areas) the employment of laccases and the impact of novel catalytic antibodies are featured.

It is stating the obvious to say that biocatalysis is not a panacea for synthetic organic chemistry. However, advances over the past thirty years mean that it would be a serious mistake not to consider the employment of a biocatalyst, in, perhaps, the key step in a sequence of transformations that turn a cheap starting material into an expensive fine chemical. If pressed to be more quantitative, I reckon that, if one totally ignores the opportunities offered by biocatalysts, the best route to a target fine chemical may be overlooked in about 10% of cases. Fortunately excellent, recently published textbooks[3] and a comprehensive database[4] are available fully to apprise chemists and other scientists of the possibilities for using Nature's catalysts in present-day organic synthesis.

It has been a pleasure for me to compile this Symposium-in-Print. Papers were forthcoming (on schedule!) from academic and industrial laboratories situated in 10 different countries. I thank all the authors for their hard work. Particularly, I thank Professor Jenny Littlechild (University of Exeter) for providing the picture for the cover. The shot shows a γ-lactamase forming a tetrahedral intermediate in a reaction involving the hydrolysis of a γ-lactam.[5] (This biotransformation formed a key step in the preparation of an optically active intermediate *en route* to the anti-HIV drug Abacavir.[6]) Finally, I have taken advantage of much positive advice from the *Tetrahedron* Editors, Professor Richard Taylor and Professor Harry Wasserman. The professional help of Dr Peter O'Brien and the *Tetrahedron* Staff in the York Office has also proved to be invaluable.

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Enantioselective acylation of chiral amines catalysed by serine hydrolases

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Contents

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1. Introduction

Enantiopure amines are used in the fine-chemicals industry as resolving agents, chiral auxiliaries and chiral synthetic building blocks for pharmaceuticals and agrochemicals. Among the methodologies that have been developed for the industrial production of enantiopure amines, lipase mediated enantioselective acylation[1] is increasing in significance compared with competing procedures, such as the crystallisation of a diastereomeric salt, enantioselective reductive amination using a chiral auxiliairy and enantioselective reductive amination mediated by a transaminase.

Lipases, the lipid hydrolysing catalysts of Nature, are highly stable enzymes with a very simple catalytic machinery (see Fig. 1). These characteristics have made them outstanding catalysts for synthetic biotransformations that involve the carboxyl group, such as esterification, transesterification, perhydrolysis and aminolysis in which the natural nucleophile—water—is replaced by an alcohol, hydroperoxide or amine.[2,3] The principle of lipase-catalysed aminolysis of carboxylic esters was first described in 1984 by Inada et al.[4] in a paper that also contributed to the revival of the use of lipases in organic media, following the largely disregarded example by Sym in the 1930's.[5] A publication by Zaks and Klibanov followed closely[6] and a multitude of publications describing the application of lipase-catalysed aminolysis has been published since.[7]

Although Nature has not designed lipases for enantioselectivity, it was recognised already 100 years ago that they are chiral and, hence, capable of enantiodiscrimination.[8] The first—incompletely documented—examples of enantioselective lipase-mediated amine acylation date from the late 1980's.[9] In the early 1990's, after highly stable lipase preparations had become freely available, papers and patent applications describing the resolution of chiral amines via lipase-catalysed enantioselective acylation (kinetic resolution) started to appear in large numbers. Less than 10 years later, the procedure is applied by BASF at a scale of over 1000 t/a[1] and an extension of the capacity has been announced.

Two other serine hydrolases, the protease subtilisin and penicillin acylase, have received much less attention, although their capability for enantioselective amine acylation was demonstrated in the late 1980's and early 1990's, respectively. In the present paper we will critically review the accomplishments in the field of the kinetic resolution of amines in the presence of serine hydrolases from three classes: lipases, subtilisin and penicillin acylase and compare their capabilities.

2. Lipase mediated aminolysis

2.1. Mechanism

Lipases, the lipid splitting catalysts of Nature (EC 3.1.1.3), operate by essentially the same mechanism as the serine proteases.[10] The active site (see Fig. 1) contains a serine residue that is activated by histidine and aspartate residues; these together form the catalytic triad. The reactant ester forms a tetrahedral acyl-enzyme intermediate by reaction with the OH group of the catalytic serine residue; the resulting excess of negative charge that develops on the carbonyl oxygen atom is stabilised by the oxyanion hole. Next, the tetrahedral intermediate collapses to the serinate

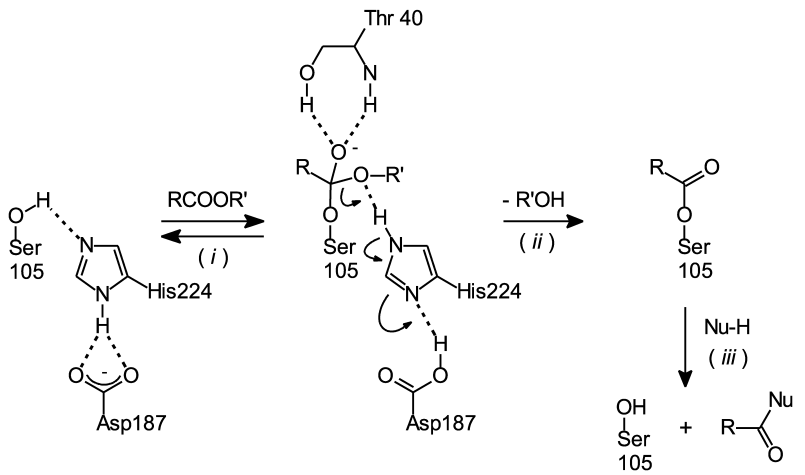


Figure 1. Reaction mechanism of lipase catalysis (numbering is for *Candida antarctica* lipase B); - - - denotes a hydrogen bond. Step (iii) is the microscopic reversal of steps (i) and (ii).

ester with elimination of the alcohol. Subsequent reaction of the acyl-enzyme intermediate with a nucleophile—the acyl acceptor—affords the product. In the case of hydrolysis, which is the natural reaction of lipases, the nucleophile is water. The reaction of the acyl-enzyme with an amine affords the corresponding carboxylic amide.[2]

Ester aminolysis can be regarded as irreversible, because the transformation is strongly exothermic under normal reaction conditions.[11] Amide hydrolysis, which is not readily mediated by lipases, can also be excluded due to the almost universally employed anhydrous reaction conditions (see below).

2.2. Practical considerations

Water, which is the natural reaction medium of enzymes, is not a good solvent for aminolysis, because hydrolysis of the donor is bound to predominate. In consequence, organic solvents are used almost universally. Even trace quantities of water in the reaction mixture will lead to undesirable hydrolysis of the acyl donor;[11] apart from the resulting loss in yield, the liberated acid may effect deactivation of the lipase. Hence, aminolysis is preferably carried out in a strictly anhydrous medium; in some cases activated zeolites have been added to the reaction mixture to ensure anhydrous reaction conditions. Lipases, in particular the ones from microbial sources that are commonly used nowadays, are exceptionally rugged enzymes and they tolerate anhydrous organic solvents rather well. In particular *Candida antarctica* lipase B (CaLB),[12,13] which actually seems to prefer anhydrous conditions,[14] has been widely used in consequence.

The efficient use of enzymes in non-aqueous media necessitates their immobilisation on a suitable carrier material,[15,16] presumably because the use of 'free' protein dispersions renders the major part of the enzyme molecules inaccessible to the reaction medium. Accordingly, in ammoniolysis lipases adsorbed on macroporous polypropylene (Accurel EP100) are more efficient than a free suspension of an equal amount of lyophilisate.[16] CaLB can be obtained from commercial sources as adsorbate on acrylic resin (Novozym 435), on Accurel EP100 (SP611 from Novozymes) and as a cross-linked enzyme crystal (Altus ChiroCLEC™-CAB). The use of sol–gel encapsulated CaLB in enantioselective aminolysis has quite recently been demonstrated.[17] We have reported the immobilisation of lipases, including CaLB, as cross-linked enzyme aggregates[18] but their use in amine resolution has not yet been published.

C. antarctica lipase A[19] (CaLA) has seen little application until now, but recently it has shown particular promise in the selective *N*-acylation of α- and β-amino acid esters (see later). CaLA was immobilised on Celite[15] in the presence of sucrose for optimum activity in aminolysis.

2.3. Lipase-catalysed synthesis of carboxylic amides

Because the subject has recently been reviewed[7] a brief introduction will suffice. Lipase-catalysed aminolysis seems to be a fairly general reaction that has been reported for a wide range of lipases and amines, including, besides the primary alkylamines, ammonia, hydroxylamine and hydrazine. Aniline derivatives, which are—similar to phenols—weak nucleophiles, hardly react. Examples of enzymatic acylation of secondary amines are rare (see later).

Lipase-catalysed esterification is well documented but the corresponding reaction of carboxylic acids and amines was thought to be not feasible due to the tendency of the reactants to form unreactive salts, in spite of ample indications to the contrary.[7] It has recently been shown, however, that the lipase catalysed condensation of carboxylic acids and ammonia is quite feasible.[20–22]

2.4. Kinetic resolution of amines

The kinetic resolution of chiral amines via enantioselective acylation by an ester is depicted in Figure 2.

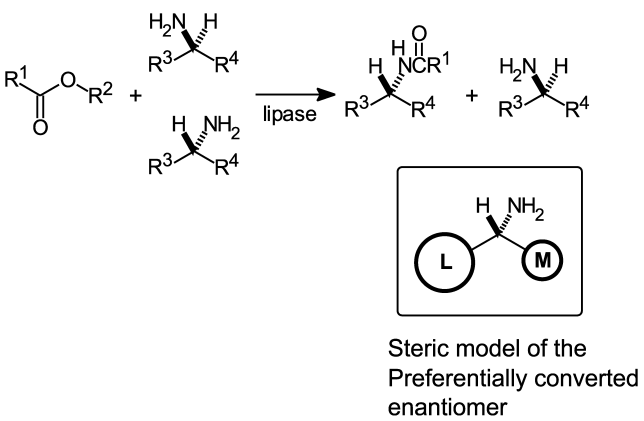


Figure 2. Lipase mediated enantioselective acylation of chiral amines. The steric model shows the preferentially acylated enantiomer.[27,28]

It is common practice to discuss a kinetic resolution in terms of its enantiomeric ratio E[23,24] which is equal to the ratio of the pseudo second order rate constants of the enantiomers according to Eq. (1). We note that formally this practice is not correct for lipase catalysed resolutions, because lipases do not obey minimal Michaelis–Menten kinetics and, hence, slight deviations of the predicted behaviour are to be expected.[25] It has often been attempted to predict enzymatic kinetic resolutions by measuring the specificity constants k_{cat}/K_m of the enantiomers separately. It has recently been shown, however, that E can only be determined reliably from experiments with the racemic nucleophile.[26]

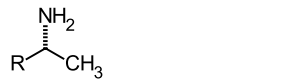
$$E = \frac{\left(\frac{k_{cat}}{K_m}\right)_R}{\left(\frac{k_{cat}}{K_m}\right)_S} \quad (1)$$

The enantiopreference of lipases in the acylation of chiral alkyl and arylalkyl amines corresponds with a steric model (see Fig. 2) that originally has been developed to predict the preferentially acylated enantiomer of secondary alcohols.[27] Interestingly, subtilisin mediates the acylation of chiral alcohols and amines with the opposite enantiopreference.[28]

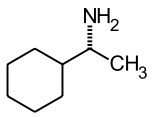
Table 1. Non-enzymatic acylation of **6** by 2,2,2-trifluoroethyl butyrate[a]

Solvent	Conversion (%)	K ($M^{-1}h^{-1}\times10^{3}$)[b]
Hexane	47	74
DIPE	25	31
Toluene	15	17
Acetonitrile	10	11
Acetone	10	11
3-Methyl-3-pentanol	3	3
tert-Amyl alcohol	2	2

[a] Compound **6** (0.32 M) and 2,2,2-trifluoroethyl butyrate (0.70 M) in the chosen solvent, 14 h at 40 °C. Data have been taken from Ref. 29.
[b] Calculated from the conversion by standard second-order kinetics.



(*R*)-**1a-e**



(*R*)-**1i**

a: R = C_2H_5 e: R = C_5H_{11}
b: R = C_3H_7 f: R = C_6H_{13}
c: R = C_4H_9 g: R = C_9H_{19}
d: R = *t*-C_4H_9 h: R = CH_3OCH_2

Figure 3. Lipase mediated enantioselective acylation of the alkylamines **1a**–**i**; the preferentially acylated enantiomers are shown.

3. Lipase-mediated enantioselective acylation of amines

The number of chiral amines that has been resolved via lipase mediated enantioselective acylation has grown explosively over the last few years; most of these have involved *C. antarctica* lipase B. A wide range of acyl donors and reaction conditions has been employed but systematic investigations are few.

It should always be kept in mind that amines, in contrast with alcohols, react spontaneously with esters[29–31] and that such an uncatalysed background reaction will erode the enantiomeric excess of the product. Hence, the acyl donor should not be too reactive[30] and the reaction conditions should be chosen with some consideration for this issue, which will be discussed more fully later.

Effects of the medium on the non-enzymatic reaction. Gutman et al. have found that the non-enzymatic reaction of 1-aminoindane and the activated ester 2,2,2-trifluoroethyl butyrate (see Table 1) is severely influenced by the solvent.[29] The rate decreased with increasing solvent polarity as the reaction was 30 times faster in hexane than in *tert*-amyl alcohol or 3-methyl-3-pentanol. One could conclude from these results that tertiary alcohols are attractive resolution media. Alcohols are lipase inhibitors,[32] however. Moreover, it is the ratio of the rates of the enzymatic and the non-enzymatic reactions, rather than either parameter in isolation, that should be optimised.

3.1. Alkylamines

2-Aminobutane (**1a**, see Fig. 3) was one of the first amines to be resolved via enantioselective acylation.[33,34] The E values that we have estimated from the—often incomplete—literature data generally are rather low, reflecting, it would seem, the difficulty of discriminating a methyl and an ethyl group. Acetic acid esters were inefficient resolution reagents,[35,36] presumably because of the small size of the acetyl group (see Table 2). Methyl methacrylate seems to give the best results (E>55), but the reaction was very slow and the conversion was low even after 11 days reaction time.[33] BASF, who has developed a robust process for the resolution of a wide range of alkyl- and arylalkylamines, has claimed a modest E of 8 for **1a**.[1]

Table 2. Resolution of chiral alkylamines **1**

Amine	Lipase	Donor	Solvent	T (°C)	E	Reference
1a	CaLB	Ethyl acetate	–	21	>4.5	36
	BpL	Methoxyacetic ester[a]	–		8	1
	CaLB	Methyl methacrylate	THF	30	55	33
	CaLB	Dimethyl succinate	Dioxane	30	35	34
	CaLB	Dimethyl succinate	Hexane	30	6	34
	CalB	1-Phenylethyl acetate	Dioxane	30	4	35
1b	CaLB	Ethyl acetate	–		>200	37
	PaL	Ethyl acetate	Diethyl ether	rt	>100	38
	BpL	Methoxyacetic ester[a]	–		50	1
	CaLB	Dibenzyl carbonate	Hexane	rt	4	42
1c	CaLB	Ethyl acetate	–	21	>31	36
1d	CaLB	Ethyl acetate	DME	rt	29	41
	CaLB	Isopropyl acetate	DME	rt	>700	41
	BpL	Methoxyacetic ester[a]	–		>1000	1
1e	CaLB	Ethyl acetate	–	21	>55	36
	CaLB	Dimethyl succinate	Dioxane	30	35	34
	CaLB	Dimethyl succinate	Hexane	30	68	34
	CaLB	1-Phenylethyl acetate	Dioxane		>200	35
1f	CaLB	Ethyl acetate	–	21	>110	36
	CaLB	Ethyl octanoate	–	39	>100	40
	CaLB	Dibenzyl carbonate	Hexane	rt	5	42
1g	CaLB	Ethyl acetate	–	21	>40	36
1h	CaLB	Isopropyl methoxyacetate	–	rt	58	39
1i	CaLB	Dibenzyl carbonate	Toluene	rt	17	30

[a] The alkyl group was not specified.

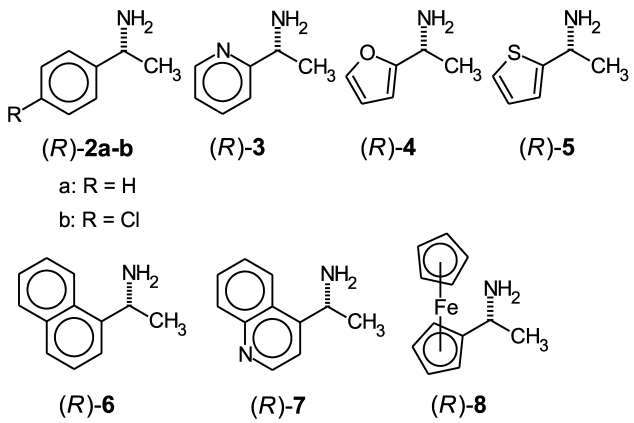


Figure 4. Lipase mediated enantioselective acylation of the 1-arylethylamines **2**–**8**; the preferentially acylated enantiomers are shown.

In contrast, the methyl and propyl groups in 2-aminopentane (**1b**) were very efficiently discriminated upon acetylation of the latter amine in the presence of CaLB[37] as well as *Pseudomonas aeruginosa* lipase (PaL).[38] Higher 2-aminoalkanes (**1c**–**1h**) have been resolved using donors such as dimethyl succinate,[34] isopropyl methoxyacetate[39] and ethyl octanoate[40] in the presence of CaLB (see Table 1 for further details). Amine **1d**, in which the **L**-group is *tert*-butyl, even was acylated with absolute enantioselectivity.[1,41] Surprisingly, the *E* ratio improved by a factor of 20 when the acyl donor was isopropyl acetate rather than ethyl acetate.[41]

1-Cyclohexylethylamine (**1i**) was acylated with only a modest *E* of 17.[30] This latter result could, perhaps, be ascribed to the acyl donor—dibenzyl carbonate—which also gave inferior results with **1b** and **1f**.[42] We note, however, that the sterically very similar **2a** (see Fig. 4) was acylated with very high enantioselectivity ($E>300$) under the conditions that failed to resolve **1i**.[30]

3.2. Arylalkylamines

3.2.1. 1-Arylethylamines. 1-Phenylethylamine (**2a**) seems to have served as a test substrate as it has been subjected to a wide variety of acylation conditions. Most of these have resulted in excellent enantiorecognition. Donor reagents (see Table 3) that have been used include ethyl acetate,[37] esters of methoxyacetic acid[43,44] and dibenzyl carbonate.[30,42] This latter reagent is particularly attractive as it affords the enantiopure Z-protected amine in a single step.

1-(4-Chlorophenyl)ethylamine (**2b**) has similarly been acylated with excellent enantiodiscrimination by a considerable range of esters in the presence of CaLB.[45,46] This work is conspicuous by the use of ethyl chloroacetate, dimethyl malonate and ethyl phenylacetate as acyl donors.[46] The enantioselectivity with ethyl chloroacetate in DME medium was very high (E=954) but high-selectivity resolutions could also be accomplished with methyl methoxyacetate, either neat (E=232) or in *tert*-amyl methyl ethyl (TAME) medium (E=458). Surprisingly, the reaction was significantly more enantioselective in TAME medium than in TBME.[45]

The resolution of the heteroaryl-substituted ethylamine derivatives **3**–**5** in the presence of CaLB illustrates the

Table 3. Resolution of the chiral 1-arylethylamines **2**–**8**

Amine	Lipase	Donor	Solvent	*T* (°C)	*E*	Reference
2a	CaLB	Ethyl acetate	–		110	37
	BpL	Ethyl methyoxyacetate	TBME		114	43
	CaLB	Isopropyl methyoxyacetate	TBME		>1000	44
	CaLB	Dimethyl succinate	Dioxane	30	95	34
	CaLB	Dimethyl succinate	Hexane	30	6	34
	CaLB	1-Phenylethyl acetate	Dioxane		>200	35
	CaLB	Dibenzyl carbonate	Toluene	rt	>300	30
	CaLB	Dibenzyl carbonate	Hexane	rt	21	30
	CaLB	Dibenzyl carbonate	Hexane	rt	50	42
2b	CaLB	Methyl methoxyacetate	–	rt	232	45
	CaLB	Methyl methoxyacetate	TBME	40	198	45
	CaLB	Methyl methoxyacetate	TAME	40	>458	45
	CaLB	Ethyl chloroacetate	DME	rt	954	46
	CaLB	Ethyl chloroacetate	TBME	rt	131	46
	CaLB	Ethyl butyrate	TAME	45	449	46
	CaLB	Ethyl phenylacetate	TBME	45	309	46
3	CaLB	Ethyl acetate	–	30	66	47
	CaLB	Ethyl acetate	–	60	41	48
	CaLB	Ethyl acetate	Dioxane	30	72	47
4	CaLB	Ethyl acetate	–	30	>100	47
	CaLB	Ethyl acetate	Dioxane	30	31	47
5	CaLB	Ethyl acetate	–	30	32	47
	CaLB	Ethyl acetate	Dioxane	30	>100	47
6	CaLB	Ethyl acetate	–		24	37
	CaLB	Ethyl acetate	–	60	5	48
	CaLB	Isopropyl acetate	DME	rt	650	41
	BcL	Isopropyl acetate	–	rt	18	41
7	CaLB	Ethyl acetate	DIPE	60	120	48
8	CaLB	Ethyl formate	Dioxane	28	30	49
	CaLB	Ethyl acetate	–	28	>200	49

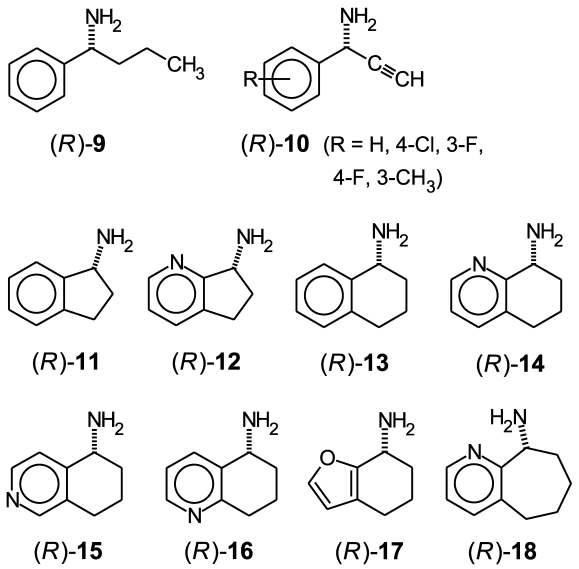


Figure 5. Lipase mediated enantioselective acylation of the 1-arylalkylamines **9**–**18**; the preferentially acylated enantiomers are shown.

capricious nature of enantioselective amine acylation. The resolution of the pyridine derivative **3** was less selective than that of its carbocyclic equivalent **2a** under comparable conditions, regardless of whether the reaction was carried out in neat ethyl acetate or dioxane medium.[47,48] 1-(2-Furyl)ethylamine (**4**) was efficiently resolved ($E>100$) by neat ethyl acetate in the presence of CaLB.[47] This latter result contrasts with the somewhat more modest enantiorecognition in the resolution of **1h**,[1] which also has an ether oxygen atom β to the amine function. The effect of the medium on the acylation of **4** is also noteworthy: in dioxane medium the enantioselectivity was much less than in neat ethyl acetate. The thiophene equivalent **5**, in contrast, was very well resolved in dioxane, but in neat ethyl acetate the enantioselectivity was mediocre.[47]

Attempts at resolving 1-(1-naphthyl)ethylamine (**6**) in the presence of CaLB produced conflicting results. Ethyl acetate was found to be an inefficient resolving agent by several authors,[37,41,48] even though one of these had resolved **2a** very efficiently under identical conditions.[37] Isopropyl acetate[41] and 1-phenylethyl acetate,[35] in contrast, gave excellent enantioselectivity. A possible effect by a heteroatom became apparent with the corresponding quinoline system (**7**), which was excellently resolved under conditions that had failed with **6**.[48]

Finally, 1-ferrocenylethylamine (**8**) was acylated with near-quantitative enantioselectivity by neat ethyl acetate in the presence of CaLB, but, in contrast, an attempt at enantioselective formylation resulted in a mediocre enantiodiscrimination.[49]

3.2.2. 1-Arylalkylamines. In all of the above examples the **M** group is methyl, but the acylation of 1-phenylbutylamine (**9**, see Fig. 5 and Table 4) demonstrated the ability of CaLB to discriminate larger **M** groups, although with a lower enantioselectivity ($E=60$), than observed with **2a** by the same authors.[35] Hence, the discrimination of a phenyl and a butyl group is less clear-cut than that between a phenyl and a methyl group but, in contrast, the CaLB mediated resolution of a range of 1-phenyl-2-propynylamines (**10**) was accomplished with high enantioselectivity ($E\gg100$).[50] Apparently, the acetylene moiety is readily accommodated in the subsite that binds the **M** group in the resolution of **2**.

The **M** subsite in CaLB is also able to accommodate an alicyclic ring, as shown by the efficient resolution, via acylation with dibut-3-enyl carbonate in toluene, of the bicyclic amine 1-aminoindane (**11**).[30] The closely related compound 1-aminotetraline (**13**), in contrast, reacted with only modest enantioselectivity ($E\sim18$) with either dibenzyl carbonate[42] or ethyl acetate[41,48] in the presence of CaLB. The effect of the leaving group that had already been noted in the resolutions of **1d** and **6** also became manifest with **13** as in the acylation of the latter with neat isopropyl acetate the reaction was highly enantioselective.[41]

Surprisingly, a series of analogs of **13** that contained a heteroaromatic nitrogen, such as **14**–**16**, were acylated with excellent enantioselectivity by ethyl acetate, but, in contrast, the E ratio of the furan equivalent **17** was only mediocre. It is also noteworthy that the expansion of the alicyclic ring in **14** by one methylene group (**18**) caused a complete loss of enantiorecognition.[48] The obvious conclusion is that the discrimination of a phenyl or pyridyl group and an alicyclic ring becomes more difficult when the latter is increased in size. The low E ratio of **17** likewise could be attributed to the

Table 4. Resolution of the chiral 1-arylalkylamines **9**–**18**

Amine	Lipase	Donor	Solvent	T (°C)	E	Reference
9	CaLB	1-Phenylethyl acetate	Dioxane	30	60	35
10	CaLB	Ethyl acetate	–		>100	50
11	CaLB	Dibut-3-enyl carbonate	Toluene	rt	>200	30
	CaLB	Dibenzyl carbonate	Hexane	rt	72	42
12	CaLB	Ethyl acetate	–	60	49	48
13	CaLB	Ethyl acetate	DIPE	60	17	48
	CaLB	Isopropyl acetate	–	rt	458	41
	CaLB	Dibenzyl carbonate	Hexane	rt	19	42
	BcL	Isopropyl acetate	–	rt	28	41
14	CaLB	Ethyl acetate	DIPE	60	>500	48
15	CaLB	Ethyl acetate	DIPE	60	210	48
16	CaLB	Ethyl acetate	DIPE	60	>500	48
17	CaLB	Ethyl acetate	DIPE	60	27	48
18	CaLB	Ethyl acetate	DIPE	60	5	48

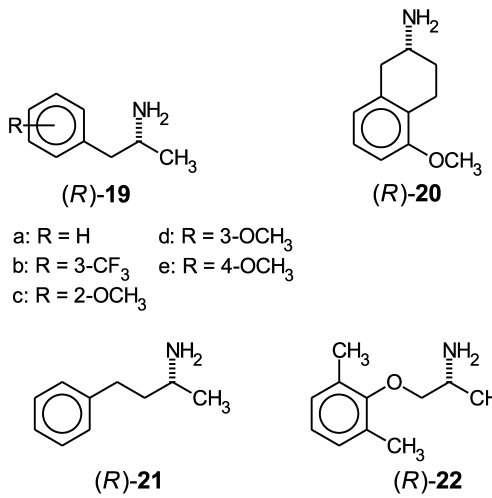


Figure 6. Lipase mediated enantioselective acylation of the arylalkylamines **19**–**22**; the preferentially acylated enantiomers are shown.

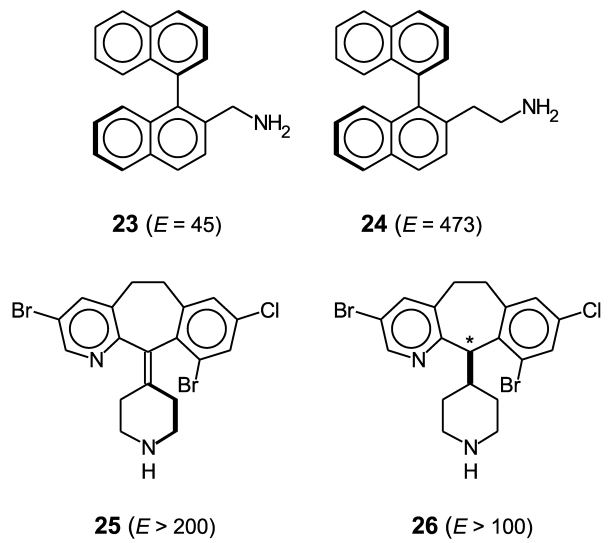


Figure 7. Lipase-mediated resolution of highly sterically demanding amines; the preferentially acylated enantiomers are shown.

somewhat smaller size of a furyl group compared with phenyl or pyridyl.

Summarising, the effect of a heteroaromatic nitrogen atom in the **L** group on the enantiorecognition is ambiguous. A positive effect on the enantiorecognition is apparent in the acylation of **7** compared with that of **6** and also with **14**–**16** in comparison with **13**. In contrast, the acylations of **3** and **12** were not more enantioselective than those of **2a** and **11**, respectively.

3.2.3. Various arylalkylamines. When an extra methylene group was inserted between the phenyl group and the amine group (**19a**, see Fig. 6), the extent of enantiodiscrimination seems to become less,[51] although the 3-trifluoromethyl derivative **19b** was resolved with near-absolute enantioselectivity (Table 5).[35]

The enantiomer discrimination by *Burkholderia cepacia* lipase (BcL) in the acylation of **20** seems to be rather modest[52]—the experimental data are incomplete—which fails to surprise as BcL did not perform very well in other amine resolutions either. The successful resolutions of **21** and **22** show that efficient enantiodiscrimination with CaLB is also possible when three atoms separate the aromatic moiety and the amine function.[45,51]

In the resolution of the very bulky binaphthyl-substituted amines **23** and **24** (see Fig. 7) PaL performed better than CaLB or any other common lipase.[53] 2,2,2-Trifluoroethyl butyrate gave the best result (E=45) in the enantioselective acylation of **23**, whereas the optimum donor for **24**, which reacted more than 25 times as fast, was ethyl butyrate. This latter resolution was accomplished with absolute enantioselectivity (E=473).[53]

PaL was also the optimum biocatalyst in the enantioselective acylation of **25** and **26** (E>200), with 2,2,2-trifluoroethyl isobutyrate in TBME.[54] The latter resolution was quite sensitive to the solvent. In THF, for example, the enantiomeric ratio only was 27. These resolutions are rare examples of the enantioselective enzymatic acylation of secondary amines.

3.3. Alkyl- and arylalkylamines in perspective

Summarising, an impressive number of alkyl- and arylalkylamines has been acylated and nearly every amine can be adequately resolved by fine-tuning the reaction conditions. In every case the enantiomeric bias was in agreement with Kazlauskas' rule. A comparison of the examples in Tables 2 and 3 seems to hint that an aromatic **L** group increases the enantiomeric bias, indicating that non-steric interactions also contribute to the enantiorecognition. The unpredictable effect of heteroatoms in the aromatic system points to the same conclusion.

Tables 2–4 contain numerous examples where the acyl

Table 5. Resolution of the chiral arylalkylamines **19**–**22**

Amine	Lipase	Donor	Solvent	T (°C)	E	Reference
19a	CaLB	Ethyl acetate	–	28	37	51
19b	CaLB	1-Phenylethyl acetate	Dioxane	30	168	35
19c	CaLB	Ethyl acetate	–	28	79	51
19d	CaLB	Ethyl acetate	–	28	70	51
19e	CaLB	Ethyl acetate	–	28	52	51
20	BcL	Ethyl acetate	Toluene		>16	52
21	CaLB	Methyl methoxyacetate	TBME	35	99	45
	CaLB	Ethyl acetate	–	28	41	51
22	CaLB	Ethyl acetate	–	28	123	51

donor and the reaction medium exert an appreciable, and sometimes even a vital, effect on the outcome of the resolution. Such effects raise the issue of a competing uncatalysed background reaction. The rate of the latter depends on the activity of the donor[30,31] and the reaction medium.[29]

3.3.1. Leaving group effects. In the kinetic resolution of **6**, mediated by CaLB or BcL, in neat alkyl acetate, reported by Reeve[41] (see Table 6), the leaving group exerts a remarkable effect on the enantiorecognition that seems difficult to explain. Taken together with the beneficial effect of dilution on *E*, however, the suspicion arises that the leaving group predominantly influences the background reaction. It is to be expected that the uncatalysed aminolysis of an acetic acid ester by **6** will be slower with the isopropyl ester than with the methyl one, due to the electropositive effect of the isopropyl group. It is also noteworthy that the enantioselectivity was lower in neat isoamyl acetate (E=28) than in neat isobutyl acetate (E=37). Apparently, the retarding effect of the increase in electropositive character of the leaving group on the uncatalysed reaction is less, in this case, than the accelerating effect of the decrease in medium polarity. Very similar effects were noted in the acylations of **1d** and **13** in the presence of two different CaLB preparations as well as BcL.[41]

Table 6. Effect of the leaving group and the medium on the enantiodiscrimination of 1(-1-naphthyl)ethylamine (**6**)

Donor	Lipase	*E* (neat)	*E* (20% in DME)
Methyl acetate	CaLB	1	10
Ethyl acetate	CaLB	15	33
Propyl acetate	CaLB	16	35
Butyl acetate	CaLB	18	43
Isopropyl acetate	CaLB	100	650
Isobutyl acetate	CaLB	37	95
Isoamyl acetate	CaLB	28	61
Ethyl acetate	BcL	3	n.d.
Isopropyl acetate	BcL	18	n.d.

In seeming contradiction, the high enantiomeric ratios that have been observed by other authors upon acylation by methyl and ethyl esters strongly indicate that the above problems are by no means universal. In our view the phenomenon is of sufficient importance to warrant a systematic investigation, which has not yet been performed, however. A somewhat speculative hint that we could offer is the possibility of an additive in the enzyme preparations catalysing a non-chiral reaction. An obvious candidate would be Tris buffer, which is known to act as a transesterification catalyst.

3.3.2. Acyl donors. Although acetic acid esters have performed adequately in many cases, the reaction is often undesirably slow. It has been shown that methoxyacetic acid esters are >100 times more active[43] and also have other characteristics that are desirable in an industrial resolution process.[1] Thus, BASF has developed a robust process for the resolution of a wide range of alkyl- and arylalkylamines in the presence of, presumably, a proprietary BpL, using an undisclosed ester of 2-methoxyacetic acid.[1]

3.3.3. Reaction media. The numerous examples of successful resolutions in neat esters of acetic and methoxyacetic acid demonstrate that a solvent is not really required, unless there is a background reaction to cope with. Such solventless procedures are particularly attractive in an industrial context as they obviate the need for a solvent recycle.

Many authors have used diisopropyl ether (DIPE), in spite of its propensity to form peroxides, which renders its large-scale use unattractive. In such applications it could easily be replaced by DME, TBME or TAME.

When different solvent systems can be compared, hexane generally gives the worst outcome. Thus, the CaLB-mediated acylations of **1a**, **1e** and **2a** with dimethyl succinate proceeded 4–15 times slower in hexane than in dioxane,[34] whereas a relatively fast non-enzymatic reaction should be expected in hexane medium.[29] These effects probably account for the mediocre enantioselectivity in hexane (E=6 and 5 for **1a** and **2a**, respectively), whereas in dioxane these values were 35 and 95.[34] Similarly, attempts at resolving **1b** and **1f** with dibenzylcarbonate in hexane met with scant success[42] and the acylations of **2a** and **11**, using the same donor, were much less enantioselective in hexane[30,42] than in toluene.[30] Only the resolution of **1e** with dimethyl succinate, which occurred with comparable enantioselectivity in hexane and dioxane, is possibly an exception.[34†] Summarising, hexane seems not a good solvent for enantioselective amine acylation, although it is an excellent medium for many other lipase-catalysed reactions.

Because of the non-enzymatic background reaction, the issue of how the enantiorecognition of amines by lipases is influenced by the medium remains obscure, as no systematic study has been performed. Extrapolating from alcohol resolutions, such an effect should be expected, however.

3.3.4. Lipases compared. CaLB is emerging as nearly everybody's first choice as amine resolution catalyst. It should be noted, however, that BASF has claimed an equally robust resolution, using its proprietary BpL biocatalyst[1] and that encouraging results have been reported with PaL.[38,53,54] The enantiorecognition of (*R*)-**2a** by BpL and CaLB seems of the same order under slightly varying reaction conditions. BcL, in contrast, which has resolved alcohols with excellent results, did not perform very well in amine resolution. Thus BcL did not discriminate the enantiomers of **6** and **13** under conditions that worked very well with CaLB.[41] The resolution of **20** is possibly an exception, but the incomplete data preclude a firm conclusion.[52]

3.4. Hydroxyalkylamines and diamines

Kazlauskas' rule correctly predicted the CaLB-mediated (*R*)-selective acylation of the *trans* aminoalcohols **27** and **28** (Fig. 8) by dimethyl glutarate.[55] The resolution of **28** was subject to a considerable solvent effect: the enantiomeric

† As the ee of the acylation products of **1a** and **1e** was calculated from the optical rotation, a considerable error is to be expected, in view of the very low specific rotation of these compounds.

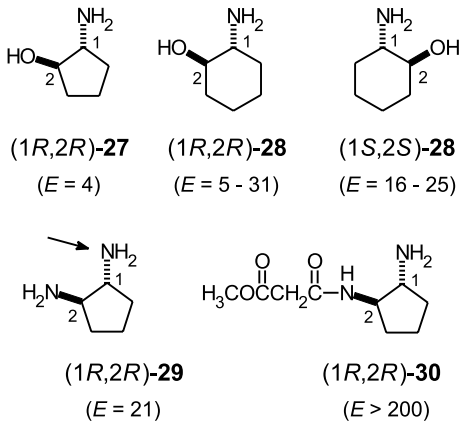


Figure 8. Lipase mediated enantioselective acylation of the hydroxyalkylamines and diamines **27**–**30**; the preferentially acylated enantiomers are shown.

ratio was 31 when the reaction was conducted in dioxane medium, whereas in toluene *E* was only 5. Surprisingly, **28** was acylated with opposite enantiopreference by dibenzyl carbonate in dioxane or toluene,[30,55] indicating that non-steric forces also play a role in determining the enantiomer discrimination.

Kazlauskas-type enantioselectivity was also found in the acylation of *trans*-1,2-diaminocyclopentane (**29**) with dimethyl malonate in the presence of CaLB (E=20); the subsequent acylation of the resulting monomalonic amide (**30**) occurred with a higher enantiomeric ratio (E=200).[56] Steric forces could account for this effect, but alternative explanations are also possible.

3.5. N-Acylation of α-amino acid derivatives

1-Amino-1-phenylacetonitrile (**31**, see Fig. 9) reacted with 2,2,2-trifluoroethyl acetate in diisopropyl ether with moderate (*S*)-selectivity (E=12–15) in the presence of PaL or *Chromobacterium viscosum* lipase (CvL), but BcL displayed a slight pro-(*R*) bias.[57] Acylation of **31** with ethyl acetate in the presence of CaLB resulted in an unexpected turnover-related racemisation of the reactant.[58] With phenylacetic acid ethyl ester, in contrast, no racemisation was observed and the reaction was highly (*S*)-selective.[58] This latter result may be explained as a beneficial effect of steric congestion in the active site.

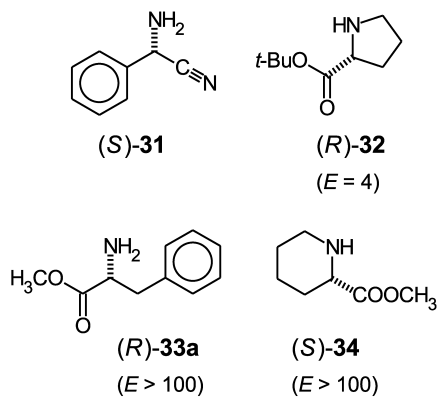


Figure 9. Lipase mediated enantioselective acylation of α-amino acid derivatives; the preferentially acylated enantiomers are shown.

A few α-amino acid esters have been acylated at the amino function. The reaction of proline *tert*-butyl ester (**32**) with dibenzyl carbonate in the presence of CaLB was slightly (E=4) (*R*)-selective.[30] It is not surprising that the *tert*-butyloxycarbonyl group in **32** is recognised as the **L** group by CaLB but in the acylation of the methyl ester of phenylalanine (**33a**) with ethyl methoxyacetate the preference of CaLB for the (*R*)-enantiomer was nearly quantitative (E>100).[59] Hence, the methoxycarbonyl group, rather than the benzyl group, is accommodated in the **L** subsite, which strongly indicates that non-steric interactions determine the enantiomeric bias in this case. It is also noteworthy that the ester group in **33a** did not react, presumably because the ethyl methoxyacetate donor is a highly active one.

The Kanerva group, which has been exploring the lipase-catalysed reactions of α and β-aminoacid esters for some years, has found that CaLB mainly catalysed interesterification reactions of the carboxyl group, whereas CaLA showed a strong preference for amine acylation. These authors surprisingly found that methyl pipecolinate (**34**) could be selectively acylated at the *N*-atom, by the highly active 2,2,2-trifluoroethyl esters, although this is a secondary amine function.[60] 2,2,2-Trifluoroethyl butanoate was the preferred acyl donor as it was nearly eight times as reactive as the acetate; E>100 was observed with both donors. Remarkably, the enantiorecognition was not significantly affected by the reaction medium as E>100 was observed in a wide range of solvents, from acetonitrile to ethers and even hexane.[60] If it is assumed that the active site geometry of CaLA is similar to that of the common lipases, the latter result indicates that the methoxycarbonyl group binds in the **M** subsite, in contrast with the acylations of **32** and **33a**.

3.6. N-Acylation of β-amino esters

A range of β-amino acid ethyl esters (**35a**–**e**, see Fig. 10) likewise reacted regioselectively at the amino function in the presence of CaLA.[61] Two acyl donors were used: 2,2,2-trifluoroethyl butyrate and butyl butyrate (see Table 5). Ethyl 3-aminobutyrate (**35a**) reacted with moderate

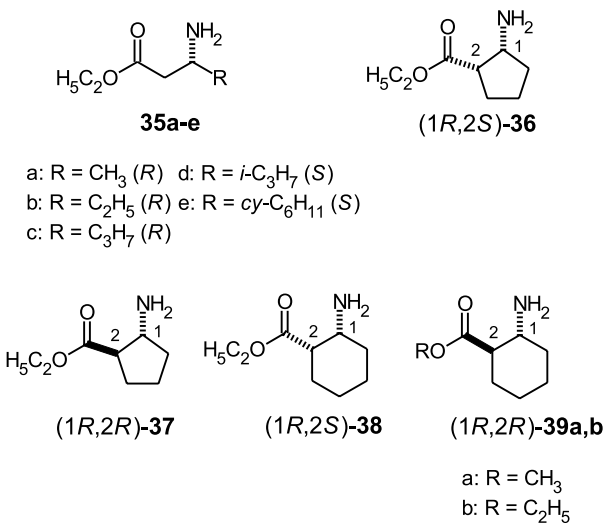


Figure 10. Lipase mediated enantioselective acylation of the β-aminoesters **35**–**39**; the preferentially acylated enantiomers are shown.

enantioselectivity (E=32) upon acylation with butyl butyrate. Acylation with the 2,2,2-trifluoroethyl ester, under otherwise similar conditions, was uncharacteristically slow and hardly enantioselective.[61] We note that **35a** was also resolved very efficiently in a CaLB-mediated acylation with isopropyl methoxyacetate.[59] The enantioselectivity of the latter procedure was nearly quantitative (E=200),[59] in favour of the same enantiomer as preferentially acylated by CaLA.[61] The acylation of **35b**, also using 2,2,2-trifluoroethyl and butyl butyrate as donors, was highly enantioselective with both and the reaction with the fluoroethyl ester was nearly 20 times faster, as would be expected.[61] This pattern was maintained with **35c** and **35d**; the latter nucleophile reacted almost 100 times faster with the trifluoroethyl ester than with the butyl ester. The acylation of the cyclohexyl derivative **35e** with 2,2,2-trifluoroethyl butyrate was highly enantioselective (E=100), but with the butyl ester the enantiomeric bias was very much reduced (E=9).[61] These results show, the confusing effects of the leaving group notwithstanding, that CaLA is able to discriminate the enantiomers of **35** efficiently under the right conditions. A systematic background reaction is not involved, as is demonstrated by the excellent enantiodiscrimination of **35d** with butyl butyrate, which required 130 h reaction time.[61] We can only guess that some batches of β-amino ester and/or acyl donor may have been contaminated with small amounts of acid chlorides, which are known to be powerful lipase inhibitors.

3.6.1. Monocyclic β-amino esters. The acylation of a range of cyclic β-amino esters (**36**–**39**) is conspicuous because some of these were rather well resolved by BcL (see Table 7).[62] The enantiomeric ratio of the latter enzyme in the acylation of **36** was high (~100) in a wide range of solvents but, in contrast, the effect of the solvent on the enantiodiscrimination of **39b** was dramatic (see Table 5). CaLA discriminated the enantiomers of the *cis*-isomers **36** and **38** rather well, but **37** and **39b**, in which the substituents are *trans*, not at all. CaLB acted with high enantioselectivity ($E \gg 100$) in the acylation of the methyl ester **39a** with isopropyl methoxyacetate.[59] In the acylation of **39a** and **39b**, CaLB and CaLA again showed a preference for the same enantiomer.

From the results obtained with **33a**, **35a** and **39a**[59] we tentatively conclude that amino esters can selectively react at the amino group rather than at the ester, in the presence of CaLB, if the acyl donor is a highly active one. It also becomes clear that CaLA and CaLB preferentially convert the same enantiomer of this type of reactants, which could indicate that the active sites have a similar structure. We note that, in the CaLA mediated resolutions of **35a**–**d**, a bulky alkyl group seems to exert a favourable effect on the enantiorecognition, which could indicate that its **M** subsite can accommodate more bulky groups than that of CaLB and that polarity, rather than steric interactions, dominates the enantiorecognition of amino esters by CaLA.

3.6.2. Bicyclic β-amino esters. CaLA also mediated the acylation of the bicyclic β-amino esters **40**–**42** with enantiomeric ratios (under optimum conditions) of approx. 30, but BcL, in contrast, was not enantioselective in these acylations.[62] Dramatic effects of the solvent on E, even when comparing different ethers, were manifest in the resolutions of **40** and **41**, in which the substituents are *exo*. Thus, the enantiomeric ratio of the acylation of **40** was 26 when the reaction was performed in TBME, which contrasts with E ratios of 10 in diethyl ether, 7 in DIPE and 1 in toluene. In the acylation of **41** the differences between the ethers were less (see Table 7), but, in contrast with **40**, the enantioselectivity was relatively high (E=41) when the reaction was performed in 3-methyl-3-pentanol (Fig. 11).[62]

3.6.3. Aryl-substituted β-amino esters. Finally a number

Table 7. Enantioselective *N*-acylation of the β-amino acid esters **35**–**42**

Amine	Lipase	Donor	Solvent	T (°C)	E	Reference
35a	CaLB	Isopropyl methoxyacetate	–		200	59
	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	6	61
	CaLA	Butyl butyrate	DIPE	rt	32	61
35b	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	168	61
	CaLA	Butyl butyrate	DIPE	rt	256	61
35c	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	72	61
	CaLA	Butyl butyrate	DIPE	rt	>100	61
35d	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	106	61
	CaLA	Butyl butyrate	DIPE	rt	115	61
35e	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	>100	61
	CaLA	Butyl butyrate	DIPE	rt	9	61
36	CaLA	2,2,2-Trifuoroethyl acetate	DIPE	rt	46	61
	BcL	2,2,2-Trifuoroethyl acetate	DIPE	rt	>100	61
37	BcL	2,2,2-Trifuoroethyl acetate	Diethyl ether	rt	12	62
38	CaLA	2,2,2-Trifuoroethyl acetate	DIPE	rt	61	62
39a	CaLB	Isopropyl methoxyacetate	TBME	rt	≫ 100	59
39b	BcL	2,2,2-Trifuoroethyl acetate	DIPE	rt	13	62
	BcL	2,2,2-Trifuoroethyl acetate	Diethyl ether	rt	33	62
	BcL	2,2,2-Trifuoroethyl acetate	Dibutyl ether	rt	4	62
40	CaLA	2,2,2-Trifuoroethyl acetate	Diethyl ether	rt	10	62
	CaLA	2,2,2-Trifuoroethyl acetate	TBME	rt	26	62
	CaLA	2,2,2-Trifuoroethyl acetate	DIPE	rt	7	62
	CaLA	2,2,2-Trifuoroethyl acetate	Toluene	rt	1	62
41	CaLA	2,2,2-Trifuoroethyl acetate	Et_2MeCOH	rt	41	62
	CaLA	2,2,2-Trifuoroethyl acetate	TBME	rt	46	62
42	CaLA	2,2,2-Trifuoroethyl acetate	Diethyl ether	rt	30	62

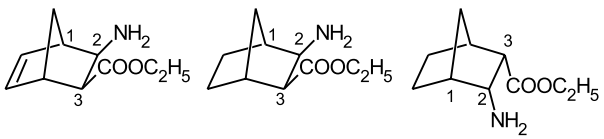


Figure 11. Lipase mediated enantioselective acylation of the bicyclic β-aminoesters **40**–**42**; the preferentially acylated enantiomers are shown.

of 3-amino-3-arylpropionic acid ethyl esters (**43**–**47**, see Fig. 12) were resolved via acylation with various butyric acid esters, in the presence of CaLA. The acylation of **43a** by 2,2,2-trifluoroethyl butyrate in DIPE was highly enantioselective ($E>100$, see Table 8); with butyl butyrate, in contrast, the reaction was approx. 200 times slower and the enantiomeric ratio was only a modest 29.[61]

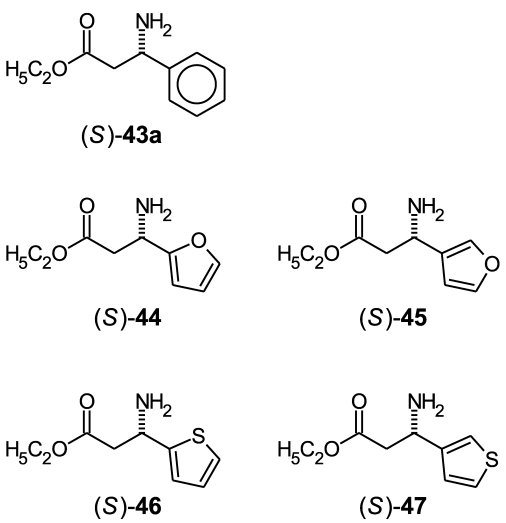


Figure 12. Lipase mediated enantioselective acylation of the ethyl 3-amine-3-arylpropionates **43**–**47**; the preferentially acylated enantiomers are shown.

The furyl-substituted β-amino esters **44** and, in particular, **45** were very well resolved via acylation with neat ethyl or butyl butyrate.[63] Surprisingly, the acylation of **44** with neat ethyl acetate was six times slower and also less enantioselective than with ethyl butyrate. The acylation of **44** with 2,2,2-trifluoroethyl butyrate in DIPE was also highly enantioselective, but much less so in ACN or TBME medium.[63] A very similar pattern was observed with the thienyl-substituted amines **46** and **47**, which were also efficiently resolved via acylation with butyl and ethyl butyrate, respectively.[63]

3.6.4. Enantiorecognition of β-amino esters by CaLA.

The enantiomer selectivity of CaLA in the *N*-acylation of β-amino esters is uniform in all of the studied examples. In terms of Kazlauskas' lipase steric model, the ester group preferentially binds in the **L** subsite and the alkyl or aryl group in the **M** subsite. The enantiomeric bias of CaLA is the same as those of CaLB and PcL, although not always to the same extent, whenever a comparison can be made. Hence, the conclusion seems justified that the **L** subsite preferentially binds a polar group, such as an ester, whereas a non-polar substituent is preferentially bound in the **M** subsite. From the available data, it seems that a bulky or aromatic substituent increases its affinity for the latter subsite in CaLA.

3.7. Lipases: enantiorecognition revisited

It is generally accepted that the enantiodiscrimination of lipase can be rationalised in terms of **L** and **M** substituents that bind in their respective subsites, and which exchange their positions when the 'wrong' enantiomer reacts (see the preceding discussion). It quite recently became clear, however, that this attractive mechanistic picture, which logically emanates from Kazlauskas' rule, is an over simplification. An X-ray analysis of *C. rugosa* lipase inhibited by (*R*)- and (*S*)-menthyl phosphonates showed that the oxygen and hydrogen atoms at C-1 in menthol exchange their positions in the active site and not the **L** and **M** groups.[64] A similar, quite recent, study of the resolution of **2a** by CaLB indicated that the hydrogen atom and the methyl group at C-1 exchange their positions.[65] It would seem that the mechanistic explanation of lipase enantioselectivity is a good deal more complex and interesting than expected.

3.8. Lipases: concluding remarks

A very wide range of chiral amines has been successfully resolved via lipase-catalysed acylation and the limit seems

Table 8. Enantioselective *N*-acylation of the 3-amino-3-arylpropionic acid esters **43a**–**47**

Amine	Lipase	Donor	Solvent	*T* (°C)	*E*	Reference
43a	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	75	61
	CaLA	Butyl butyrate	DIPE	rt	29	61
44	CaLA	Ethyl acetate	–	rt	100	63
	CaLA	2,2,2-Trifuoroethyl butyrate	ACN	rt	90	63
	CaLA	2,2,2-Trifuoroethyl butyrate	TBME	rt	60	63
	CaLA	2,2,2-Trifuoroethyl butyrate	DIPE	rt	210	63
	CaLA	Ethyl butyrate	–	rt	150	63
	CaLA	Butyl butyrate	–	rt	220	63
45	CaLA	Ethyl butyrate	–	rt	460	63
46	CaLA	Ethyl acetate	–	rt	60	63
	CaLA	Ethyl butyrate	–	rt	305	63
	CaLA	2,2,2-Trifuoroethyl butyrate	ACN	rt	130	63
	CaLA	2,2,2-Trifuoroethyl butyrate	TBME	rt	380	63
	CaLA	Butyl butyrate	–	rt	580	63
47	CaLA	Ethyl butyrate	–	rt	220	63

nowhere in sight. CaLB is a highly enantioselective and stable biocatalyst, which tends to eclipse many other potentially useful lipases. The, hereto, little-used CaLA has shown unexpected strength in the enantioselective acylation of α- and β-amino esters. The enantioselectivity often, sometimes vitally, depends on the proper selection of the acylating agent and the reaction medium. The role of the non-enzymatic background reaction in these phenomena is still obscure, but the conclusion that it is a major actor seems inescapable.

4. Subtilisin as an amine resolution catalyst

4.1. Subtilisin

The subtilisin-type endoproteases (EC 3.4.21.14) are structurally very similar to serine proteases. The Ser-His-Asp triad in the active site is sterically arranged as the mirror image of the lipase active site. The subtilisins also act as esterases on *N*-acyl-L-amino acid esters and short-chain fatty acid esters, such as butyrates.

Subtilisin is, similar to the lipases, a very stable enzyme that maintains its activity in anhydrous organic media. For optimum catalytic efficiency subtilisin should be immobilised, for example via adsorption on glass beads[29] or Accurel EP100.[66] Immobilised subtilisin is commercially available as a cross-linked enzyme crystal (Altus ChiroCLEC™-BL).

4.2. Subtilisin-catalysed enantioselective amine acylation

4.2.1. Alkyl- and arylalkylamines. The potential of subtilisin as an amine resolution catalyst was already demonstrated in an early stage in the development of non-natural enzymology. With few exceptions, the acylations were conducted with 2,2,2-trifuoroethyl butyrate in an anhydrous organic solvent. The solvent exerted a very considerable effect on the enantiomeric ratio, as measured in separate experiments with pure enantiomers,‡ of the acylation of **2a** and **6** (see Fig. 13).[67] *E* ranged from 1.0 in octane to 22 in 3-methyl-3-pentanol but, in hindsight, it would seem that the uncatalysed background reaction[29] (cf. Table 1) accounts for at least some of these solvent effects.

Subsequently, 3-methyl-3-pentanol was adopted as the solvent for the resolution of a range of alkyl- and arylalkylamines (see Fig. 13). Most of these were resolved with $E>20$, as calculated from the experimental data.[29,67,68] It would seem that a subtilisin CLEC[68] afforded a somewhat better enantiorecognition than a suspension of lyophilisate, although a reduction of the non-enzymatic reaction, perhaps due to a more active biocatalyst, could also account for the improvement.

The enantioselective acylation of **11** was scaled up to 300 g scale and that of **6** to 1.6 kg scale, using a tube reactor containing subtilisin immobilised on glass beads, with a reactant-to-enzyme ratio of 90 and 280 (g/g), respectively.[29] Thus, racemic **6** was resolved into unconverted (*R*)-**6**

‡ This is now known not to be the most reliable approach.[26]

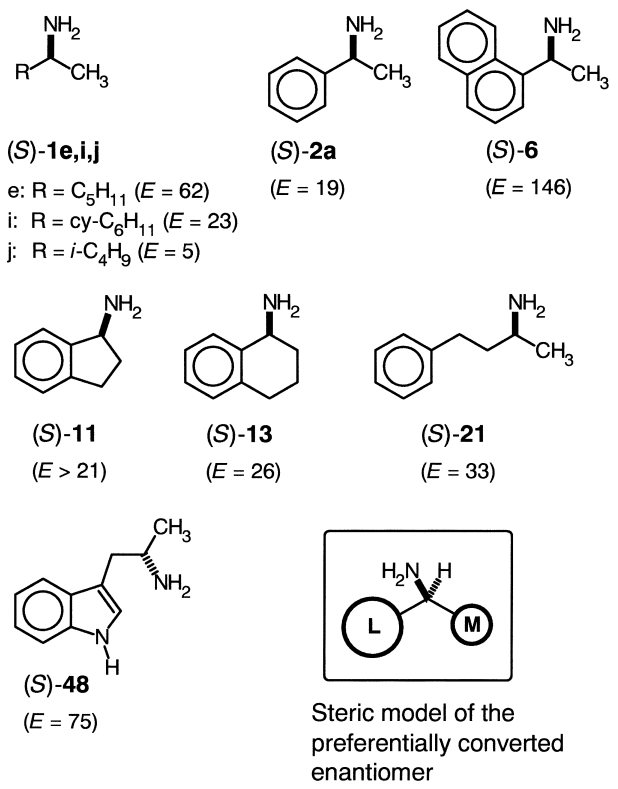


Figure 13. Subtilisin mediated enantioselective acylation of alkyl- and arylalkylamines; the preferentially acylated enantiomers and the seric model[28] are shown. The *E* ratios have been calculated from the literature data: **1e**,**i**,**j**, **2**, **13**, **21**;[67] **6**, **48**;[68] **11**.[29]

(ee>95%) and (*S*)-amide, which afforded (*S*)-**6** with ee>90% upon hydrolysis.

The enantiomeric bias of subtilisin in the acylation of the alkyl- and arylalkylamines corresponds with a steric model[28] that is the mirror image of the one that predicts the enantiomeric preference of the lipases (see Fig. 13). These authors have also correlated the steric model with the three-dimensional structure of subtilisin.

4.2.2. Functionalised amines. A few amines bearing various functional groups have also been resolved in the presence of subtilisin. An attempt at the acylation of **28** (Fig. 14) with neat diallyl carbonate was hampered by a significant background reaction. It was surprisingly found, however, that the acylations of **28** and **49** could be carried out, without any background reaction, in aqueous buffer at pH 8.[69] Only a modest excess of donor was required; hence,

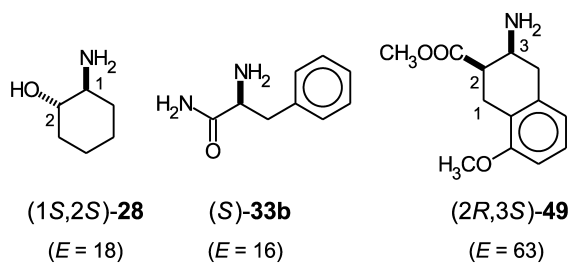


Figure 14. Subtilisin mediated enantioselective acylation of the α- and β-amino acid derivatives **28**[69], **33b**[67], **49**[69]; the preferentially acylated enantiomers are shown.

these amines seem to compete efficiently with water in the final enzymatic reaction step (step iii in Fig. 1). Subtilisin favoured the (*S*)-L-enantiomer of phenylalanine amide (**33b**) upon acylation with 2,2,2-trifluoroethyl butyrate,[67] as would be expected for a protease.

The correlation in enantiomeric bias of CaLB and subtilisin with these functionalised amines is striking, although the proteins have completely different structures. With both enzyme classes the hydroxyl group in **28** as well as the ester and amide groups in **33a** (Fig. 8), **33b** and **49** bind in the **L**-subsite.

4.3. Subtilisin: concluding remarks

Summarising, subtilisin is a promising amine resolution catalyst with an enantiomeric bias complementary to that of the lipases. The *E* values of subtilisin in these resolutions may seem quite modest in comparison with those of the lipases, but as subtilisin somehow was neglected early on there could be considerable room for improvement. Resolutions that employ subtilisin, and perhaps other proteases as well, could prove particularly useful in cases where the lipases convert the wrong enantiomer.

5. Penicillin acylase as amine resolution catalyst

5.1. Penicillin acylase

Penicillin acylase (EC 3.5.1.11) is a serine hydrolase; its mechanism is very similar to that of the lipases and serine proteases. Structurally, penicillin acylase is completely different as it belongs to the class of the N-terminal nucleophile hydrolases, which have no catalytic triad but an N-terminal serine that is activated by a bridging water molecule[70] (see Fig. 15).

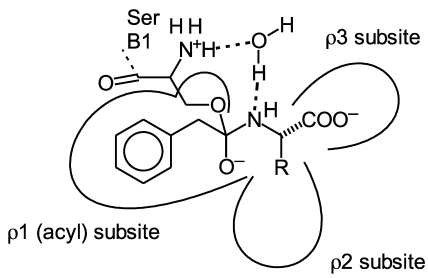


Figure 15. A schematic depiction of the active site of penicillin acylase; - - - denotes a hydrogen bond.

Penicillin acylase has a somewhat complex substrate specificity. The acyl binding (ρ1) subsite is highly specific for phenylacetic acid. Only small groups, such as hydroxyl or amino, are allowed at the 2-position and the enantiomer specificity is low. The ρ2 subsite mainly interacts with the reactant through hydrophobic and steric forces. The ρ3 subsite, in contrast, specifically binds negatively charged groups, which explains the high specificity of penicillin acylase for L-amino acid residues, but it also recognises ester groups.[71] The ρ2 and ρ3 sites together form the penicillin-recognising part of the active site.

Penicillin acylase is an industrial catalyst that is mainly used in the manufacture of the β-lactam building block 6-aminopenicillanic acid from penicillin G. The commonly used penicillin acylase from *E. coli* is commercially available in various carrier-bound formulations, such as PGA 400 from Roche, as well as a cross-linked enzyme crystal (Altus ChiroCLEC™-EC). The relatively unknown penicillin acylase from *Alcaligenes faecalis*[72,73] has recently attracted attention because it suffers less from parasitic hydrolysis in amine acylation and is more stable than the *E. coli* enzyme. Penicillin acylases are, compared with CaLB or subtilisin, fragile enzymes that lose their catalytic activity upon dehydration. Cross-linked enzyme aggregates of penicillin acylase maintained their activity in high concentrations of organic solvents,[74] but a few percent of water was an absolute requirement for activity.[75]

5.2. Use of penicillin acylase

In the preceding examples, our discussion of the kinetic resolution of amines has remained restricted, with few exceptions, to anhydrous reaction conditions. Hence, the issue of donor hydrolysis could be disregarded. With an enzyme that becomes inactive when dehydrated, such as penicillin acylase, some water must be present. In hydrophobic media, penicillin acylase requires a water activity of approx. 0.5.[76] Alternatively, the resolution can be carried out in a fully aqueous medium. Three kinetic control mechanisms contribute to the outcome of such procedures (see Fig. 16).[77] First, water and the amine compete for the acylated enzyme, causing some donor hydrolysis. Next, the amine enantiomers compete for the acylated enzyme and, hopefully, one is preferentially acylated. Finally, the enantiomerically enriched amide competes with the donor for the enzyme's active site, causing product hydrolysis. This latter process is particularly deleterious, because the enantiomer that is preferentially formed will also react the fastest in the backwards reaction, causing a rapid erosion of

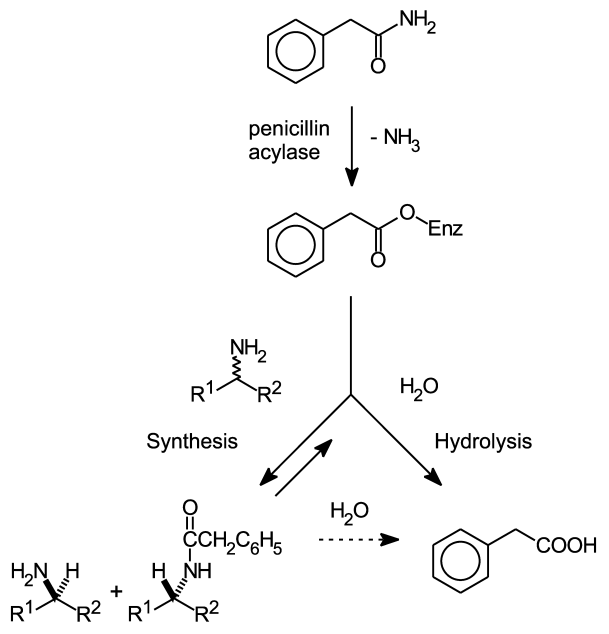


Figure 16. In a (partially) hydrous medium enantioselective aminolysis and hydrolysis compete.

the product ee. To be successful, a kinetic resolution in hydrated or aqueous medium absolutely requires an excess of a highly activated donor that monopolises the active site. To restrict the donor losses a good synthesis/hydrolysis ratio is desirable. Both methodologies: reaction in an aqueous buffer[78] as well as in partly hydrated ethyl acetate[79] or toluene[79,80] have been used in amine resolution.

The choice of acyl donor is restricted by the substrate tolerance of penicillin acylase. Simple esters of phenylacetic acid or phenylacetamide are obvious choices; the latter has the advantage of not suffering from spontaneous hydrolysis or aminolysis. Esters and amides of mandelic acid,[81] 4-hydroxyphenylacetic acid[80] or 2-phenylglycine[82] have the advantage of a much better solubility in water.

5.3. Amine resolution mediated by penicillin acylase

5.3.1. Alkyl- and arylalkylamines. Attempts to resolve alkylamines, such as **1**, **2** and **11** with phenylacetamide in the presence of *E. coli* penicillin acylase met with scant success due to a low enantioselectivity and prevailing donor hydrolysis.[83a] The penicillin acylase from *A. faecalis*, in contrast, required only a modest excess of acyl donor and it was also much more enantioselective.[83] The reaction was performed at pH 11 to deprotonate the amine and increase its competitiveness vs. water. Thus, a number of alkyl- and arylalkylamines (see Fig. 17) were acylated with, in general, the same enantiomeric bias as observed with the lipases. The enantiorecognition of the alkylamines **1b** and **1e** was quite modest however, and **1e** was even acylated with a slight preference for the (*S*)-enantiomer.[83a] The resolution of **1b**, in particular, could be improved by performing the reaction in 10–25% aqueous acetonitrile.[83a] A much better enantiomeric ratio was observed with 1-arylethylamines, which may indicate a specific interaction with the ρ2 binding site in *A. faecalis* penicillin acylase. Interestingly, such an effect was not observed with the *E. coli* enzyme. 1-Aminoindane (**11**) remained an exception as it was not very well resolved by either penicillin acylase, perhaps because the planar bicyclic systems prevents the proper orientation of the aromatic ring in the ρ2 subsite.

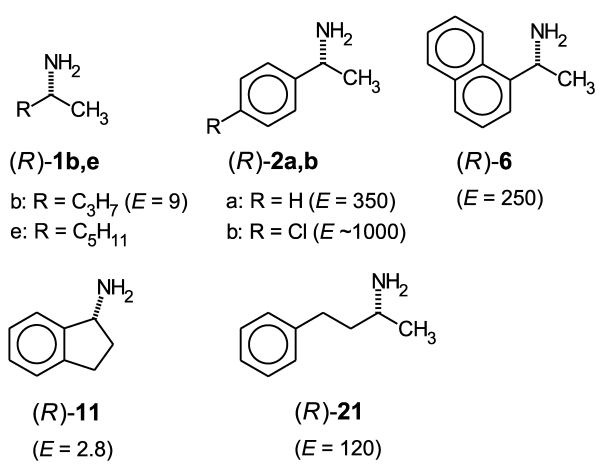


Figure 17. The enantioselective acylation of alkyl- and arylalkylamines in the presence of *A. faecalis* penicillin acylase (acyl donor: fenylacetamide); the preferentially acylated enantiomers are shown.[83]

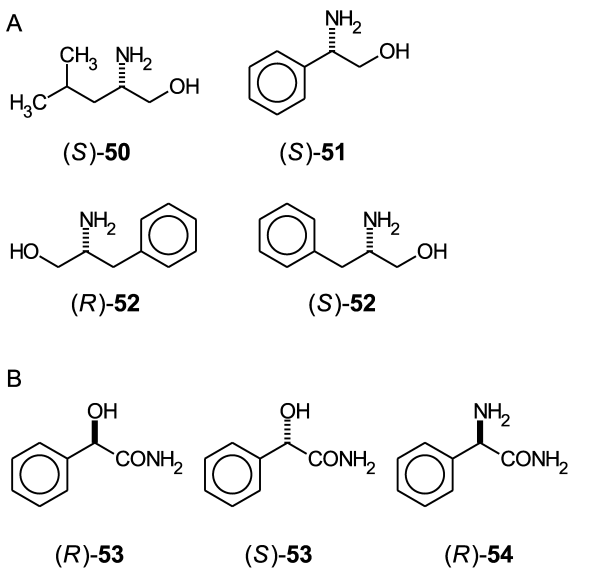


Figure 18. (A) The enantioselective acylation of the hydroxyalkylamines **50**–**52** in the presence of penicillin acylase; the preferentially acylated enantiomers are shown. (B) The acyl donors **53**–**54**. For further details see Table 9.

5.3.2. Aminoalcohols. The acylation of 2-amino-4-methylpentanol (leucinol, **50**, see Fig. 18 and Table 9) with phenylacetamide in the presence of *A. faecalis* penicillin acylase was much more enantioselective than that of the alkylamines **1b** and **1e**.[82] When comparing the enantioselectivities of 2-amino-2-phenylethanol (**51**) and **2a**, the opposite effect is observed.[83b] The enantiomeric bias shows that the primary alcohol groups in **50** and **51** selectively bind in the ρ3 subsite, similar to the methyl groups in **1b** and **2**. Surprisingly, the enantioselectivity was lost and a slight pro-(*R*) bias developed when the phenyl group in **2a** was replaced by benzyl (**52**).[82] 4-Phenyl-2-aminobutane (**21**), in which two C atoms separate the amino and phenyl groups (Fig. 17), in contrast, was acylated with a high selectivity for the (*R*)-enantiomer, similar to **2a**.

5.3.3. Donor effects in (hydroxy)alkylamine resolution. Švedas and co-workers surprisingly found that subtle changes in the nature of the acyl moiety of the donor exerted a dramatic effect on the enantiorecognition of alkylamines and aminoalcohols by *A. faecalis* penicillin acylase.[82] These concerned, besides phenylacetamide, (*R*)-mandelic amide ((*R*)-**53**), (*S*)-mandelic amide ((*S*)-**53**) and (*R*)-phenylglycinamide ((*R*)-**54**). The enantiomeric ratio increased, on average, by a factor of eight when phenylacetamide was replaced by (*R*)-**53**; the effect of (*S*)-**53** was somewhat less (see Table 9). The acylation of

Table 9. Effect of the acyl donor on amine resolution in the presence of *A. faecalis* penicillin acylase[a]

	Amine (*E*)				
Donor	**2a**	**21**	**50**	**51**	**52**
Phenylacetamide	350 (*R*)	120 (*R*)	40 (*S*)	>150 (*S*)	2.3 (*R*)
(*R*)-**53**	950 (*R*)	800 (*R*)	350 (*S*)	>1000 (*S*)	50 (*S*)
(*S*)-**53**	800 (*R*)	180 (*R*)	120 (*S*)	>1000 (*S*)	
(*R*)-**54**	1200 (*R*)	500 (*R*)			

[a] Data taken from Ref. 82.

2-amino-3-phenylpropanol (phenylalaninol, **52**) presents a special case as a slight preference for the (*R*)-enantiomer upon acylation with phenylacetamide changed into an (*S*)-selectivity (*E*=50) when the donor was (*R*)-**53**.

5.3.4. Amino acid derivatives. The nucleophile subsite of pencillin acylase is known to be highly specific for (*S*)-L-α-amino acid derivatives from hydrolysis studies.[71] The same enantiomeric bias was observed in the synthetic direction, upon the N-acylation of alaninamide (**55**, see Fig. 19) with (*S*)-**53** mediated by *E. coli* penicillin acylase in aqueous medium at pH 9.[81] The conversion was far from complete, however, because no excess of (*S*)-**53** was used and parasitic hydrolysis was prominent. The amino group in **56** likewise was acylated by methyl 4-hydroxyphenylacetate with a high selectivity in favour of the (*S*)-enantiomer.[80] The reaction was performed in partially hydrated (a_W 0.7–0.8) toluene and dichloromethane, in the presence of immobilised *E. coli* penicillin acylase.

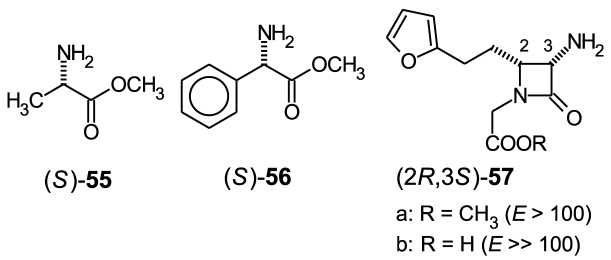


Figure 19. The enantioselective acylation of the α-amino acid derivatives **55**–**57** in the presence of the penicillin acylase from *E. coli*; the preferentially acylated enantiomers are shown.

The enantioselective acylation of the amino-β-lactam derivative **57a** with methyl phenylacetate was carried out in the presence of immobilised *E. coli* penicillin acylase in water at pH 6.[78] The enantiodiscrimination was excellent and no product hydrolysis was observed, presumably because the product precipitated. The corresponding product derived from the free acid **57b** remained in solution and some product hydrolysis indeed occurred. A fast and highly enantioselective reaction was, surprisingly, observed with methyl phenoxyacetate as the donor. Product hydrolysis did not occur, as should be expected, because phenoxyacetamides are known not to be substrates for penicillin G acylase. In seeming contradiction, phenoxyacetic acid ester can act as donor nevertheless.

N-acyl-β-amino acid derivatives are known to be hydrolysed with the same enantiomeric preference as their α-amino counterparts.[71] Hence, the carboxymethyl moiety is accommodated in the ρ3 subsite. The same enantiopreference was observed in an acylation study, using methyl phenylacetate and ChiroCLEC™-EC biocatalyst in partially hydrated ethyl acetate or toluene.[79] The enantiomer discrimination with the alkyl and alkenyl-substituted compounds **35a** and **35f** (see Fig. 20) was modest. In contrast, **43b** and **43c** were resolved with high enantioselectivity, presumably because of a favourable interaction with the aromatic substituent. A lower *E* was found with the more heavily substituted **43d**, however.

There are few examples of amine resolution using a

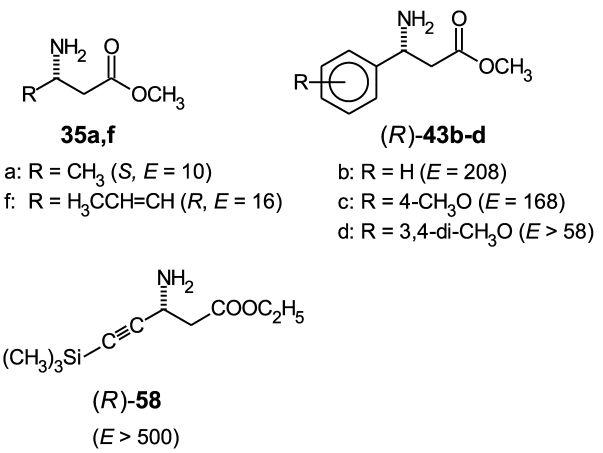


Figure 20. The enantioselective acylation of the β-amino acid esters **35**, **43** and **58** in the presence of the penicillin acylase from *E. coli*; the preferentially acylated enantiomers are shown.

carboxylic acid as the acyl donor. The reason is that product hydrolysis, which comes into the play when equilibrium is approached, would erode the ee. It has surprisingly been demonstrated, however, that **58** could be acylated with near-quantitative enantioselectivity via condensation with phenylacetic acid in the presence of immobilised *E. coli* penicillin acylase.[84] The procedure was scaled up to 7 kg and the desired, unconverted (*S*)-**58** was obtained in 43% yield (based on the racemate) and 99% ee.

5.4. Penicillin acylase: concluding remarks

Penicillin acylases have shown a surprising capability for the enantioselective acylation of chiral amines in aqueous media. The relatively unknown penicillin acylase from *A. faecalis* was, in contrast with the *E. coli* enzyme, an efficient resolution catalyst with alkyl- and, particularly, arylalkylamines with, in general, the same enantiomeric preference as the lipases. *E. coli* penicillin acylase is an efficient and highly enantioselective catalyst for the resolution of α- and β-amino acid derivatives. Its enantiomeric preference with these latter compounds is the opposite of that observed with the lipases.

6. Racemisation and dynamic kinetic resolution

Only one of the pure enantiomers that result from a kinetic resolution will, in general, be required, which renders the racemisation of the unwanted enantiomer a necessity to improve the process economy. BASF has disclosed a procedure that involves racemisation via a Schiff's base derivative.[1] It has also been shown that **9**, as well as its *N*-acetyl derivative, could be racemised by heating at 150°C for 30 min.[48] The racemisation of a range of 1-arylethylamines has been accomplished using a binuclear ruthenium catalyst at 110°C.[85] The formation of side-products was suppressed by the addition of a hydrogen donor. The racemisation conditions were not compatible with lipase-catalysed resolution, however, which means that racemisation has to be performed ex situ.

It would be desirable, however, to integrate racemisation

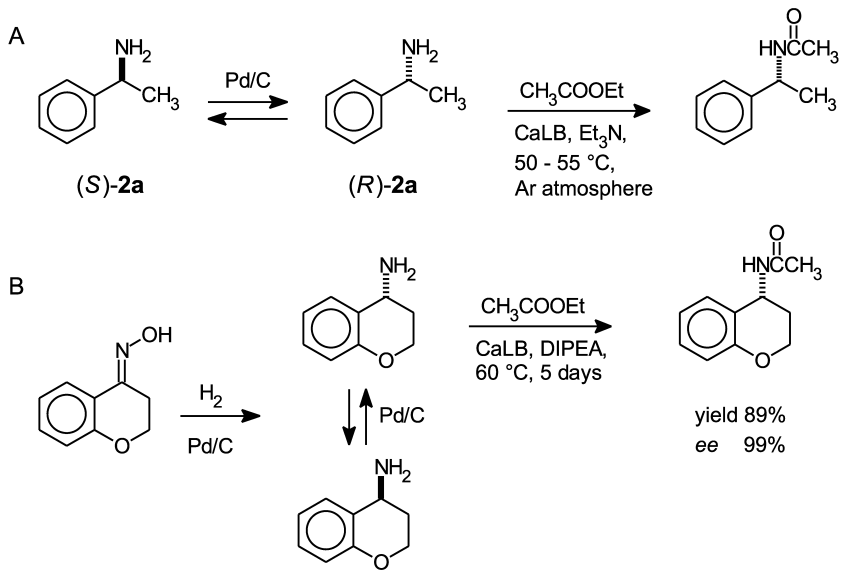


Figure 21. Dynamic kinetic resolution of chiral amines combined with in situ racemisation of the slow-reacting enantiomer.

and kinetic resolution, provided, of course, that the preferentially converted enantiomer is the desired one. Such a dynamic kinetic resolution (DKR) is particularly attractive because it requires a much lower enantiomeric ratio for the same product ee than a normal kinetic resolution. The reason is the 50:50 ratio of the reactant enantiomers that is maintained throughout the reaction.

Dynamic kinetic resolution of secondary alcohols is already well developed[86] but, in contrast, that of amines is still in its infancy. The DKR of **2a** has been accomplished by combining its CaLB mediated acylation with palladium catalysed racemisation of the unreacted amine in one pot (Fig. 21(A)).[87] The long reaction time required by the latter procedure could be remedied by starting from the corresponding oxime rather than the racemic amine (Fig. 21(B)).[88]

7. Conclusion

In the last decade much progress has been made in the enzymatic resolution of chiral amines, both from the viewpoint of practical utility and of understanding the influence of various parameters on the enantioselectivity. There are two fundamental differences between alcohol and amine resolution. With amines there is a blank non-enzymatic acylation, which has to be taken into account. This seems to have been forgotten by some authors. Secondly, hydrolysis of the acylated product is much more difficult in the case of amides compared to esters. Most of the studies have involved the use of lipases but subtilisin and penicillin acylase are also promising enzymes for amine resolution. Finally, the ultimate goal is an effective method for the dynamic kinetic resolution of amines. This necessitates the development of effective racemisation catalysts that are compatible with the enzymatic resolution step. We are confident that such systems will be developed in the future.

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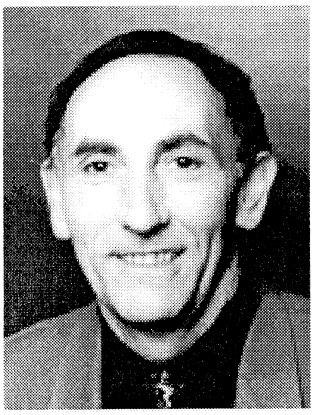
Biographical sketch

Fred van Rantwijk (1943) studied organic chemistry at the Delft University of Technology where he remainded as a staff-member. He received his PhD in 1980 under the direction of Professor H. van Bekkum. Since the late eighties he works on the application of enzymes in organic synthesis. His particular research interests are the use of enzymes in non-natural reactions, enzyme immobilisation and transformations using multi-enzyme systems.



Roger Sheldon was born in Nottingham (UK) in 1942. He has a PhD in organic chemistry (1967) from the University of Leicester (UK), for work on organophosphorus chemistry under the joint supervision of S. Trippett and R. S. Davidson. Following post-doctoral studies with Jay Kochi in the US on reactions of metal ions with free radicals, he joined Shell Research in Amsterdam in 1969 where he carried out research on various catalytic processes, particularly liquid phase oxidations. From 1980 to 1990 he was R & D Director of Andeno (a subsidiary of DSM) in Venlo (Netherlands). In 1991 he moved to his present position as Professor of organic chemistry and catalysis at the Delft University of Technology (Netherlands). His primary research interests are in the application of catalytic methodologies—homogeneous, heterogeneous and enzymatic—in organic synthesis, particularly in relation to fine chemicals production. He is well known for his devevelopment of the of E factor for assessing the environmental impact of chemical processes.

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Synthesis of (+)-goniothalamin and its enantiomer by combination of lipase catalyzed resolution and alkene metathesis

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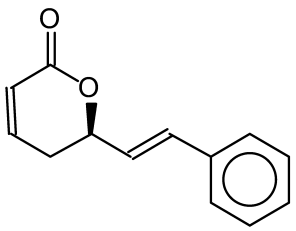
Abstract—(+)-Goniothalamin has been synthesized by lipase catalyzed resolution of (1*E*)-1-phenylhexa-1,5-dien-3-ol using vinyl acrylate as acyl donor followed by ring closing metathesis of the formed (1*R*)-1-[(*E*)-2-phenylvinyl]but-3-enyl acrylate. The unreacted alcohol from the resolution, (1*E*,3*S*)-1-phenylhexa-1,5-dien-3-ol, was esterified non-enzymatically, and used for synthesis of (−)-goniothalamin.

1. Introduction

Chiral unsaturated lactones are structural elements commonly found in natural products of medicinal interest. Furthermore, they are often used as intermediates in the syntheses of natural products. (+)-Goniothalamin (**1**) was first isolated in 1967 from dried bark of *Cryptocarya caloneura*[1] and given (*S*)-configuration. The configuration of its stereocenter has been revised and established as being (*R*).[2] Later **1** has been obtained from several other sources as well. The chemistry of naturally occurring 6-substituted 5,6-dihydro-α-pyrones including goniothalamin, has been reviewed.[3] This class of compounds shows several biological effects. (+)-Goniothalamin, for instance, exhibits antifungal effect,[4] immunosuppressive and anti-inflammatory activity.[5]



(+)-Goniothalamin (**1**)

Other natural products which contain the α,β-unsaturated δ-lactone moiety, such as (−)-argentilactone,[6–8] parasorbic acid and ratjadone,[9] have also been targets for several syntheses. Syntheses of (+)-goniothalamin[2,9–16] and its non-natural (−)-enantiomer have previously been reported.[2,11,17–19]

Acrylates as acyl donors in lipase-catalyzed transesterifications have been reported; e.g. for making starting materials for sugar-based polymers,[20,21] glycolipids,[22] pantolactone acrylate,[23] and (*R*)-(+)-1-phenylethyl acrylate.[24] The efficiency of vinyl acrylate as acyl donor has been investigated.[25] Combination of enzymatic transesterification and alkene metathesis have been reported,[26] but not directly combined in two steps as in the present work.

2. Results and discussion

The alcohol **2** was prepared by Grignard reaction between allylmagnesium bromide and cinnamaldehyde. The racemic alcohol was kinetically resolved by a transesterification reaction in hexane using vinyl acrylate as acyl donor and *Candida antarctica* lipase B (CALB) as catalyst (Scheme 1). Optical rotation values confirmed that the (*R*)-enantiomer was the faster reacting enantiomer,[27] in accordance with the stereopreference of CALB.[28] The resolution proceeded with an *E*-value of 65.

The transesterification reaction was stopped after 45% conversion, and the ester and remaining unreacted alcohol were separated by column chromatography affording (1*R*)-1-[(*E*)-2-phenylvinyl]but-3-enyl acrylate [(*R*)-**3**], with 93%

Keywords: (+)-Goniothalamin; (−)-Goniothalamin; *Candida antarctica* lipase B; Resolution; Alkene metathesis; Grubbs' catalyst.
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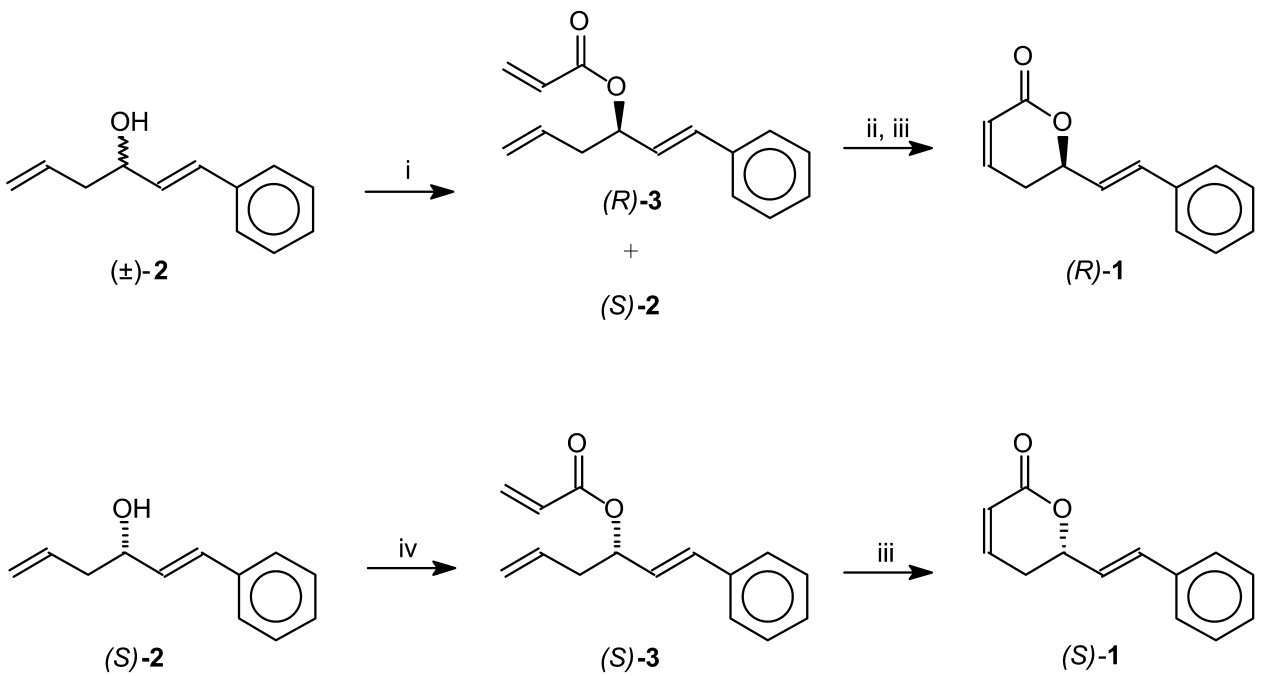

Scheme 1. (i) CALB, vinyl acrylate, hexane. (ii) Separation by chromatography, (iii) Grubbs'cat., CH_2Cl_2, δ, (iv) Acryloyl chloride, Et_3N, THF.

ee and (1*E*,3*S*)-1-phenylhexa-1,5-dien-3-ol [(*S*)-**2**], with 74% ee. The configuration was established by comparing the measured optical rotation which was $[\alpha]_D^{20}=+10.70$ (*c* 2.0, Et_2O), with the rotation of a previously reported value of an (*S*)-enriched sample of **2** with 24% ee and $[\alpha]_D^{20}=+3.60$ (*c* 10.08, Et_2O).[29] The alcohol (*S*)-**2** was esterified with acryloyl chloride to give (1*S*)-1-[(*E*)-2-phenylvinyl]but-3-enyl acrylate, [(*S*)-**3**], with no change of enantiomeric excess.

Treatment of (*R*)-**3** with 8 mol% of Grubbs' catalyst[30] gave the unsaturated lactone, (*R*)-(+)-goniothalamin [(*R*)-**1**] via a ring closing metathesis reaction in 92% yield. It was observed that during this reaction the ee-value increased to >99% in comparison to 93% ee of (*R*)-**3**. The ee was determined by HPLC analysis using a chiral stationary phase. Chiral analysis of the enantiomers of **1**–**3** was laborious; attempts with three different chiral stationary phases on GLC columns failed. Derivatization of (±)-**2** using (*R*)-4-methoxyphenylacetyl chloride, (*S*)-(1)-naphthylethylisocyanate or (*R*)-1-phenylethyl isocyanate also failed. Careful selection of chiral stationary phase for HPLC (Table 2) allowed accurate chiral analyses of **1**, **2** and **3**.

Table 1. Enantiomeric excesses before and after the ring-closing reaction

Acrylate	% ee	Product	% ee
(*R*)-**3**	88	(*R*)-**1**	96
(*R*)-**3**	93	(*R*)-**1**	>99
(*S*)-**3**	74	(*S*)-**1**	85
(±)-**3**	0	(±)-**1**	0

The absolute configuration was determined as (*R*) by comparing the measured specific rotation, $[\alpha]_D^{20}=+172.2$ (*c* 0.8, $CHCl_3$), with that of a previously reported value for (+)-(*R*)-goniothalamin (**1**) with 92% ee, $[\alpha]_D^{20}=+160.2$ (*c* 0.8, $CHCl_3$).[16] The spectral data were in accordance with those reported earlier. When (*S*)-**3** with an ee of 74% was treated in the same manner, (*S*)-**1** was obtained, also with increased ee (85%). The increase of enantiomeric excess values triggered further investigations, and the results are summarized in Table 1. Two samples of (*R*)-**3** and one of (*S*)-**3** with different ee-values led to products with increased ee-values. Racemic **3** furnished racemic goniothalamin (**1**).

One might expect that these results were due to inaccuracy of the chiral analysis. However, both racemic **1** and **3** were baseline separated by HPLC, giving optimum R_S-values of 2.78 and 3.54, respectively (see Table 2, entries 1 and 5). As a further check, the samples were also analysed using

Table 2. Chiral HPLC chromatography of **1**–**3**

Entry	Compound	Column	2-Propanol:hexane:EtOH	t_S (min)	t_R (min)	R_S
1	**1**	Chirasil AD	5:91:4	50.90	59.90	2.78
2	**1**	Chirasil AD	3:95:2	34.27	36.72	1.39
3	**1**	Chirasil OD-H	5:95:0	27.21	29.10	1.46
4	**2**	Chirasil AD	5:95:0	28.10	26.49	1.61
5	**3**	Chirasil AD	2:98:0	11.88	13.94	3.54
6	**3**	Chirasil AD	3:95:2	24.13	27.01	2.16
7	**3**	Chirasil OD-H	5:95:0	67.20	20.13	1.37

different conditions. No change of the ee-values were observed (Table 2, entries 2, 3, 6 and 7).

The reaction is homogeneous, and no crystallization is expected to occur during reaction since **1** and **3** are oils. In previously reported syntheses of analogs of goniothalamin, a similar increase of the ee-values during the ring-closing reaction were not observed, since the ee-values of the final products were assumed to be the same as the ee-values of the intermediates.[16]

3. Conclusion

In conclusion, we have carried out the synthesis of (+)-goniothalamin with high enantiomeric excess and its (−)-enantiomer with moderate enantiomeric excess by lipase catalyzed kinetic resolution of (1*E*)-1-phenylhexa-1,5-dien-3-ol, followed by separation and non-enzymatic esterification of the remaining (*S*)-alcohol. Subsequent ring-closing metathesis produced both lactones. This efficient procedure can easily be extended to the synthesis of other chiral natural products with an α,β-unsaturated δ-lactone moiety.

4. Experimental

4.1. General

Immobilized lipase B from *Candida antarctica*, CALB (Novozyme 435) with an activity of approx. 10,000 PLU/g and a water content of 1–2% was used. Solvents were distilled and dried over molecular sieves. Column chromatography was performed on silica gel 60 from Fluka. First generation Grubbs' catalyst, benzylidenebis-(tricyclohexylphosphine)-dichlororuthenium, was purchased from Aldrich. Enzyme reactions were performed in a shaker incubator (New Brunswick, Edison, NJ, USA). Optical rotations were determined using a Perkin–Elmer 243B polarimeter, concentrations are given in g/100 mL. NMR spectra were recorded on a Bruker DPX 400 instrument, using $CDCl_3$ as solvent. ^1H and ^{13}C spectra were recorded at 400 and 100 MHz, respectively. Chemical shifts are in ppm rel. to TMS and coupling constants in Hertz. Analytical GLC analyses were performed on a Supelco SPB™-5 column (30 m×0.25 mm, film thickness of 0.25 μm) at 10 psi, split ratio 60 mL/min. The inlet temperature was 250 °C and the FID temperature was 270 °C for all samples. The temperature program was as follows: 100 °C (0)–250 °C (2 min hold), 15 °C/min. Chiral HPLC analyses were performed on a Varian 9010 HPLC with a Varian 2550 variable λ detector, equipped with a Chiralcel AD column (25×0.46 cm) or Chiralcel OD-H column (25×0.46 cm) from Daicel Chemical Industries, LTD). The flow rate was 0.5 mL/min. Enantiomeric ratios, *E*, were calculated using the computer program *E* and *K* calculator version 2.1b (http://bendik.chembio.ntnu.no).

4.1.1. (1*E*)-1-Phenylhexa-1,5-dien-3-ol [(±)-2]. Allylmagnesium bromide (Aldrich, 1 M, 24 mL, 24 mmol) was added dropwise to a cooled (0 °C) solution of cinnamaldehyde (2.64 g, 20 mmol) in dry THF (30 mL). The cooling bath was removed and the reaction mixture stirred for 4 h. The reaction was monitored by GLC. Saturated NH_4Cl (30 mL) and Et_2O (50 mL) was added. The water phase was extracted with Et_2O (3×50 mL), and the combined organic fractions washed with brine (50 mL) and dried (Na_2SO_4). Removal of solvent afforded the racemic alcohol (±)-**2** (3.22 g, 93%). ^1H NMR: δ 1.9 (br s, OH), 2.37–2.43 (m, 2H), 4.35 (q, *J*=6.4 Hz, 1H), 5.14–5.20 (m, 2H), 5.84 (m, 1H), 6.23 (dd, *J*=16.0, 6.4 Hz, 1H), 6.60 (d, *J*=16.0 Hz, 1H), 7.23–7.39 (m, 5H). ^{13}C NMR: δ 42.43, 72.15, 118.86, 126.91, 128.08, 129.00, 130.76, 132.02, 134.50, and 137.10.

4.1.2. Lipase catalyzed kinetic resolution of (±)-2. The alcohol (±)-**2** (400 mg, 2.3 mmol) and vinyl acrylate (1.13 g, 11.5 mmol) were dissolved in hexane (60 mL). Immobilized CALB (300 mg) was added and the reaction mixture was shaken at 30 °C for 54 h when 45% conversion was reached. The enzyme was filtered off and the solvent removed in vacuo. The remaining alcohol (*S*)-**2**, and the ester (*R*)-**3** were separated by column chromatography (hexane/acetone, 4:1).

4.1.3. (1*R*)-1-[(*E*)-2-Phenylvinyl]but-3-enyl acrylate [(*R*)-3]. Yield: 200 mg (38%), 93% *ee*, $[\alpha]_D^{20}$=+66.1 (*c* 2.0, $CHCl_3$). ^1H NMR: δ 2.53 (m, 2H), 5.09–5.16 (m, 2H), 5.56 (q, *J*=6.6 Hz, 1H), 5.75–5.85 (m, 2H), 6.11–6.21 (m, 2H), 6.43 (dd, *J*=17.4, 1.4 Hz, 1H), 6.64 (d, *J*=16.0 Hz, 1H), 7.24–7.39 (m, 5H).

4.1.4. (3*S*)-(1*E*)-1-Phenylhexa-1,5-dien-3-ol [(*S*)-2]. Yield: 170 mg (42%), 74% ee, $[\alpha]_D^{20}$=+10.7 (*c* 2.0, Et_2O). Spectroscopic data as for (±)-**2**.

4.1.5. (1*S*)-1-[(*E*)-2-Phenylvinyl]but-3-enyl acrylate [(*S*)-3]. The alcohol (*S*)-**2**, (170 mg, 0.98 mmol) was dissolved in dry THF (10 mL), to this solution was added Et_3N (two drops) and the solution was cooled to 0 °C. Acryloyl chloride (111 mg, 1.3 mmol) in THF (5 mL) was added dropwise to the solution. Stirring at 0 °C was continued for 3 h. Water (5 mL) and HCl (0.05%, 10 μL) was added and THF was removed in vacuo. The product was extracted with CH_2Cl_2 (2×20 mL), the extract was dried over Na_2SO_4 and the solvent evaporated affording 216 mg of (*S*)-**3**, yield 97%, ee 74% by HPLC analysis, $[\alpha]_D^{20}$=−50.6 (*c* 2.1, $CHCl_3$).

4.1.6. 1-[(*E*)-2-Phenylvinyl]but-3-enyl acrylate [(±)-3]. The alcohol (±)-**2** (300 mg) was treated in the same manner as described for (*S*)-**2** to give the racemic ester (±)-**3**, yield 370 mg (94%), spectroscopic data in accord with (*S*)-**3**.

4.1.7. (+)-Goniothalamin. 6-(6*R*)-[(*E*)-2-phenylvinyl]-5,6-dihydro-2*H*-pyran-2-one [(+)-1]. Grubbs' catalyst (30 mg, 8 mol%,) was dissolved in dry CH_2Cl_2 (5 mL) and this solution was added dropwise to a refluxing solution of the ester (*R*)-**3** (100 mg, 0.47 mmol, ee 93%) in dry CH_2Cl_2 (15 mL). The mixture was heated under reflux in an argon atmosphere for 3 h. The solvent was removed in vacuo and the mixture purified by column chromatography (acetone/hexane, 1:4) to afford 86 mg (92%) of (+)-goniothalamin, (*R*)-**1**, ee>99% as determined by HPLC analysis, $[\alpha]_D^{20}$=+172.2 (*c* 0.8, $CHCl_3$), ^1H NMR: δ 2.55 (m, 2H), 5.11 (m, 1H), 6.1 (dt, *J*=9.8, 1.6 Hz, 1H), 6.29 (dd, *J*=16.0, 6.3 Hz, 1H), 6.73 (d, *J*=16.0 Hz, 1H), 6.93 (dt,

J=9.7, 4.5 Hz, 1H), 7.3 (m, 5H); ^{13}C NMR: δ 30.07, 78.13, 121.86, 125.83, 126.88, 128.54, 128.88, 133.31, 135.94, 144.82, and 164.07.

4.1.8. (−)-Goniothalamin, 6-(6*S*)-[(*E*)-2-phenylvinyl]-5,6-dihydro-2*H*-pyran-2-one [(−)-1]. Using the same procedure as under Section 4.1.7 afforded 141 mg (81%) of (−)-**1**, ee 85% by HPLC analysis, $[\alpha]_D^{20}=-146.0$ (c 0.8, $CHCl_3$) with spectroscopic properties in accord with (+)-**1**.

4.1.9. (±)-Goniothalamin, 6-[(*E*)-2-phenylvinyl]-5,6-dihydro-2*H*-pyran-2-one [(±)-1]. Using the same procedure as described under Section 4.1.7 afforded 261 mg (86%) of (±)-**1** with spectroscopic properties in agreement with (+)-**1**.

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Alkene epoxidation catalyzed by cytochrome P450 BM-3 139-3

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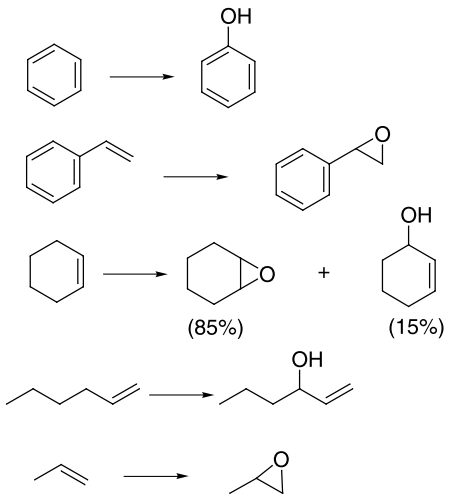
Abstract—We recently reported conversion of cytochrome P450 BM-3, a medium-chain (C_{12}–C_{18}) fatty acid monooxygenase, into a highly efficient alkane hydroxylase by directed evolution [*Nat. Biotechnol.* 2002, *20*, 1135]. P450 BM-3 mutant 139-3 exhibited high activity towards a variety of fatty acid and alkane substrates, including C_3–C_8 alkanes. We report here that mutant 139-3 is also active on benzene, styrene, cyclohexene, 1-hexene, and propylene. Benzene is converted to phenol, while styrene is converted to styrene oxide. Propylene oxidation generates only propylene oxide, but cyclohexene oxidation produces a mixture of cyclohexene oxide (85%) and 2-cyclohexene-1-ol (15%), and 1-hexene is converted to the allylic hydroxylation product, 1-hexene-3-ol. Initial rates of NADPH oxidation for 139-3 in the presence of the substrates greatly (17- to >100-fold) surpass the wild-type in all cases. However, NADPH consumption is only partially coupled to product formation (14–79%). This cytochrome P450 epoxidation catalyst is a suitable starting point for further evolution to improve coupling and activity.

1. Introduction

Catalyzing a wide range of oxidative reactions under mild conditions in aqueous solutions, cytochrome P450 monooxygenases (P450s) are interesting potential 'green' catalysts.[1] It has been shown that many of their limitations, including poor activities towards non-natural substrates,[2–9] limited stability,[10] inability to withstand organic solvent,[12] and even their cofactor requirements[11,13] can be improved by protein engineering, particularly directed evolution.

This group recently described directed evolution of the highly active, soluble fatty acid hydroxylase P450 BM-3 for selective alkane oxidation.[9] Five generations of random mutagenesis/recombination and screening transformed P450 BM-3 into an efficient alkane hydroxylase, which we named 139-3. The evolved gene contained 13 nucleotide base substitutions in the region coding for the heme domain, resulting in 11 amino acid changes. Mutant 139-3 displayed higher turnover rates than any reported enzyme for the (subterminal) hydroxylation of alkanes with chain lengths between C_3 and C_8 (3600 min^{-1} for hexane). It also exhibited ~2-fold higher initial activity for lauric and palmitic acid, which are already good substrates for the wild-type. Here we report that the broadly-active 139-3 mutant is also a good epoxidation catalyst. Much more active than the wild-type P450 BM-3, mutant 139-3 is a convenient platform for further directed evolution to optimize epoxidation of specific substrates.



Scheme 1. Substrates oxidized by BM-3 variant 139-3 and the corresponding products. For benzene, styrene, cyclohexene, or 1-hexene oxidations, the reaction mixture contained enzyme, substrate, and methanol in potassium phosphate buffer. For propylene oxidation, the buffer was first saturated with propylene. Reactions were initiated by addition of NADPH, and products were analyzed by GC/MS.

Keywords: Cytochrome P450; Epoxidation; Monooxygenase; Biocatatysis; Directed evolution.
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Table 1. Products, turnover rates, and coupling during oxidation catalyzed by cytochrome P450 BM-3 mutant 139-3

Substrate	Products[a]	Maximum initial rate[b]	Coupling (%)
Benzene	Phenol (100%)[d]	200±64 (0)[e]	14
Styrene	Styrene oxide (100%)	1100±87 (17±15)	35
Cyclohexene	Cyclohexene oxide (85%) 2-Cyclohexene-1-ol (15%)	1200±130 (0)	79
1-Hexene	1-Hexene-3-ol (100%)	1300±160 (60±8)	22
Propylene	Propylene oxide (100%)	700±62 (40±31)	nd[c]

[a] The product yields and distributions were determined by gas chromatography/mass spectrometry as described in Section 4.
[b] Maximum initial rates are given in nmoles NADPH consumed/min/nmoles P450.
[c] nd=Not determined.
[d] The value in the parenthesis represents the ratio of product formed/total product.
[e] The value in the parenthesis represents the maximum initial rate for the wild-type.

2. Results and discussion

The activity of BM-3 mutant 139-3 was evaluated against the substrates shown in Scheme 1 and Table 1. Benzene was converted to phenol, presumably via epoxidation of the aromatic ring,[14] with a maximum initial rate of NADPH oxidation of 200 mol/min/mol enzyme. The wild-type enzyme in the presence of benzene shows no NADPH consumption above background (Table 1). For styrene, cyclohexene, 1-hexene, and propylene, the maximum rates of NADPH oxidation by 139-3 also greatly surpassed those for wild-type (between 17- and >100-fold) (Table 1). Styrene and propylene oxidation by 139-3 yielded styrene oxide and propylene oxide, respectively. Cyclohexene oxidation yielded cyclohexene oxide (85%) and 2-cyclohexene-1-ol (15%).

The sole product of 1-hexene oxidation was the allylic hydroxylation product, 1-hexene-3-ol. This was not unexpected, since wild-type P450 BM-3 preferentially oxidizes ω-unsaturated fatty acids at the allylic position, and the corresponding terminal epoxide is not formed.[15] It has been proposed that the structure of the BM-3 substrate binding pocket prevents terminal oxidation.[16] The pocket resembles a long funnel[16–18] at the end of which a small hydrophobic pocket sequesters the substrate terminus, rendering it unavailable for oxidation. The selectivity also reflects the different C–H bond strengths: the ω-2 secondary allylic C–H bond (ΔH^0_{298}~83 kcal/mol) is weaker than the ω-3 secondary C–H bond (ΔH^0_{298}~98 kcal/mol).

NADPH oxidation is not necessarily an accurate measure of P450 catalytic activity, because electron equivalents from NADPH can be diverted to produce reduced oxygen species (H_2O or H_2O_2). Using NADPH as a limiting reagent, the 139-3 'uncoupling' reaction was measured for all the substrates (Table 1). In all cases, NADPH oxidation was only partially coupled to substrate oxidation, with efficiencies between 14 and 79%. The amount of H_2O_2 detected using the ABTS/HRP assay was less than 1% in all cases; therefore, it is assumed that H_2O is the uncoupled product. It has been proposed that high substrate mobility in the active site and the presence of excess H_2O near the heme iron cause uncoupling and reflect poor substrate binding.[19]

Substrate binding can be monitored spectroscopically. The substrate-free structure of wild-type P450 BM-3 shows between 17 and 21 water molecules in the substrate access channel.[16] The P450 resting state contains a heme iron as a low-spin six-coordinate ferric species with a dissociable H_2O *trans* to the proximal cysteinate[20] and has a characteristic absorption maximum at 419 nm. The channel dehydrates upon substrate binding, resulting in a high-spin five-coordinate species, and the absorption maximum shifts to 390 nm. In the presence of the alkenes, mutant 139-3 displays only slight spectral shifts, reflecting poor substrate binding or the presence of water in the active site. On the other hand, no detectable spectral shifts are observed when the substrates are added to the wild-type enzyme.

P450s are known to epoxidize a broad range of alkenes and arenes, with rates ranging from 1 min^{-1} for microsomal proteins to 3200 min^{-1} for bacterial enzymes.[1] Using styrene for comparison, P450cam from *Pseudomonas putida* is reported to have an initial rate of NADH consumption of 51 min^{-1}, with only 2% coupling to styrene oxide formation (1.1 min^{-1}).[21] Mutant 139-3, despite its not being 100% coupled, has an initial rate of styrene oxide formation of 385 min^{-1} and is more active than any reported P450 for this reaction.[21,22]

Caldariomyces fumago chloroperoxidase (CPO) performs some P450-like oxygen insertion reactions, including epoxidation.[23] The reported initial rate of CPO-catalyzed styrene epoxidation is 288 min^{-1}.[24] CPO utilizes peroxide in the catalytic cycle and does not require expensive cofactors such as NADPH or additional electron transfer proteins. However, CPO is not functionally expressed in *Escherichia coli*, presumably due to the bacterium's inability to do the processing or post-translational modifications to CPO that occur in the natural, fungal host. Recombinant CPO can be expressed in *C. fumago*, but the fungus does not provide a convenient transformation or expression system for rational protein design or directed evolution experiments.[24–26] In contrast, P450 BM-3 and its mutants, including 139-3, are soluble and easily expressed in *E. coli.* Cirino and Arnold have recently shown that P450 BM-3 heme domain alone can be evolved to use the peroxide shunt pathway, much like CPO.[13] Thus it should be possible to engineer mutants of the P450 heme domain that catalyze epoxidation, using peroxide.

Chiral epoxides are useful synthetic intermediates for the pharmaceutical and chemical industries, and P450s are potential asymmetric epoxidation catalysts.[1] However, slow reaction rates and low expression yields have hampered the synthetic utility of enzymes. For the oxidation of styrene by

P450cam, P450terp from *Pseudomonas* sp., and P450 BM-3, the ratios of *S*/*R* styrene oxide produced are 12:88, 83:17, and 40:60, respectively.[22] Mutant 139-3, which was evolved to hydroxylate alkanes, shows no enantioselectivity in this reaction (*S*/*R* styrene oxide=50:50). With a suitable high throughput screen, however, enantioselectivity is also a good target for optimization by directed evolution.[27]

3. Conclusion

The broadly-active laboratory-evolved cytochrome P450 BM-3 mutant 139-3 is far more active than the wild-type enzyme as a catalyst for epoxidation reactions. The activity of wild-type P450 BM-3 on the substrates investigated here is too low to monitor in high throughput and therefore too low for improvement by directed evolution. The evolved enzyme is a suitable parent for further directed evolution to improve rates and coupling efficiency, both of which can be optimized by appropriate changes in the catalyst. It is also possible to modify enantioselectivity. This relatively stable, efficient, and easily-produced enzyme has potential as an epoxidation catalyst.

4. Experimental

All chemicals were purchased from commercial sources and used as received.

4.1. Expression of P450 BM-3 mutant 139-3

The mutant P450 BM-3 gene was cloned behind the double *tac* promoter of the expression vector pCWori.[9,28] For enzyme production, supplemented terrific broth medium[29] (500 ml) was inoculated with 0.5 ml of an overnight culture of *E. coli* DH5α containing the expression plasmid. After shaking for 10 h at 35 °C, δ-aminolevulinic acid hydrochloride (ALA, 0.5 mM) was added, and expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG, 1 mM). The cells were cultivated for 30 h at 30 °C. The enzyme was purified following published procedures,[29] and the enzyme concentration was determined from the CO-difference spectra.[30]

4.2. Determination of maximum initial rates of oxidation

The mutant was purified and quantified as described above. The initial rate of oxidation was determined as described.[9] A typical reaction solution contained enzyme (1.0 ml, 1.0 μM) in potassium phosphate buffer (0.1 M, pH 8.0), substrate (2 mM), and methanol (1% v/v). The reaction was initiated by the addition of NADPH (200 μl, 200 μM), and the absorbance was monitored at 340 nm.

4.3. Product characterization

Product distributions and yields were characterized by gas chromatography/mass spectrometry. For benzene, styrene, cyclohexene, or 1-hexene oxidations, the reaction solution contained substrate (2 mM) in methanol (1% v/v) and enzyme (1.0 μM) in potassium phosphate buffer (1.5 ml, 0.1 M, pH 8.0). For propylene oxidation, the buffer was first saturated with propylene. The solution was stirred for 5 min at room temperature, and the reaction was initiated by the addition of NADPH (200 μM). After completion of the reaction, the solution was extracted once with $CHCl_3$ (300 μl) containing 1-hexanol (0.5 mM) as an internal standard. The organic layer was analyzed by GC/MS using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass selective detector. The GC was fitted with an Agilent Technologies Innowax column (30 m×0.25 mm with a film thickness of 0.25 μm). The conditions are as follows: (i) 35 °C for 5 min, (ii) 35–200 °C at 20 °C/min, (iii) isothermic at 200 °C for 5 min. Authentic standards were used to identify the retention times and to prepare standard curves. Products were further verified by matching the fragmentation distributions with a database in the software provided with the instrument. Chiral resolution of styrene oxide was determined using a GC fitted with a chiral column (25×0.25 mm Hydrodex β-6 TBDM, Macherey-Nagel), and the conditions are as follows: (i) 100 °C for 10 min, (ii) 100–170 °C at 20 °C/min, (iii) isothermic at 170 °C for 6 min, (iv) 170–100 °C at 20 °C/min, (v) isothermic 100 °C for 1 min.

Percent coupling is determined from the ratio of moles of product formed to the moles of NADPH added to the reaction. NADPH was the limiting reagent, and the substrate concentration was 10× that of NADPH to ensure complete consumption of NADPH. The total product formed was determined by GC/MS, as described above. The concentration of NADPH was determined spectroscopically by measuring the absorbance at 340 nm (ε =6210 M^{-1} cm^{-1}) of the stock solution. The amount of H_2O_2 generated by the reaction was determined using the 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]/horseradish peroxidase (ABTS/HRP) assay according to published procedures.[31,32]

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Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor

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Abstract—Cyclodextrin glucosyltransferase (CGTase) from *Thermoanaerobacter* sp. was covalently immobilized on Eupergit C and used for the synthesis of maltooligosyl fructofuranosides employing soluble starch as donor and sucrose as acceptor. Using a weight ratio starch–sucrose of 1:2, the conversion of starch into acceptor products catalyzed by soluble and immobilized CGTases was higher than 80% in 48 h. Under these conditions, the reaction was selective for the formation of maltosyl fructofuranoside.

1. Introduction

Cyclodextrin glucosyltransferase (CGTase, EC 2.4.1.19) is a bacterial enzyme belonging to the α-amylase family (or glycoside hydrolase family 13)[1] that catalyzes four reactions.[2,3] Cyclization is an intramolecular transglycosylation in which the CGTase converts starch into cyclodextrins (CDs). Coupling is an intermolecular transglycosylation between a CD molecule and a linear oligosaccharide. Disproportionation is an intermolecular transglycosylation between two carbohydrates. In addition, CGTase displays a weak starch hydrolysis activity (where water acts as acceptor).

Either via coupling or disproportionation, CGTases are able to perform the so-called acceptor reaction allowing the synthesis of products of industrial interest. The main requirement for strong acceptor properties is the presence of a D-glucopyranose structure (chair form) with equatorial hydroxyl groups at C-2, C-3 and C-4.[4] Different carbohydrates (such as xylose, sucrose, glucose, maltose and lactose), glycosides, sugar alcohols, vitamins and flavonoids, have been successfully used as CGTase acceptors.[5] The resulting transglycosylated products usually exhibit improved functionality compared with the parent acceptor molecules, e.g. increased water solubility or stability, bifidogenic properties, etc.[6]

In this context, when sucrose is employed as acceptor, maltosyl fructofuranoside ([α-D-Glu-(1→4)-α-D-Glu-(1→2)-β-D-Fru) and higher polymerization products are obtained.[7] A mixture of maltooligosyl fructofuranosides ('maltooligosyl fructose') with a degree of polymerization of 3–7 is currently being commercialized as coupling sugar, and is obtained using starch as donor and sucrose as acceptor, in a process catalyzed by CGTase.[8] Coupling sugar, a non-cariogenic sweetener, is also used as a viscosity modifier, being more stable than other reducing sugar mixtures.[7]

Thermoanaerobacter sp. (a thermophilic anaerobic bacterium) produces a highly thermostable CGTase that displays a high disproportionation activity.[9–11] This enables the enzyme to be used in several acceptor reactions.[12]

In this work, we have studied the synthesis of maltooligosyl fructose employing soluble starch as donor and sucrose as acceptor, catalyzed by CGTase from *Thermoanaerobacter* sp. immobilized on Eupergit C. The reaction rates using the soluble and immobilized CGTases were compared. The main reaction products were isolated by preparative column chromatography, and characterized using NMR and MS analysis.

Keywords: Cyclodextrin glucanotransferase; Glycosyltransferases; Transglycosylation; *Thermoanaerobacter* sp; Coupling sugar; Glucosyl sucrose.
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2. Results and discussion

CGTase is able to use starch[12] or cyclodextrins[13] as glucose donor in transglycosylation processes. Starch is a cheap, abundant and renewable agroproduct, therefore it is preferred to expensive cyclodextrins for industrial purposes. CGTases do not require sugar nucleotides (as all the glycosyltransferases of the Leloir pathway) nor sugar–phosphates (as most glycosyltransferases of the non-Leloir pathway) as donors, since it uses the free energy of cleavage of the readily available starch.[3]

For these acceptor reactions, the immobilization of glucosyltransferases may allow for their stabilization as well as enabling the separation of the biocatalyst. Different approaches have been applied to the immobilization of CGTases, based on adsorption or covalent coupling.[14] We have recently reported CGTase immobilization by covalent binding to aminated silica, activated Sepharose 4B and epoxy-activated acrylic polymers such as Eupergit C.[15] The latter support represents one of the best candidates for CGTase immobilization displaying a half life at 95 °C five times higher that that of the soluble enzyme, as was demonstrated elsewhere.[15b]

The acceptor reaction with soluble starch (Paselli SA2, with an average degree of polymerization of 50) as donor and sucrose as acceptor was studied with soluble and CGTase immobilized on Eupergit C. This enzyme has a higher affinity for disaccharides as acceptors compared with monosaccharides.[3,6,16] When sucrose is employed as acceptor, maltosyl fructose (**1**) is initially formed (Scheme 1). Maltosyl fructose also acts as acceptor, yielding maltotriosyl fructose (**2**). A series of maltooligosyl fructosides is subsequently formed.

The effect of the weight ratio starch–sucrose (from 2:1 to 1:2) on product distribution, using 10% (w/v) soluble starch and variable amounts of sucrose, was tested (Table 1). The maximum conversion to oligosaccharides, regardless of the donor/acceptor ratio, was achieved at 48 h. There are no commercially available standards of maltosyl fructofuranosides. Thus, the main reaction products were isolated by column chromatography (purities higher than 95%) and characterized using NMR and MALDI-TOF mass spectrometry. Maltosyl fructose (**1**), maltotriosyl fructose (**2**) and maltotetraosyl fructose (**3**) were confirmed to be the major products. Spectral data correlated well with those reported for maltosyl fructose by Munksgaard,[17] and for maltotriosyl and maltotetraosyl fructose by Monthieu.[13] However, small amounts of maltooligosyl fructosides with a degree of polymerization up to 7–8 were also observed by TLC and HPLC (Fig. 1).

In the absence of sucrose, the consumption of starch led to the formation of CDs.[12] The presence of co-substrate molecules such as sucrose that are strong acceptors (and, consequently, strong promoters of the formation of linear transfer products) inhibited CD formation. As shown in Table 1, in the presence of the same weight of starch and sucrose, the formation of CDs was almost negligible. Reaction selectivity can be modulated by varying the donor/acceptor ratio. Thus, using a starch/sucrose ratio 2:1 (w/w) the yield of maltooligosyl fructose was 72% and minor amounts of CDs (7–10%) were formed. Under the above conditions, the selectivity to maltosyl fructose was quite low. In contrast, using a starch/sucrose ratio of 1:2 (w/w) the yield of oligosaccharides was 80%, and the formation of maltosyl fructose was enhanced (60%).

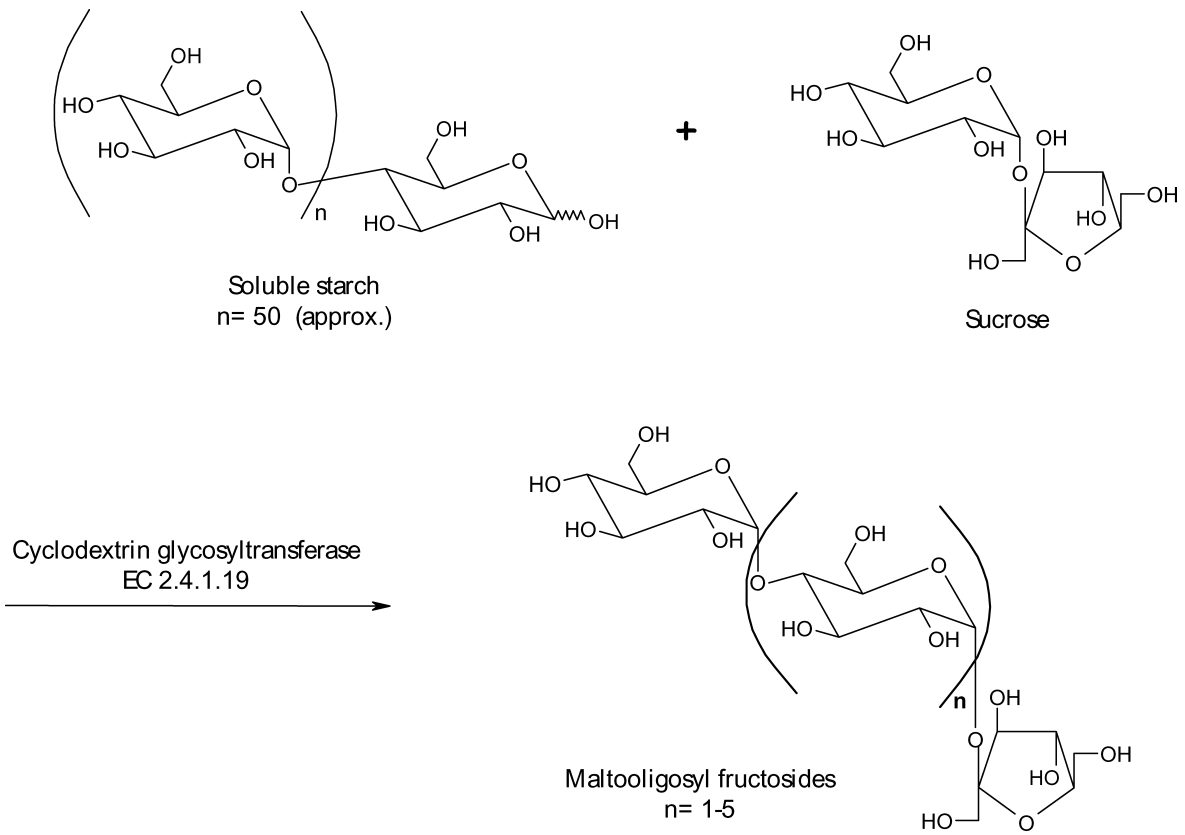
No significant differences were found concerning yield and product distribution when analysing the effect of enzyme immobilization on the reaction (Table 1). However, the reaction rate was higher with the immobilized CGTase (Fig. 2). The initial rate of maltosyl fructose formation with the Eupergit C-immobilized CGTase (14.6 g/l h) was about 3-fold higher as compared with the soluble enzyme (5.6 g/l h). This could be due to improved thermostability of CGTase immobilized on Eupergit C compared to the soluble enzyme.[15]

In conclusion, CGTase immobilized on Eupergit C is able to use starch as donor and sucrose as acceptor, resulting in excellent yields of maltooligosyl fructose (92%). The starch/sucrose ratio is of great importance for inhibiting cyclodextrin synthesis and for modulating the selectivity of the process. Two industrial processes for the CGTase-catalyzed synthesis of coupling sugar have been patented using the enzyme from *Bacillus macerans*[8] and *Thermoanaerobacter*.[18] Our Eupergit-CGTase can become an attractive alternative to the soluble enzyme for these processes. In addition, new applications may emerge from employing distinct acceptors in reactions catalyzed by CGTase from *Thermoanaerobacter* sp.

Table 1. Maltooligosyl fructosides and cyclodextrin production using different starch/sucrose (w/w) ratios catalyzed by soluble and immobilized CGTase. Conditions: 10% (w/v) soluble starch, 60 °C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$, 48 h reaction. The conversion of starch into maltooligosyl fructose and cyclodextrins is also presented

Ratio starch/sucrose (w/w)	Enzyme	Products formed (g/l)[a]						Starch conversion (%)[b]	
		1	**2**	**3**	αCD	β-CD	γCD	Maltooligosyl-fructose	Cyclodextrins
2:1	Soluble	28	21	11	3.3	6.8	—	72	10.1
	Immobilized	35	26	13	2	5.2	—	72	7.2
1:1	Soluble	51	35	15	0.6	2.5	—	78	3.1
	Immobilized	51	34	14	—	—	—	79	—
1:2	Soluble	88	48	15	—	—	—	83	—
	Immobilized	95	50	16	—	—	—	92	—

[a] Nomenclature of the different maltooligosyl fructosides: **1**, maltosyl fructose; **2**, maltotriosyl fructose; **3**, maltotetraosyl fructose.
[b] Based on starch consumption.



Scheme 1. Acceptor reaction using soluble starch as donor and sucrose as acceptor catalyzed by *Thermoanaerobacter* CGTase. Nomenclature used for maltoolygosyl fructofuranosides: maltosyl fructose (**1**) where n=1; maltotriosyl fructose (**2**) where n=2; maltotetraosyl fructose (**3**) where n=3. Reaction conditions given in Section 3.

3. Experimental

3.1. General

CGTase from *Thermoanaerobacter* sp. was kindly provided by Novo Nordisk (Toruzyme 3.0 L, batch ACN00019), and purified as described.[15] Cyclodextrins (α-, β- and γ-) were purchased from Sigma. Potato soluble starch (Paselli SA2) was kindly donated by Avebe (Foxhol, The Netherlands).

NMR spectra were recorded on a Varian UNITY spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 30 °C for solutions in D_2O. The identity of the protons and carbons of the different units were obtained through 2D- homo- (DQCOSY and TOCSY) and hetero- (HMQC and HMBC) experiments. Chemical shifts are referred to the residual signal of HOD at 4.71 ppm for ^{1}H NMR, and that of acetone as external reference at 31.07 ppm for ^{13}C NMR. Mass spectral analyses were obtained on a a Bruker (Bremen,

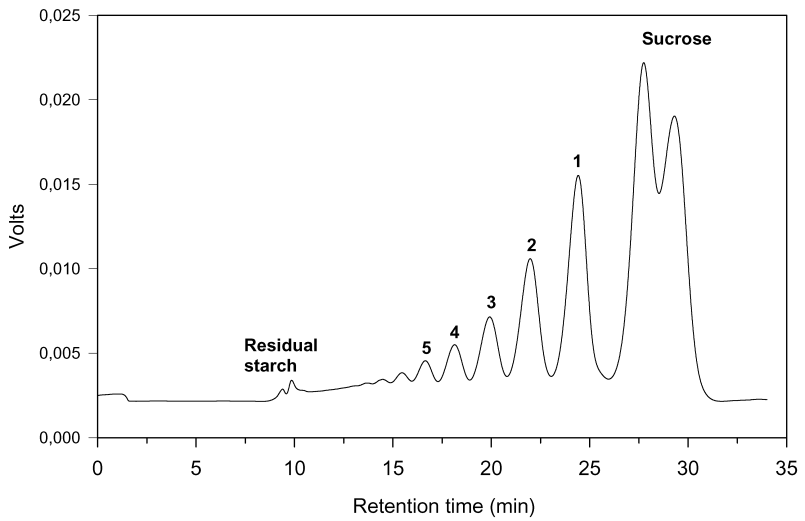


Figure 1. HPLC chromatogram of the reaction mixture at 48 h using soluble starch as donor and sucrose as acceptor catalyzed by CGTase immobilized on Eupergit C. Conditions: 10% soluble starch (w/v), 20% (w/v) sucrose, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$, 60 °C. The sucrose peak appeared split in two peaks. **1** to **5** represents the homologous series of acceptor products where **1** is maltosyl fructose.

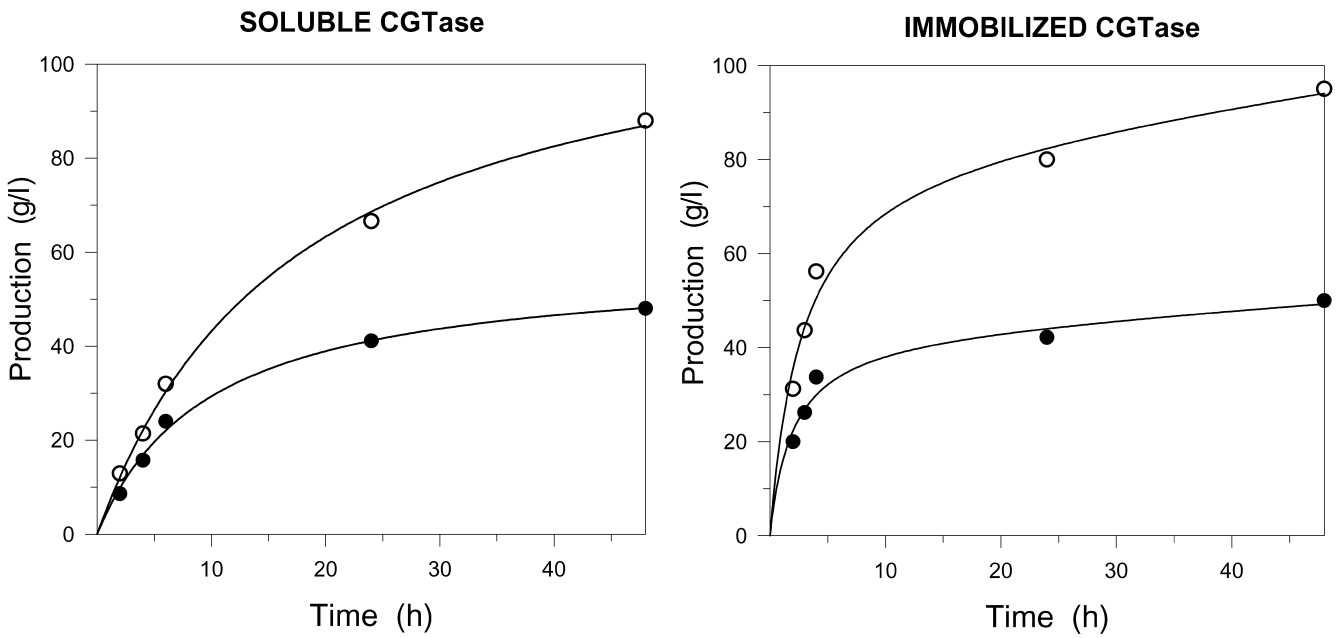


Figure 2. Maltosyl fructose (○) and maltotriosyl fructose (●) production employing a starch:sucrose (w/w) ratio of 1:2 catalyzed by soluble *Thermoanaerobacter* CGTase and immobilized CGTase on Eupergit C. Reaction conditions described in Section 3.

Germany) Reflex III MALDI-TOF mass spectrometer equipped with an ion source with visualization optics and a N_2 laser (337 nm). Mass spectra were recorded using a dihydroxybenzoic acid matrix and methanol as eluent.

Cyclization assays. The production of cyclodextrins was detected spectrophotometrically via the formation of inclusion complexes with several organic compounds. Paselli SA2 (partially hydrolyzed potato starch with an average degree of polymerization of 50) was used as substrate at final concentrations of 5% (w/v) for β- and γ-CD, and 2% (w/v) for α-CD. α-CD was determined at 490 nm on the basis of its ability to form a stable, colorless inclusion complex with methyl orange.[19] β-CD was determined at 552 nm on the basis of its ability to form a stable, colorless inclusion complex with phenolphthalein.[20] γ-CD was determined measuring the color increase at 630 nm due to the formation of an inclusion complex with bromocresol green.[21] The CGTase activity was measured at 85 °C by incubating the enzyme (0.03–0.4 U/ml) in the presence of soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. The formation of the corresponding CD was followed for 5 min. One unit of activity was defined as that catalyzing the production of 1 μmol of CD per min under the above conditions.

The progress of the acceptor reaction was analyzed by high-performance liquid chromatography (HPLC). 1.7 β-CD units of soluble (140 U/mg protein) or immobilized CGTase (147 U/g support) were incubated at 60 °C with 10% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$ in the presence of different amounts of sucrose -studied ratio starch–sucrose (w/w): 2:1, 1:1 and 1:2. The final volume was 10 ml. At different times, aliquots were taken and mixed with the same volume of 0.4 M NaOH to quench the reaction. Samples were centrifuged during 15 min at 8500*g*. HPLC was performed using a ternary pump 9012 (Varian) and two Aminex HPX-42A columns (300×7.8 mm, Bio-Rad) connected in series. Two deashing cartridges (30×4.6 mm, Bio-Rad) were used prior to the column. Water was used as mobile phase (0.7 ml/min). The column temperature was maintained at 85 °C using a Timberline oven. A refractive-index detector 9040 (Varian) was employed. Integration was carried out using the Varian Star 4.0 software.

Thin-layer chromatography (TLC) was carried out on aminated silica gel plates (Lichroprep-NH_2, 25–40 μm, Merck) using water/butanol/ethanol 2:3:5 (v:v.v) as eluent.[22] Spots were detected by immersion of plates into a mixture formed by 45 ml of 1 g urea dissolved in 85% H_3PO_4 aqueous, 48 ml butanol and 50 ml ethanol,[23] drying and heating at 120 °C for 5 min.

Immobilization on Eupergit C was carried out at 25 °C with gentle orbital shaking (150 rpm) in 1.2 M potassium phosphate buffer pH 7.0. A 1:100 (w/w) enzyme–support ratio was employed. After incubation for 72 h, the beads were collected by vacuum filtration using a porous glass filter. The beads were rinsed thoroughly on the same filter with approximately 3×20 ml of diluted potassium phosphate and 2×20 ml of 10 mM sodium citrate buffer, pH 5.5.[15]

3.2. Synthesis and purification of maltooligosyl fructofuranosides

1.7 β-CD units of immobilized CGTase (147 U/g support) were incubated at 60 °C with 10% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$ in the presence of 20% (w/v) sucrose. The final volume was 10 ml. The reaction was maintained for 48 h. The immobilized CGTase and the remaining starch were removed by centrifugation. The aqueous supernatant was evaporated azeotropically with methanol under reduced pressure. After evaporation, the resulting residue was

fractionated by column chromatography using Lichroprep-NH_2 silica gel (25–40 μm, Merck) as adsorbent and an adsorbent–sample ratio of approx. 30:1 (w/w). The reaction products were eluted with a mixture H_2O–ethanol–1-butanol 2:5:3 (v/v/v). The order of elution was maltosyl fructose (**1**), maltotriosyl fructose (**2**), maltotetraosyl fructose (**3**) and higher polymerization products, as shown by TLC and HPLC. Solvents were removed under vacuum from collected fractions of the respective oligosaccharides and characterized.

3.2.1. Maltosyl fructose (1) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.40 (d, 1H, H-1′, $J_{1'2'}$=3.7 Hz), 5.39 (d, 1H, H-1″, $J_{1''2''}$=3.7 Hz), 4.20 (d, 1H, H-3, $J_{3,4}$=8.7 Hz), 4.02 (m, 2H, H-4+H-3′), 3.98 (m, 1H, H-5′), 3.88 (m, 1H, H-5), 3.88–3.76 (m, 6H, H-6a+H-6b+H-6′a+6′b+H-6″a+6″b), 3.76–3.64 (m, 2H, H-3″+H-5″), 3.69 (m, 1H, H-4′), 3.67 (s, 2H, H-1a+H-1b), 3.58 (m, 2H, H-2′+H-2″), 3.40 (t, 1H, H-4″, $J_{3,4}$=$J_{4,5}$=9.2 Hz); ^{13}C NMR (δ, ppm): 104.5 (C-2), 100.7 (C-1″), 92.3 (C-1′), 82.2 (C-5), 77.6 (C-4′), 77.3 (C-3), 74.9 (C-4), 73.9+73.8 (C-3′+C-3″), 73.6 (C-5″), 72.6 (C-2″), 71.8 (C-5′), 71.7 (C-2′), 70.2 (C-4″), 63.2 (C-6), 62.2 (C-1), 61.3 (C-6″), 61.0 (C-6′). MS (MALDI-TOF): calcd for $C_{18}H_{32}O_{16}Na$ 527.3, found 527.1; calcd for $C_{18}H_{32}O_{16}K$ 543.4, found 543.1.

3.2.2. Maltotriosyl fructose (2) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.38 (d, 1H, H-1′, $J_{1'2'}$=3.7 Hz), 5.36+5.35 (2d, H-1″+H-1‴, J=3.7 Hz), 4.18 (d, 1H, H-3, $J_{3,4}$=8.6 Hz), 4.05 (t, 1H, H-4, $J_{4,5}$=8.6 Hz), 4.00 (dd, 1H, H-3′, J=8.9+10 Hz), 3.94 (m, 1H, H-5′), 3.86 (m,1H,H-5), 3.86–3.70 (m, 8H, H-6a+6b+H-6′a+6′b+H-6″a+6″b+H-6‴a+6‴b), 3.68 (m, 2H, H-5″+H-5‴), 3.64 (s, 2H, H-1a+H-1b), 3.70–3.60 (m, 2H, H-4′+H-4″), 3.60–3.50 (m, 3H, H-2′+H-2″+H-2‴), 3.38 (t, 1H, H-4‴, J=9.6+9.2 Hz); ^{13}C NMR (δ, ppm): 104.6 (C-2), 100.7+100.6 (C-1″+C-1‴), 92.9 (C-1′), 82.3 (C-5), 77.9+77.7 (C-4′+C-4″), 77.5 (C-3), 75.0 (C-4), 74.3+73.9+73.8+73.7 (C-3′+C-3″+C-3‴+C-5″), 72.7+72.5+72.2 (C-2′+C-2″+C-2″), 71.9 (C-5′), 71.8 (C-5‴), 70.3 (C-4‴), 63.3 (C-6), 62.3 (C-1), 61.5 (2)+61.1 (C-6′+C-6″+C-6‴). MS (MALDI-TOF): calcd for $C_{24}H_{42}O_{21}Na$ 689.3, found 689.2; calcd for $C_{24}H_{42}O_{21}K$ 705.4, found 705.2.

3.2.3. Maltotetraosyl fructose (3) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.40–5.38 (m, 4H, H-1′+H-1″+H-1‴+H-1^{IV}), 4.20 (d, 1H, H-3, $J_{3,4}$=8.7 Hz), 4.03 (m, 2H, H-4+H-3′), 3.96 (m, 3H, H-5+H-5′+H-3^{IV}), 3.86–3.70 (m, 10H, H-6a+6b+H-6′a+6′b+H-6″a+6″b+H-6‴a+6‴b+H-6^{IV}a+6^{IV}b), 3.67 (s, 2H, H-1a+H-1b), 3.65–3.55 (m, 4H, H-2′+H-2″+H-2‴+H-2^{IV}), 3.42 (t, 1H, H-4^{IV}, J=9.5+9.7 Hz); ^{13}C NMR (δ, ppm): 104.5 (C-2), 100.6+100.4+100.3 (C-1″+C-1‴+C-1^{IV}), 92.8 (C-1′), 82.2 (C-5), 77.8+77.4 (2) (C-4′+C-4″+C-4‴), 76.8 (C-3), 74.9 (C-4), 74.2 (2)+73.8+73.7+73.6 (C-3′+C-3″+C-3‴+C-3^{IV}), 72.6+72.4 (2)+72.1+71.8+71.7 (2) (C-2′+C-2″+C-2‴+C-2^{IV}+C-5′+C-5″+C-5‴), 70.2 (C-4^{IV}), 63.2 (C-6), 62.3 (C-1), 61.4 (3)+61.0 (C-6′+C-6″+C-6‴+C-6^{IV}). MS (MALDI-TOF): calcd for $C_{30}H_{52}O_{26}Na$ 851.4, found 851.3; calcd for $C_{30}H_{52}O_{26}K$ 867.5, found 867.2.

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A chemoenzymatic synthesis of the linear triquinane (−)-hirsutene and identification of possible precursors to the naturally occurring (+)-enantiomer

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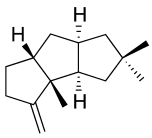
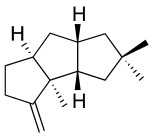
Abstract—An enantiomerically pure *cis*-1,2-dihydrocatechol, which is readily obtained via a toluene dioxygenase-mediated dihydroxylation of toluene in a whole-cell biotransformation process, has been converted over 17 steps into the linear triquinane (−)-hirsutene. Since the enantiomer of the starting material is also available this work constitutes a formal total synthesis of the naturally occurring (+)-form of hirsutene. Furthermore, minor modifications of the route used here offer the possibility of accessing (+)-hirsutene from the original starting material.

1. Introduction

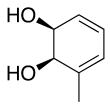
The linear triquinane-type[1] sesquiterpene (+)-hirsutene [(+)-**1**)][2] is the biogenetic precursor to a range of oxygenated derivatives, including hirsutic acid C,[3] complicatic acid,[3] coriolin[4] and hypnophilin,[5] many of which display significant biological properties. As such, this hydrocarbon has been a popular synthetic target and many ingenious approaches to it have been devised.[1,6] Remarkably, only one enantioselective total synthesis of (+)-hirsutene has been achieved,[6a] although others have claimed formal preparations[6b,c,e] of the same target. Weinges et al. have reported[7] the synthesis of the non-natural (−)-enantiomer **1** from (−)-carvone using Curran's tandem radical cyclization strategy[8] as a key element. Herein we report full details of our recently communicated[9] synthesis of (−)-hirsutene (**1**) from the *cis*-1,2-dihydrocatechol **2**, a compound available in enantiomerically pure form via the toluene dioxygenase (TDO)-mediated whole-cell biotransformation of toluene.[10] Since the enantiomeric form of this starting material, viz. *ent*-**2**, is also available[11] the present work constitutes a formal total synthesis of (+)-hirsutene, viz. (+)-**1**. Moreover, minor modifications to the route used here (and discussed below) would appear to offer the possibility that compound **2** could also serve as a precursor to (+)-hirsutene, thus highlighting the potential for the enantiodivergent synthesis of various terpenoids from this readily available chiron.[12]



(+)-**1**



(−)-**1**



2

2. Results and discussion

The retrosynthetic analysis of (−)-hirsutene [(−)-**1**)] employed in the present work is shown in Figure 1. Thus, the closing stages of the synthesis would involve reductive cleavage of the three-membered ring associated with the cyclopropa-fused triquinane **3**, followed by deletion of the carbonyl group and, finally, manipulation of the protected hydroxyl group so as to install the exocyclic methylene unit associated with target **1**. Formation of compound **3** was to involve a photochemically-induced oxa-di-π-methane rearrangement of the cyclopentannulated bicyclo[2.2.2]-oct-5-en-2-one **4**, a protocol for triquinane synthesis originally enunciated by Demuth[1c] and recently exploited by Singh et al.[6e] in their preparation of (±)-hirsutene. Compound **4** was, in turn, to be generated via a Diels–Alder cycloaddition reaction between *cis*-1,2-dihydrocatechol **2** and the *gem*-dimethylated cyclopentenone **5**, the last compound being available by established routes[13] from the dimethylketene dimer **6**.

On the basis of recent studies carried out in our laboratories (and to be reported shortly), we anticipated that a high pressure promoted Diels–Alder reaction between diene **2**

Keywords: Chemoenzymatic; Hirsutene; Sesquiterpene; Triquinane; Oxa-di-π-methane; Enantiodivergence; Cycloaddition; *cis*-1,2-Dihydrocatechol.
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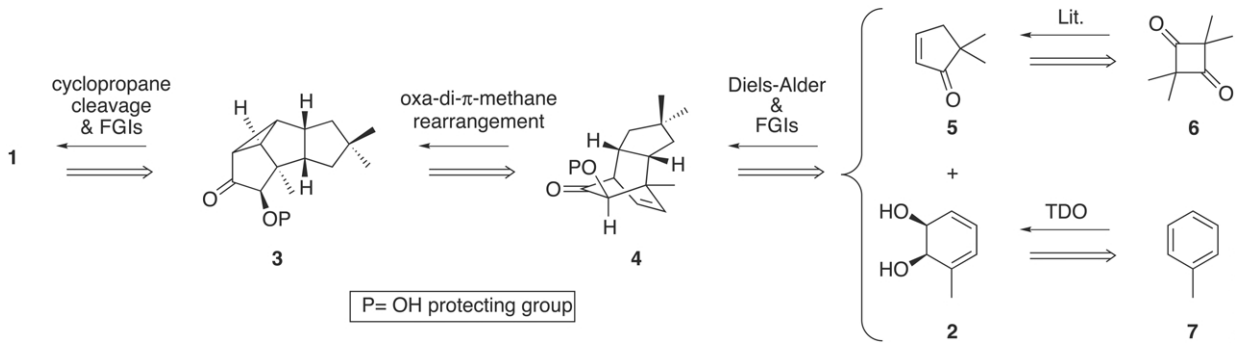
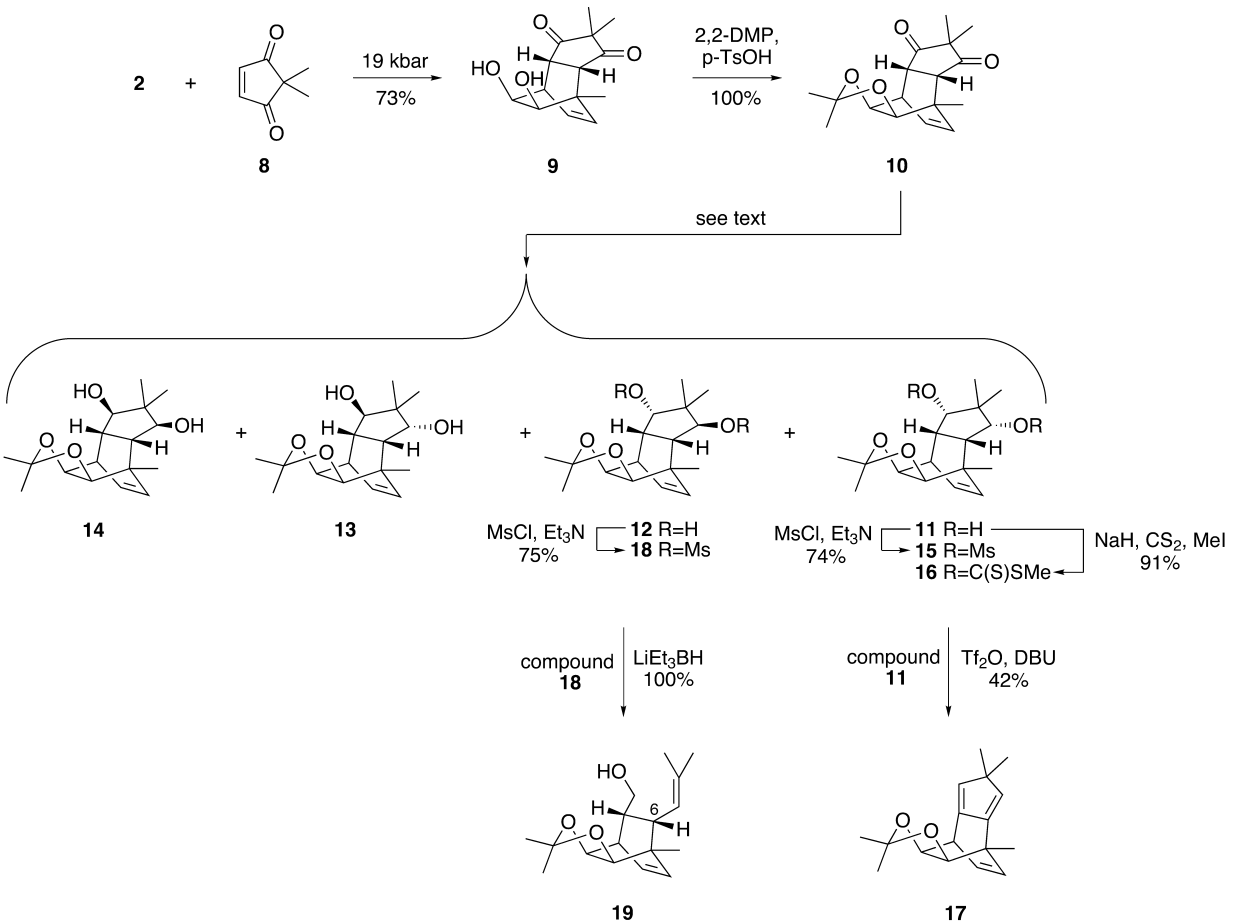


Figure 1.

and dienophile **5** should proceed efficiently and in a *syn*-selective fashion.[14,15] However, all efforts to implement such a reaction failed to produce preparatively useful quantities of the desired cycloaddition product or any other adduct. On the basis that the steric inhibition to this process caused by the *gem*-dimethyl unit of the dienophile could be offset by further carbonyl activation, the reaction of the readily available cyclopentenedione **8** with diene **2** at 19 kbar was examined (Scheme 1). Gratifyingly, the anticipated adduct **9** was obtained and its structure established by X-ray crystallographic studies, details of which will be reported elsewhere. The readily derived acetonide **10** (100%) was prepared in anticipation of examining protocols for the reductive removal, via the corresponding alcohols, of the two now redundant ketone carbonyl units associated with the Diels–Alder adduct. Exposure of compound **10** to various reducing agents led to varying combinations (Table 1) of some or all of the four possible diols **11**–**14**, the first three of which could be purified and then subjected to comprehensive characterization.

Compound **11** was obtained as a crystalline solid such that an X-ray crystallographic analysis could be undertaken, the results of which are presented in Figure 2 and the Experimental section. Unfortunately, all efforts to exploit this diol in the required deoxygenation process have failed. Thus, the derived *bis*-mesylate **15** only produced complex mixtures of products on exposure to hydride donors such as lithium aluminium hydride while the derived *bis*-xanthate



Scheme 1.

Table 1. Product distributions from reduction of dione **10** under various conditions

Entry	Reductant/conditions	% Diol **11**	% Diol **12**	% Diol **13**	% Diol **14**
1	$LiAlH_4$, THF, Δ	10	75	5	9
2	$LiAlH_4$, Et_2O, Δ	28	31	26	10
3	DIBALH, THF, −78 °C	99	0	0	0

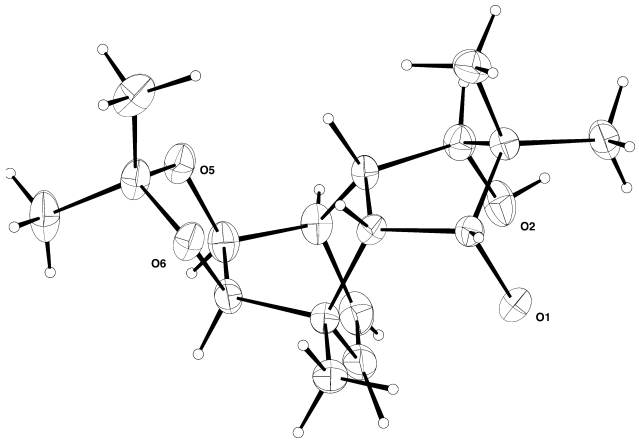
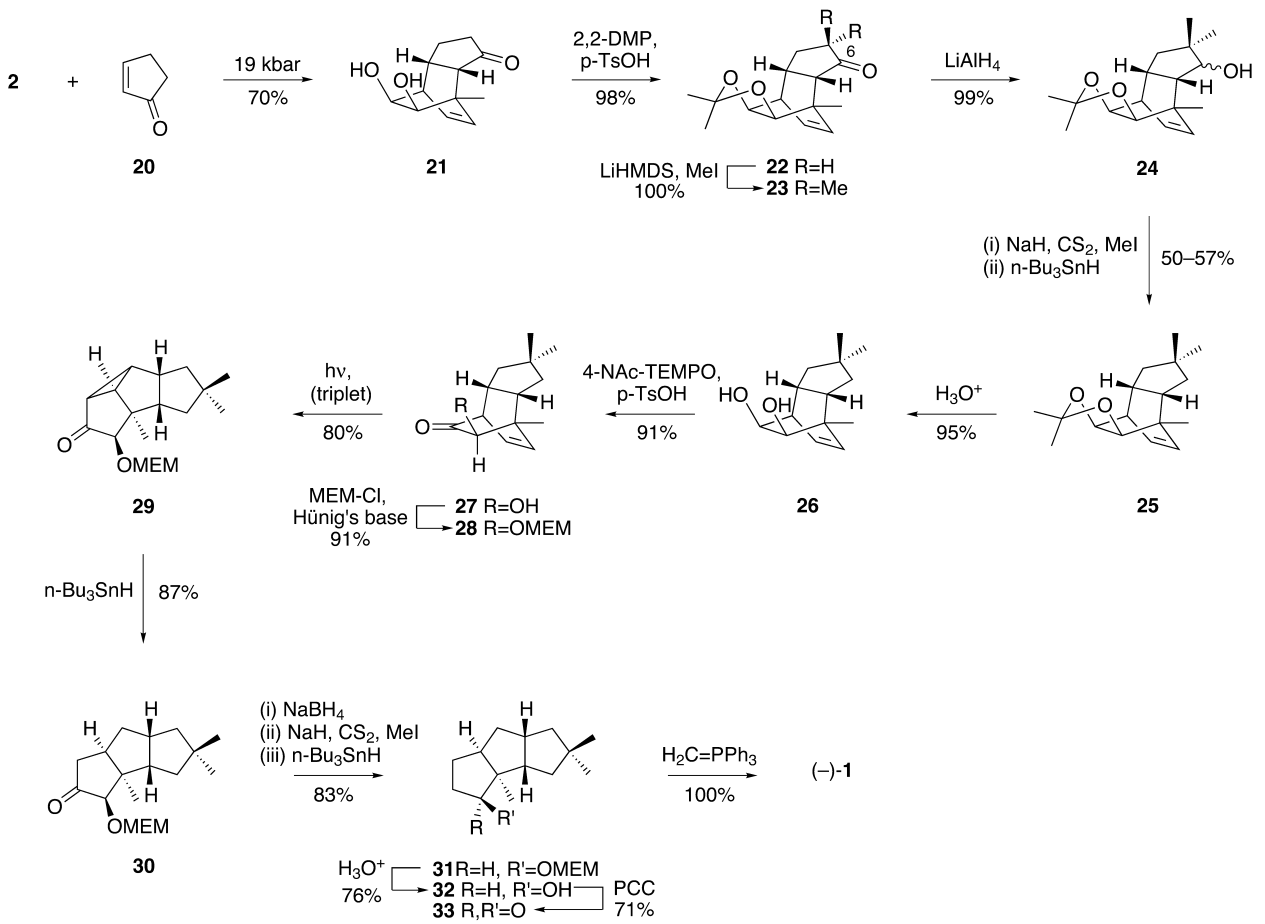


Figure 2. ADEP derived from single-crystal X-ray analysis of diol **11**.

suffered a similar fate during attempts to engage it in Barton–McCombie-type deoxygenation[16] processes. The related *bis*-triflate could not be isolated but underwent two-fold elimination under the conditions of its formation and thus providing the cyclopentadiene **17** (42%) as the only isolable reaction product. In principle, this triene could be subjected to a selective dihydrogenation reaction so as to provide a product capable of being carried forward to (−)-**1**, but the issues of regio- and stereo-selectivity that would need to be addressed in achieving such ends looked too formidable to warrant serious consideration. In a final attempt to effect deoxygenation, the *bis*-mesylate, **18**, derived from diol **12** was subject to reaction with lithium triethylborohydride (Super-Hydride®) but the only product of reaction was the fragmentation product **19** (100%) the structure of which follows from detailed NMR spectroscopic analysis, including the use of NOESY techniques, which revealed an interaction between H6 and the bridgehead methyl protons. The formation of compound **19** from precursor **18** highlights the potential for fragmentation during attempts to reductively deoxygenate 1,3-dioxygenated systems[17] and led us to abandon this approach to substrates (viz. **4**, Fig. 1) suitable for studying the foreshadowed oxa-di-π-methane rearrangement.

The ultimately successful route to (−)-hirsutene [(−)-**1**] is shown in Scheme 2 and the abovementioned problems were addressed by reverting to a protocol that avoids the need for using a 1,3-deoxygenation regime but now requiring a *gem*-dimethylation step after the Diels–Alder cycloaddition reaction. Thus, *cis*-1,2-dihydrocatechol **2** was reacted with cyclopentenone **20** at 19 kbar and the *syn*-addition product **21** (70%) was obtained, together with small quantities (ca. 9%) of the corresponding *anti*-isomer. These adducts were readily separated by flash chromatography and the structure of the former established by single-crystal X-ray analysis, details of which will be published elsewhere. The *cis*-1,2-diol moiety associated with compound **21** was protected, using standard methods, as the corresponding acetonide **22** (98%) which could then be subjected to *gem*-dimethylation at C6 using LiHMDS as base and methyl iodide as the alkylating agent. The resulting ketone **23** (100%) was reduced with lithium aluminium hydride to give a chromatographically separable and 9:1 mixture of the two epimeric forms of alcohol **24** (99% combined yield) which were each converted into their respective xanthates by standard methods and in essentially quantitative yield. In contrast to the difficulties detailed earlier, these esters each underwent a smooth Barton–McCombie deoxygenation reaction to give the, by now, long sought after compound **25** (57%). As observed in related systems,[18] acid-catalysed removal of the acetonide group within this latter compound proved a sluggish process and even when forcing conditions and extended reaction times were used, complete conversion of substrate **25** into diol **26** (95% at 44% conversion) could not be achieved. Nevertheless, preparatively useful quantities of compound **26** were available by such means and the less hindered hydroxy group associated with this compound could be selectively oxidized to the corresponding ketone using the sterically demanding oxoammonium ion derived from 4-acetamido-TEMPO.[19] The acyloin **27** (91% at 96% conversion) thus obtained proved rather unstable and the product resulting from its oxa-di-π-methane rearrangement was even more so. Consequently, compound **27** was protected as the corresponding and now completely stable MEM-ether[20] **28** (91%).

Subjection of compound **28** to triplet sensitized photolysis resulted in the anticipated oxa-di-π-methane rearrangement process and thus provided the tetracyclic product **29** (80% at 71% conversion) as a crystalline solid suitable for single crystal X-ray analysis, results of which have been reported previously.[9] Whilst a number of methods are available for the reductive cleavage of carbonyl-conjugated cyclopropyl groups[21] we favored one using tri-*n*-butyltin hydride[22] as successfully employed by Singh et al. in their recently reported[6e] synthesis of (±)-hirsutene. Thus, treatment of compound **29** with this hydride in the presence of AIBN resulted in smooth reduction to the triquinane **30** (87% at 81% conversion) the carbonyl group of which was reduced, with sodium borohydride, to the corresponding alcohol (98%) which was obtained as a single diastereoisomer and presumably that possessing a β-hydroxy group as a result of hydride delivery to the *exo*-face of the precursor ketone. The readily derived xanthate ester (92%) was then deoxygenated under Barton–McCombie conditions to give the MEM-ether **31** (92% from **30**). Cleavage of the MEM-group within the latter compound could be achieved under the conditions described by Monti et al.[23] and so affording the extremely volatile and, therefore, refractory alcohol **32** (76%), the racemic form of which has been employed in previous syntheses of (±)-hirsutene. PCC-promoted oxidation of



Scheme 2.

compound **32** afforded the corresponding ketone **33** (71%), which also proved a difficult compound to handle because of its high vapor pressure. In the final step of the synthesis compound **33** was methylenated using methylene triphenylphosphorane under standard conditions. The sterically hindered nature of ketone **33** led to difficulties in driving this reaction to completion with the result that whilst near quantitative yields of target **1** were obtained this could only be accomplished at modest (32%) conversions. Nevertheless, by such means, very clean samples of (−)-hirsutene could be obtained and the derived spectral data were in complete accord with the assigned structure and a good match for the excellent data presented by Weinges et al.[7] In particular, there was excellent agreement between the specific rotations, viz. $[\alpha]_D=-26$ (*c* 0.2, $CDCl_3$) vs $[\alpha]_D=-29.4$ (*c* 1.0, pentane).[7]

As noted earlier, since the enantiomer of *cis*-1,2-dihydrocatechol **2** is known,[11] the present work also represents a formal total synthesis of the naturally occurring or (+)-form of hirsutene. However, there are also interesting possibilities for accessing (+)-hirsutene from compound **2** itself. In particular, we have observed (Fig. 3) that the known[24] acetonide derivative, **34**, of diol **2** engages in an efficient high pressure-promoted Diels–Alder cycloaddition reaction with cyclopentenone **20** to give the adduct **35** in 56% yield and incorporating a bicyclo[2.2.2]oct-5-en-2-one residue that is enantiomerically related to the one associated with isomer **22**. In other words, compounds **20** and **35** can be regarded as pseudo-enantiomers, each of which is accessible from the common precursor **2** by controlling the facial selectivity of its reaction with the dienophile **20**. Such control is achieved by simply employing either the

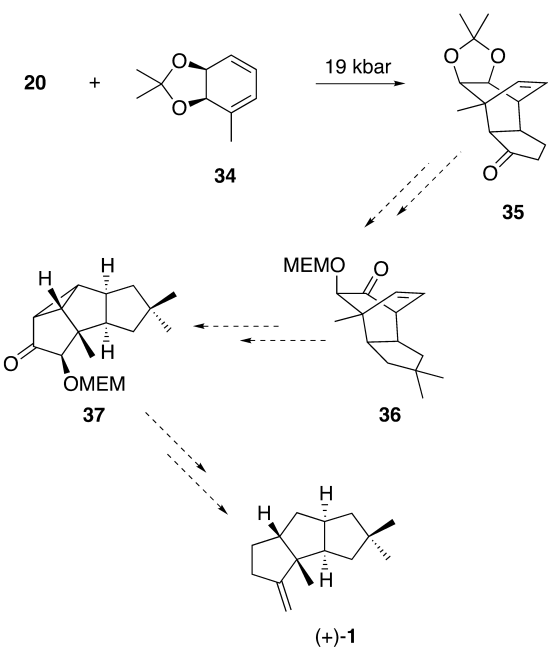


Figure 3.

unprotected or protected forms of **2** in the Diels–Alder reaction. Given the foregoing and considering the application of the chemistry defined in Scheme 2 to adduct **35**, it might reasonably be anticipated that the acyloin **36** (Fig. 3) could be obtained and that this would engage in an oxa-di-π-methane rearrangement, thus, delivering the cyclopropa-fused triquinane **37**, a pseudo-enantiomer of compound **29** and a seemingly realistic precursor to (+)-**1**. The pursuit of such possibilities will be the subject of further reports from these laboratories although it is already clear from earlier work, described by us,[12b,c,18,25] that the *cis*-1,2-dihydrocatechol **2** offers significant opportunities for the enantiodivergent synthesis of a range of biologically relevant carbocyclic frameworks. A further noteworthy aspect of the work described in Schemes 1 and 2 is that the strategies and chemistries defined therein should lend themselves to the synthesis of various oxygenated and, therefore, biologically interesting linear triquinane-type natural products such as those mentioned in the introduction.

3. Experimental

3.1. General

Melting points were measured on a Reichert hot-stage microscope apparatus and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on either a Varian Inova 600 spectrometer operating at 600 MHz for proton or a Gemini 300 NMR spectrometer, operating at 300 MHz (for proton) and 75 MHz (for carbon). Unless otherwise specified spectra were acquired at 20 °C in deuterochloroform ($CDCl_3$) which had been filtered through basic alumina prior to use. Chemical shifts were recorded as δ values in parts per million (ppm). Infrared spectra (ν_{max}) were recorded on a Perkin–Elmer 1800 Series FTIR Spectrometer and samples were analysed as thin films on NaCl plates. Low resolution mass spectra were recorded on a Micromass-Waters LC-ZMD single quadrupole liquid chromatograph-MS or VG Quattro II triple quadrupole MS instrument using electron impact techniques. High resolution mass spectra were acquired by liquid secondary ion MS methods on a Kratos Analytical Concept ISQ instrument located at the University of Tasmania. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at the sodium-D line (589 nm) and the concentrations (*c*) (g 100 mL^{-1}) indicated using spectroscopic grade $CHCl_3$ unless otherwise specified. The measurements were carried out between 17 and 28 °C in a cell with a path length (*l*) of 1 dm. Specific rotations $[\alpha]_D$ were calculated using the equation $[\alpha]_D=100\ \alpha/(c\ l)$ and are given in 10^{-1} deg cm^2 g^{-1}. Elemental analyses were performed by the Australian National University's Microanalytical Services Unit based at the Research School of Chemistry, Canberra, Australia. All reactions were performed under a nitrogen atmosphere. Anhydrous solvents were obtained by distillation from appropriate drying agents under a nitrogen atmosphere.

3.2. Synthetic studies

3.2.1. (3a*R*,4*S*,4a*S*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-dione (10). A magnetically stirred solution of diol **9** (1.07 g, 4.27 mmol) in 2,2-dimethoxypropane (7.5 mL, 61 mmol, 14 mole equiv.) and dichloromethane (7.5 mL) was treated with *p*-TsOH·H_2O (12.2 mg, 1.5 mole%) and the resulting mixture stirred at 18 °C for 24 h then concentrated under reduced pressure. Subjection of the ensuing deep-red oil to flash chromatography (silica, 1:4 v/v ethyl acetate–hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.9 in 1:1 v/v ethyl acetate–hexane), the title compound *acetonide* **10** (1.24 g, 100%) as a clear, colourless oil, $[\alpha]_D=+29$ (*c* 0.2, $CHCl_3$) (Found: M$^{+\cdot}$, 290.1524. $C_{17}H_{22}O_4$ requires M$^{+\cdot}$, 290.1518). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.02 (dd, *J*=8.4, 6.6 Hz, 1H), 5.82 (d, *J*=8.4 Hz, 1H), 4.11 (dd, *J*=8.1, 3.9 Hz, 1H), 3.68 (d, *J*=8.4 Hz, 1H), 3.52 (dd, *J*=10.2, 3.0 Hz, 1H), 3.42–3.40 (m, 1H), 3.22 (d, *J*=10.2 Hz, 1H), 1.57 (s, 3H), 1.48 (s, 3H), 1.32 (s, 3H), 1.06 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.4, 219.7, 138.7, 132.1, 112.5, 80.8, 75.1, 54.6, 48.0, 45.4, 42.1, 37.4, 26.9, 24.7, 23.6, 20.5, 16.8; IR, ν_{max} 2976, 2930, 1759, 1720, 1460, 1375, 1265, 1206, 1163, 1109, 1065, 1049, 880, 710 cm^{-1}; MS, *m*/*z* (EI, 70 eV) 290 (M$^{+\cdot}$, 11%), 275 (16), 232 (28), 189 (28), 161 (87), 134 (100), 105 (88), 100 (75), 70 (98).

3.2.2. (3a*R*,4*S*,4a*S*,5*R*,7*S*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol (11), (3a*R*,4*S*,4a*S*,5*S*,7-*R*,4*S*,4a*S*,5*S*,7*S*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol (12), (3a*R*,4*S*,4a*S*,5*R*,7*R*,7a*S*,8-*R*,4*S*,4a*S*,5*R*,7*R*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol (13) and (3a*R*,4*S*,4a*S*,5*S*,7*R*,7a*S*,8-*R*,4*S*,4a*S*,5*S*,7*R*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol (14).

3.2.2.1. Method A. A magnetically stirred solution of the dione **10** (102.6 mg, 0.35 mmol) in THF (1.7 mL) maintained at −78 °C (acetone-dry ice bath) was treated, dropwise, with DIBAL-H (1.55 mL of a 1 M solution in hexane, 0.155 mmol). After 3 h the reaction mixture was warmed to 18 °C, stirring continued for a further 3 h, then water (10 mL), NH_4Cl (10 mL of a saturated aq. solution) and diethyl ether (10 mL) were added (CAUTION!). The separated aqueous phase was extracted with diethyl ether (4×10 mL) and the combined organic phases washed with brine (1×10 mL) then dried ($MgSO_4$), filtered and concentrated under reduced pressure to give an oily solid. Subjection of this material to flash chromatography (silica, 1:1 v/v ethyl acetate–hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.4), the title compound *diol* **11** (103 mg, 99%) as a white crystalline solid, mp 106–107 °C (with sublimation), $[\alpha]_D=+45$ (*c* 0.8, $CHCl_3$) (Found: M$^{+\cdot}$, 294.1826. C, 69.1; H, 8.6. $C_{17}H_{26}O_4$ requires M$^{+\cdot}$, 294.1831. C, 69.4; H, 8.9%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.08 (dd, *J*=8.1, 6.9 Hz, 1H), 5.88 (d, *J*=8.1 Hz, 1H), 4.11 (dd, *J*=8.1, 3.9 Hz, 1H), 3.75 (d, *J*=8.1 Hz, 1H), 3.71–3.68 (m, 2H), 3.10 (ddd, *J*=11.4, 6.0, 2.4 Hz, 1H), 2.95–2.92 (m, 1H), 2.81 (dd, *J*=11.4, 5.7 Hz, 1H), 1.91 (broad s, 1H), 1.62 (broad s, 1H), 1.47 (s, 3H), 1.36 (s, 3H), 1.34 (s, 3H), 1.12 (s, 3H), 0.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.4, 131.6, 112.1, 84.1, 83.2,

81.8, 76.8, 49.7, 47.5, 43.2, 40.8, 36.6, 26.8, 24.7, 24.6, 20.4, 17.8; IR, ν_{max} 3288, 2946, 2890, 1455, 1378, 1257, 1208, 1160, 1061, 1030, 979, 876 cm^{-1}; MS, *m/z* (EI, 70 eV) 294 ($M^{+\cdot}$, 2%), 279 (12), 265 (64), 194 (89), 135 (93), 134 (92), 84 (100).

3.2.2.2. Method B. A magnetically stirred solution of dione **10** (103 mg, 0.35 mmol) in THF (20 mL) was treated with $LiAlH_4$ (ca 1.00 g, 26.7 mmol). The resulting mixture was heated at reflux for 48 h then cooled and treated with NH_4Cl (10 mL of a saturated aq. solution—CAUTION!) and the separated aqueous phase extracted with diethyl ether (4×10 mL). The combined organic phases were washed with brine (1×10 mL) then dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a light-yellow oil. Subjection of this material to flash chromatography (silica, 1:4→3:7 v/v ethyl acetate–hexane gradient elution) afforded three fractions, A–C.

Concentration of fraction A (R_f 0.4 in 1:1 v/v ethyl acetate–hexane) afforded diol **11** (10 mg, 10%) identical, in all respects, with the material obtained via Method A.

Concentration of fraction B [R_f 0.2(3) in 1:1 v/v ethyl acetate–hexane] afforded a clear, colourless glass (40 mg) tentatively identified as diol **12** (75 mg, 75%), $[\alpha]_D=-9$ (*c* 0.2, $CHCl_3$) (Found: $M^{+\cdot}$, 294.1826. C, 69.3; H, 9.2. $C_{17}H_{26}O_4$ requires $M^{+\cdot}$, 294.1831. C, 69.4; H, 8.9%). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.30 (dd, *J*=8.1, 6.6 Hz, 1H), 5.82 (dm, *J*=8.1 Hz, 1H), 4.09 (dd, *J*=8.1, 3.9 Hz, 1H), 3.77 (d, *J*=8.4 Hz, 1H), 3.66 (d, *J*=6.6 Hz, 1H), 3.43 (d, *J*=9 Hz, 1H), 3.07 (ddd, *J*=11.1, 6.6, 2.4 Hz, 1H), 2.87–2.82 (m, 1H), 2.36–2.29 (m, 1H), 1.47 (s, 3H), 1.35 (s, 3H), 1.32 (s, 3H), 0.97 (s, 3H), 0.88 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 137.3, 135.2, 112.3, 81.7, 81.2, 80.8, 76.5, 48.7, 46.0, 41.3, 40.4, 37.2, 26.7, 24.7, 20.7, 20.4, 20.1; IR, ν_{max} 3467, 2958, 2930, 2873, 1457, 1381, 1371, 1262, 1207, 1160, 1061, 1032, 876, 710 cm^{-1}; MS, *m/z* (EI, 70 eV) 294 ($M^{+\cdot}$, <1%), 297 (7), 265 (16), 236 (29), 194 (100), 134 (73), 84 (94).

Concentration of fraction C [R_f 0.1(7) in 1:1 v/v ethyl acetate–hexane] afforded an oily solid (15 mg) tentatively identified as a ca. 4:1 mixture of diols **13** and **14** (14%). IR, ν_{max} 3434, 2959, 2930, 2873, 1464, 1381, 1372, 1263, 1207, 1164, 1155, 1082, 1045, 882, 731 cm^{-1}.

The semi-solid obtained on concentration of fraction C was triturated (ethyl acetate) to afford *diol* **13** (11.6 mg. 11%) as white crystalline masses, mp 107–108 °C (with sublimation), $[\alpha]_D=+27$ (*c* 0.1 in $CHCl_3$) (Found: $M^{+\cdot}$, 294.1829. C, 69.1; H, 9.0. $C_{17}H_{26}O_4$ requires 294.1831. C, 69.4; H, 8.9%). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.16 (t, *J*=7.8 Hz, 1H), 5.90 (dm, *J*=7.8 Hz, 1H), 4.15 (dd, *J*=8.1, 4.2 Hz, 1H), 3.76 (d, *J*=8.1 Hz, 1H), 3.22–3.13 (complex m, 2H), 2.98–2.93 (m, 1H), 2.55–2.47 (m, 1H), 2.24 (dd, *J*=11.4, 8.7 Hz, 1H), 1.47 (s, 3H), 1.33 (s, 3H), 1.31 (s, 3H), 1.27 (dd, *J*=7.8, 4.5 Hz, 1H), 0.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 139.7, 133.6, 112.1, 81.1, 80.9, 80.7, 76.2, 46.5, 44.0, 43.9, 41.7, 37.4, 26.7, 24.7, 24.3, 20.4, 14.8; IR, ν_{max} 3335, 2974, 2936, 2873, 1381, 1369, 1262, 1205, 1059, 1040, 880, 748 cm^{-1}.

3.2.3. (3a*R*,4*S*,4a*S*,5*R*,7*S*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol *bis*-methanesulfonate (15). A magnetically stirred solution of diol **11** (40 mg, 0.14 mmol) and pyridine (1 mL) in dichloromethane (1.4 mL) was cooled to 0 °C (ice-water bath) then treated with methanesulfonyl chloride (23 μL, 0.30 mmol) and triethylamine (42 μL, 0.30 mL). The resulting mixture was warmed to 18 °C and stirred at this temperature for 72 h, then concentrated under reduced pressure. The residue thus obtained was partitioned between ethyl acetate (5 mL) and water (5 mL) and the separated aqueous phase extracted with ethyl acetate (5×5 mL). The combined organic phases were dried (Na_2SO_4), filtered and concentrated under reduced pressure and the ensuing light-brown oil subjected to flash chromatography (silica, 1:1 v/v ethyl acetate–hexane elution). Concentration of the appropriate fractions (R_f 0.5) gave the *bis-mesylate* **15** (45 mg, 74%) as a clear, light-yellow oil, $[\alpha]_D=+47$ (*c* 0.7, $CHCl_3$) (Found: $M^{+\cdot}$, 450.1381. $C_{19}H_{30}O_8S_2$ requires $M^{+\cdot}$, 450.1382). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.05 (dd, *J*=8.1, 6.3 Hz, 1H), 5.86 (d, *J*=8.1 Hz, 1H), 4.94 (d, *J*=6.0 Hz, 1H), 4.76 (dd, *J*=6.6, 0.9 Hz, 1H), 4.09 (dd, *J*=8.1, 3.9 Hz, 1H), 3.76 (d, *J*=8.1 Hz, 1H), 3.30 (ddd, *J*=11.1, 6.6, 2.1 Hz, 1H), 3.05 (s, 3H), 3.05–2.97 (m, partially obscured, 2H), 2.99 (s, 3H), 1.45 (s, 3H), 1.40 (s, 3H), 1.33 (s, 3H), 1.22 (s, 3H), 1.10 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 137.3, 130.6, 112.4, 91.9, 91.4, 81.4, 76.1, 50.0, 47.7, 41.9, 40.4, 39.9, 38.8, 36.7, 27.0, 25.4, 24.7, 20.4, 19.9; IR, ν_{max} 2980, 2936, 1373, 1334, 1207, 1170, 1063, 932, 894, 730 cm^{-1}; MS, *m/z* (EI, 70 eV) 450 ($M^{+\cdot}$, 1%), 435 (6), 421 (26), 392 (14), 350 (22), 296 (15), 200 (100), 171 (47), 100 (45).

3.2.4. (3a*R*,4*S*,4a*S*,5*R*,7*S*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol *bis*-*S*-methyl xanthate (16). A magnetically stirred mixture of diol **11** (14.4 mg, 0.05 mmol), NaH (5.8 mg of a 60% dispersion in mineral oil, 0.15 mmol) and imidazole (two crystals) in THF (1 mL) maintained at 18 °C was treated, after 1 h, with CS_2 (18 μL, 0.30 mmol). Stirring was continued for a further 1 h then methyl iodide (11 μL, 0.18 mmol) was added and the resulting mixture stirred overnight at 18 °C. The reaction mixture was quenched with acetic acid (glacial, 20 μL) then partitioned between ethyl acetate (10 mL) and water (10 mL) and the separated aqueous phase extracted with ethyl acetate (3×10 mL). The combined organic phases were washed with $NaHCO_3$ (1×5 mL of a saturated aqueous solution) then dried ($MgSO_4$), filtered and concentrated under reduced pressure. The ensuing yellow oil was subjected to flash chromatography (silica, 1:9 v/v ethyl acetate–hexane elution) and concentration of the appropriate fractions (R_f 0.9 in 1:4 v/v ethyl acetate–hexane) gave the title compound **16** (21.1 mg, 91%) as colourless crystals, mp 124–126 °C, $[\alpha]_D=+49$ (*c* 0.1, $CHCl_3$) (Found: $M^{+\cdot}$, 474.1024. $C_{21}H_{30}O_4S_4$ requires $M^{+\cdot}$, 474.1027). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.19 (d, *J*=6.3 Hz, 1H), 6.04–5.97 (complex m, 2H), 5.85 (d, *J*=8.1 Hz, 1H), 4.06 (dd, *J*=8.4, 4.2 Hz, 1H), 3.73 (d, *J*=8.4 Hz, 1H), 3.39 (ddd, *J*=11.1, 6.0, 2.1 Hz, 1H), 3.10 (dd, *J*=11.1, 6.0 Hz, 1H), 2.99–2.95 (m, 1H), 2.62 (s, 3H), 2.59 (s, 3H), 1.47 (s, 3H), 1.32 (s, 3H), 1.17 (s, 6H), 1.17 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.4, 215.4, 137.6, 130.3, 112.3, 93.2, 91.9, 81.3, 76.3, 51.0, 46.7, 42.6, 40.5, 36.2, 26.9, 25.2, 24.7, 20.4, 19.4, 19.2, 18.1; IR, ν_{max} 2966,

2931, 1369, 1220, 1205, 1184, 1058, 1034 cm^{-1}; MS, *m/z* (EI, 70 eV) 474 ($M^{+\cdot}$, 4%), 427 (10), 383 (16), 309 (10), 201 (43), 159 (64), 91 (100).

3.2.5. (3a*R*,4*S*,8*R*,8a*S*)-3a,6,8,8a-Tetrahydro-2,2,4,6,6-pentamethyl-4,8-etheno-6*H*-indeno[5,6-*d*]-1,3-dioxole (17). A chilled (ice-water bath) magnetically stirred solution of diol **11** (50.4 mg, 0.17 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (169 mg, 0.82 mmol) in dichloromethane (3 mL) was treated with triflic anhydride (64 μL, 0.38 mmol). The reaction mixture was then allowed to warm to 18 °C and after 48 h treated with triethylamine (1 mL) then water (10 mL) and dichloromethane (10 mL). The separated aqueous phase was extracted with dichloromethane (4×10 mL) and the combined organic phases washed with brine (1×5 mL) before being dried (Na_2SO_4), filtered and concentrated under reduced pressure. The light-brown residue thus obtained was subject to flash chromatography (silica, 0:1→1:19 v/v ethyl acetate–hexane gradient elution) and concentration of the appropriate fractions (R_f 0.9 in 1:1 v/v ethyl acetate–hexane elution) afforded the title *triene* **17** (18.6 mg, 42%) as a clear, colourless oil (Found: $M^{+\cdot}$, 258.1620. $C_{17}H_{22}O_2$ requires $M^{+\cdot}$, 258.1620). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.25 (dd, *J*=8.0, 6.3 Hz, 1H), 5.96 (dd, *J*=8.0, 1.0 Hz, 1H), 5.77 (broadened s, 1H), 5.68 (broadened s, 1H), 4.34 (dd, *J*=7.7, 4.0 Hz, 1H), 4.03 (d, *J*=7.7 Hz, 1H), 3.62–3.58 (m, 1H), 1.43 (s, 3H), 1.29 (4) (s, 3H), 1.28 (5) (s, 3H), 1.25 (s, 3H), 1.11 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 144.7, 141.0, 139.3, 135.0, 133.7, 132.7, 112.3, 82.0, 77.6, 55.5, 43.5, 40.1, 26.5, 24.8, 23.3, 23.2, 17.3; IR, ν_{max} 2978, 2931, 1740, 1701, 1461, 1381, 1266, 1207, 1064, 706 cm^{-1}; MS, *m/z* (EI, 70 eV) 258 ($M^{+\cdot}$, 6%), 243 (8), 200 (8), 171 (90), 158 (100), 156 (46), 143 (34), 141 (25).

3.2.6. (3a*R*,4*S*,4a*S*,5*R*,7*R*,7a*S*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole-5,7-diol *bis*-methanesulfonate (18). Reaction of diol **12** (26 mg, 0.09 mmol) with methanesulfonyl chloride (15 μL, 22 mg, 0.19 mmol, 2.2 mole equiv.) under the same conditions as employed during the conversion **11→15** (see above) afforded a light-brown oil on work-up. Subjection of this material to flash chromatography (silica, 1:4 v/v ethyl acetate–hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.6), the title *bis-mesylate* **18** (29 mg, 75%) as a clear, colourless oil, $[\alpha]_D$=+36 (*c* 0.3, $CHCl_3$) (Found: $M^{+\cdot}$, 450.1380. $C_{19}H_{30}O_8S_2$ requires $M^{+\cdot}$, 450.1382). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.27 (dd, *J*=8.4, 6.6 Hz, 1H), 5.78 (d, *J*=8.1 Hz, 1H), 4.73 (d, *J*=6.6 Hz, 1H), 4.65 (d, *J*=9.3 Hz, 1H), 4.08 (dd, *J*=8.1, 3.9 Hz, 1H), 3.79 (d, *J*=8.1 Hz, 1H), 3.27 (ddd, *J*=11.4, 6.9, 2.4 Hz, 1H), 3.06 (s, 3H), 3.00–2.95 (m, 1H), 2.99 (s, 3H), 2.71–2.64 (m, 1H), 1.48 (s, 3H), 1.33 (s, 3H), 1.32 (s, 3H), 1.18 (s, 3H), 1.08 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 135.7, 134.7, 112.4, 89.3, 88.3, 81.5, 76.0, 46.1, 45.8, 40.5, 39.8, 39.0, 38.9, 37.2, 26.8, 24.6, 21.1, 21.0, 20.6; IR, ν_{max} 2981, 2937, 1336, 1173, 1059, 933, 881, 860, 527 cm^{-1}; MS, *m/z* (EI, 70 eV) 450 ($M^{+\cdot}$, 2%), 435 (6), 421 (7), 392 (10), 200 (100), 185 (34), 171 (52), 158 (44), 100 (54).

3.2.7. (3a*R*,4*S*,7*R*,7a*S*,8*R*,9*S*)-3a,4,7,7a-Tetrahydro-8-(hydroxymethyl)-2,2,4-trimethyl-6-(2-methyl-1-propenyl)-4,7-ethano-1,3-benzodioxole (19). A magnetically stirred mixture of the *bis*-mesylate **18** (20 mg, 0.05 mmol) in THF (3 mL) maintained at 18 °C was treated with $LiEt_3BH$ (178 μL of a 1 M solution in THF, 0.18 mmol). The resulting mixture was heated at reflux for 4 h then cooled and quenched with NH_4Cl (5 mL of a saturated aqueous solution). The resulting mixture was extracted with ethyl acetate (4×10 mL) and the combined organic phases washed with brine (1×2 mL) then dried (Na_2SO_4), filtered and concentrated under reduced pressure. Subjection of the ensuing light-brown oil to flash chromatography (silica, 1:4 v/v ethyl acetate–hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.7 in 2:3 v/v ethyl acetate–hexane), the title *dienol* **19** (12.4 mg, 100%) as a clear, colourless oil, $[\alpha]_D$=+24 (*c* 0.4, $CHCl_3$) [Found: $(M-CH_3\cdot)^+$, 263.1640. $C_{17}H_{26}O_3$ requires $(M-CH_3\cdot)^+$, 263.1647]. ^1H NMR ($CDCl_3$, 300 MHz) δ 6.14 (t, *J*=7.7 Hz, 1H), 5.86 (d, *J*=8.1 Hz, 1H), 4.92 (dt, *J*=11.5, 1.2 Hz, 1H), 4.08 (dd, *J*=8.1, 3.6 Hz, 1H), 3.78 (d, *J*=8.1 Hz, 1H), 3.43 (d, *J*=11.1, 8.5 Hz, 1H), 3.24 (dd, *J*=11.1, 6.8 Hz, 1H), 3.10 (t, *J*=11.1 Hz, 1H), 2.76–2.60 (complex m, 2H), 1.71 (d, *J*=1.1 Hz, 3H), 1.71–1.63 (m, partially obscured, 1H), 1.67 (d, *J*=1.2 Hz, 3H), 1.55 (s, 3H), 1.34 (s, 3H), 1.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.7, 135.2, 132.1, 124.5, 112.0, 80.7, 76.2, 64.7, 42.6, 39.0, 38.5, 37.8, 26.6, 26.2, 24.8, 20.5, 18.5; IR, ν_{max} 3436, 2963, 2927, 1452, 1373, 1262, 1207, 1061, 1045, 875, 711 cm^{-1}; MS, *m/z* (EI, 70 eV) 278 ($M^{+\cdot}$, 1%), 263 (11), 220 (30), 147 (32), 112 (100), 94 (92), 79 (46), 69 (38), 56 (60).

3.2.8. (3a*R*,4*S*,4a*S*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-Octahydro-2,2,4-trimethyl-4,8-etheno-5*H*-indeno[5,6-*d*]-1,3-dioxol-5-one (22). A magnetically stirred solution of diol **21** (2.38 g, 11.5 mmol) and *p*-$TsOH\cdot H_2O$ (24.6 mg, 0.13 mmol) in dichloromethane (20 mL) maintained at 0 °C was treated with 2,2-dimethoxypropane (10 mL, 81.3 mmol). The resulting mixture was stirred at 0 °C for 3 h then warmed to 18 °C and maintained at this temperature for 72 h. The reaction mixture was then concentrated under reduced pressure and the ensuing deep-red residue subjected to flash chromatography (silica, 1:4→3:7 v/v ethyl acetate–hexane gradient elution). Concentration of the appropriate fractions (R_f 0.7 in 1:1 v/v ethyl acetate–hexane) afforded the title *acetonide* **22** (2.80 g, 98%) as a clear, colourless oil, $[\alpha]_D$=−122 (*c* 0.6, $CHCl_3$) (Found: $M^{+\cdot}$, 248.1412. C, 72.6; H, 8.1. $C_{15}H_{20}O_3$ requires $M^{+\cdot}$, 248.1412. C, 72.6; H, 8.1%). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.17 (dd, *J*=8.1, 6.6 Hz, 1H), 5.84 (dd, *J*=8.1, 0.9 Hz, 1H), 4.07 (dd, *J*=9.0, 3.9 Hz, 1H), 3.70 (d, *J*=8.1 Hz, 1H), 3.13–3.05 (m, 1H), 2.89–2.85 (m, 1H), 2.55 (d, *J*=9.0 Hz, 1H), 2.15–1.95 (complex m, 3H), 1.58–1.48 (complex m, 1H), 1.49 (s, 3H), 1.45 (s, 3H), 1.33 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 222.5, 138.5, 132.1, 112.2, 80.9, 76.2, 49.9, 42.3, 41.4, 39.7, 32.8, 26.7, 25.1, 24.8, 19.9; IR, ν_{max} 2987, 2962, 2933, 2904, 2880, 1731, 1458, 1381, 1372, 1261, 1207, 1165, 1067, 1050, 1034, 879, 715 cm^{-1}; MS, *m/z* (EI, 70 eV) 248 ($M^{+\cdot}$, 6%), 233 (20), 190 (47), 161 (58), 134 (68), 119 (56), 105 (100), 100 (95), 91 (76), 75 (75).

3.2.9. (3a*R*,4*S*,4a*S*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-5*H*-indeno[5,6-*d*]-1,3-dioxol-5-one (23). A magnetically stirred solution of

the ketone **22** (2.77 g, 11.2 mmol) in THF (20 mL) maintained at 0 °C was treated, dropwise over 5 min., with LiHMDS (11.7 mL of a 1 M solution in THF, 11.7 mmol). The resulting mixture was stirred at 0 °C for 0.75 h then warmed to 18 °C over 1.25 h. The reaction mixture was re-cooled to 0 °C then treated, dropwise, with MeI (0.73 mL, 11.73 mmol), allowed to stir for 0.75 h, then warmed to 18 °C over a period of 1.25 h. The reaction mixture was then re-cooled to 0 °C and treated with a further aliquot of LiHMDS (11.7 mL of a 1 M solution in THF, 11.7 mmol) then MeI (0.73 mL, 11.73 mmol) using the warming and cooling cycle mentioned above. This process was repeated twice more then the reaction mixture treated, at 18 °C, with NH_4Cl (20 mL of a saturated aqueous solution) and $Na_2S_2O_3$ (20 mL of a saturated aqueous solution). The separated aqueous phase was extracted with ethyl acetate (4×30 mL) and the combined organic phases were dried (Na_2SO_4), filtered and concentrated under reduced pressure to give a dark-yellow oil containing small amounts of lithium iodide. This material was subjected to flash chromatography (silica, 1:4→2:3 v/v ethyl acetate–hexane gradient elution) and concentration of the appropriate fractions (R_f 0.8 in 1:1 v/v ethyl acetate–hexane) afforded the title *ketone* **23** (3.08 g, 100%) as a white crystalline solid, mp 70–72 °C, $[\alpha]_D=-47$ (*c* 0.6, $CHCl_3$) (Found: $M^{+\cdot}$, 276.1724. C, 73.5. H, 8.5. $C_{17}H_{24}O_3$ requires $M^{+\cdot}$, 276.1725. C, 73.5. H, 8.5%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.00 (broad t, *J*=7.4 Hz, 1H), 5.88 (dd, *J*=8.1, 0.6 Hz, 1H), 4.13 (dd, *J*=8.1, 3.9 Hz, 1H), 3.68 (d, *J*=8.4 Hz, 1H), 3.08 (ddd, *J*=19.8, 9.3, 2.1 Hz, 1H), 2.88–2.78 (complex m, 2H), 1.85 (dd, *J*=12.9, 9.0 Hz, 1H), 1.55 (s, 3H), 1.50 (s, 3H), 1.34 (s, 3H), 1.25 (dd, *J*=12.9, 9.3 Hz, 1H), 1.01 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 223.9, 138.9, 132.0, 112.0, 81.8, 76.0, 47.1, 46.1, 41.6, 40.4, 40.2, 29.6, 26.8, 24.8, 22.5, 19.9 (one signal obscured or overlapping); IR, ν_{max} 2961, 2933, 2899, 1736, 1381, 1373, 1263, 1207, 1063, 1052, 881, 711 cm^{-1}; MS, *m/z* (EI, 70 eV) 276 ($M^{+\cdot}$, 4%), 261 (10), 218 (27), 176 (55), 134 (67), 105 (100), 91 (37), 75 (57).

3.2.10. (3a*R*,4*S*,4a*S*,5*S*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-5*H*-indeno[5,6-*d*]-1,3-dioxol-5-ol (24a) and (3a*R*,4*S*,4a*S*,5-*R*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-5*H*-indeno[5,6-*d*]-1,3-dioxol-5-ol (24b).

A magnetically stirred solution of lithium aluminium hydride (852 mg, 22.4 mmol) in THF (80 mL) maintained at 0 °C (ice bath) was treated, dropwise over 4 h, with a solution of ketone **23** (6.07 g, 22.0 mmol) in THF (60 mL). After a further 2 h at 0 °C the reaction mixture was heated at 50 °C for 18 h then cooled to 0 °C and treated with Na_2SO_4 (3 mL of a saturated aqueous solution, CAUTION!). The resulting grey-white precipitate was removed by filtration and washed with ethyl acetate (multiple small washings to a total volume of 250 mL). The combined filtrate was concentrated under reduced pressure to give a clear, colourless oil. Subjection of this material to flash chromatography (silica, 1:4 v/v ethyl acetate–hexane elution) afforded two fractions, A and B.

Concentration of fraction A (R_f 0.5 in 3:7 v/v ethyl acetate–hexane), afforded the β-epimeric form of the title *alcohol* **24a** (502 mg, 8%) as a white crystalline solid, mp 61–62 °C, $[\alpha]_D=+75$ (*c* 0.2, $CHCl_3$) [Found: $(M-CH_3\cdot)^+$, 263.1648.C, 73.0; H, 9.2. $C_{17}H_{26}O_3$ requires $(M-CH_3\cdot)^+$, 263.1647. C, 73.4; H, 9.4%]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.14–6.03 (complex m, 2H), 4.09 (dd, *J*=8.1, 3.9 Hz, 1H), 3.73 (d, *J*=8.1 Hz, 1H), 3.56 (dd, *J*=10.5, 6.0 Hz, 1H), 2.93–2.82 (m, 1H), 2.78–2.70 (m, partially obscured, 1H), 2.72 (dd, *J*=10.8, 6.0 Hz, 1H), 1.48 (s, 3H), 1.44–1.35 (m, partially obscured, 1H), 1.37 (s, 3H), 1.34 (s, 3H), 1.12 (d, *J*=10.8 Hz, 1H), 1.08 (t, *J*=11.7 Hz, 1H), 0.96 (s, 3H), 0.93 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 141.4, 131.6, 112.1, 83.0, 81.3, 47.1, 43.9, 41.6, 41.0, 39.0, 37.2, 26.8, 26.2, 24.9, 23.0, 20.6 (one signal overlapping or obscured); IR, ν_{max} 3500, 2935, 1464, 1380, 1371, 1261, 1207, 1081, 1056, 1028, 880 cm^{-1}; MS, *m/z* (EI, 70 eV) 279 [$(M+H)^+$, 2%], 263 (5), 249 (15), 220 (23), 178 (42), 106 (48), 105 (37), 93 (39), 75 (100).

Concentration of fraction B (R_f 0.4 in 3:7 v/v ethyl acetate–hexane), afforded the α-epimeric form of the title *alcohol* **24b** (5.54 g, 91%) as a white crystalline solid, mp 88–89 °C, $[\alpha]_D=+18$ (*c* 0.4, $CHCl_3$) [Found: $(M-CH_3\cdot)^+$, 263.1648. C, 73.4; H, 9.3. $C_{17}H_{26}O_3$ requires $(M-CH_3\cdot)^+$, 263.1647. C, 73.4; H, 9.3%]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.10 (broad t, *J*=7.2 Hz, 1H), 5.87 (dm, *J*=8.1 Hz, 1H), 4.11 (dd, *J*=8.1, 3.9 Hz, 1H), 3.77 (d, *J*=8.1 Hz, 1H), 3.25 (t, *J*=7.2 Hz, 1H), 2.83–2.72 (m, 1H), 2.67–2.62 (m, 1H), 2.17 (dd, *J*=10.5, 9.0 Hz, 1H), 1.51–1.44 (m, partially obscured, 1H), 1.48 (s, 3H), 1.33 (s, 3H), 1.32 (s, 3H), 1.24 (d, *J*=7.2 Hz, 1H), 0.95 (m, partially obscured, 1H), 0.93 (s, 3H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 139.1, 133.5, 111.9, 83.5, 81.2, 76.7, 47.9, 41.9, 41.6, 40.6, 39.7, 33.9, 26.7, 26.6, 24.8, 21.5, 20.6; IR, ν_{max} 3494, 2948, 2930, 1457, 1380, 1371, 1263, 1205, 1064, 1053, 1038, 877, 729, 711 cm^{-1}; MS, *m/z* (EI, 70 eV) 279 [$(M+H)^+$, 1%], 278 ($M^{+\cdot}$, <1), 263 (1), 220 (23), 187 (26), 178 (100), 106 (61), 105 (43), 91 (35).

3.2.11. (3a*R*,4*S*,4a*S*,7a*R*,8*R*,8a*S*)-3a,4a,5,6,7,7a,8,8a-Octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-4*H*-indeno[5,6-*d*]-1,3-dioxole (25).

3.2.11.1. Step (i). THF (10 mL) was cooled to 0 °C and treated with alcohol **24a** (407 mg, 1.46 mmol) and sodium hydride (298 mg of a 60% dispersion in mineral oil, 7.44 mmol). The resulting mixture was heated at reflux for 6 h then cooled to 18 °C and treated rapidly with carbon disulfide (880 μL, 14.6 mmol). After 11 h the reaction mixture was heated at reflux for 2 h then cooled to 18 °C again and treated with methyl iodide (1.00 mL, 16.08 mmol). After 2 h the reaction mixture was heated at reflux for 6 h then cooled to 18 °C. The reaction mixture was then quenched with acetic acid (0.5 mL). The resulting mixture was filtered through a short pad of Celite® and the solids thus retained washed with ethyl acetate (4×10 mL). The combined filtrates were washed with $NaHCO_3$ (2×10 mL of a saturated aqueous solution) then dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow oil. Subjection of this material to flash chromatography (silica, 0:4→1:4 v/v ethyl acetate–hexane gradient elution) and concentration of the appropriate fractions (R_f 0.4 in 1:9 v/v ethyl acetate–hexane) afforded *(3a*R*,4*S*,4a*S*,5*S*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-5*H*-indeno[5,6-*d*]-1,3-dioxol-5-ol* S-*methyl xanthate* (538 mg, 87%) as a clear, colourless

oil, $[\alpha]_D$=+19 (*c* 0.3, $CHCl_3$) (Found: $M^{+\cdot}$, 368.1487. C, 61.7; H, 7.9; S, 17.5. $C_{19}H_{28}O_3S_2$ requires $M^{+\cdot}$, 368.1480. C, 61.9; H, 7.7; S, 17.4%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.00–5.92 (complex m, 3H), 4.14 (dd, *J*=8.1, 3.9 Hz, 1H), 3.72 (d, *J*=8.1 Hz, 1H), 2.95–2.82 (complex m, 2H), 2.81–2.76 (m, 1H), 2.57 (s, 3H), 1.47 (s, 3H), 1.44–1.34 (complex m, 2H), 1.33 (s, 3H), 1.12 (s, 3H), 1.02 (s, 3H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.3, 140.6, 128.6, 112.0, 92.9, 81.4, 76.6, 45.9, 45.0, 42.4, 40.9, 38.4, 36.6, 26.8, 25.8, 24.7, 22.8, 20.5, 19.3; IR, ν_{max} 2962, 2932, 1456, 1380, 1369, 1261, 1233, 1224, 1185, 1050, 1029, 879, 713 cm^{-1}; MS, *m/z* (EI, 70 eV) 368 ($M^{+\cdot}$, 18%), 353 (12), 261 (54), 203 (95), 185 (50), 160 (42), 95 (100).

Reaction of alcohol **24b** under the same conditions as described above for congener **24a** afforded a yellow oil on work-up. Subjection of this material to flash chromatography (silica, 0:4→1:4 v/v ethyl acetate–hexane gradient elution) and concentration of the appropriate fractions (R_f 0.4 in 1:9 v/v ethyl acetate–hexane) afforded *(3a*R*,4*S*,4a*S*,5*R*,7a*S*,8*R*,8a*S*)-3a,4,4a,6,7,7a,8,8a-octahydro-2,2,4,6,6-pentamethyl-4,8-etheno-5*H*-indeno-[5,6-*d*]-1,3-dioxol-5-ol* S-*methyl xanthate* (100%) as a clear, colourless oil, $[\alpha]_D$=+77 (*c* 0.2, $CHCl_3$) (Found: $M^{+\cdot}$, 368.1484. C, 61.3; H, 7.9; S, 17.3. $C_{19}H_{28}O_3S_2$ requires $M^{+\cdot}$, 368.1480. C, 61.9; H, 7.7; S, 17.4%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.17 (broad t, *J*=7.5 Hz, 1H), 5.94 (d, *J*=7.5 Hz, 1H), 5.66 (d, *J*=8.7 Hz, 1H), 4.11 (dd, *J*=8.1, 3.9 Hz, 1H), 3.78 (d, *J*=8.4 Hz, 1H), 2.97–2.86 (m, 1H), 2.71–2.64 (m, 2H), 2.56 (s, 3H), 1.58–1.52 (m, partially obscured, 1H), 1.52 (s, 3H), 1.33 (s, 3H), 1.17 (s, 3H), 1.00 (s, 3H), 0.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.1, 139.1, 133.5, 112.0, 91.9, 81.2, 76.4, 45.2, 42.6, 42.1, 41.2, 39.7, 33.7, 27.0, 26.8, 24.7, 23.0, 20.3, 19.2; IR, ν_{max} 2957, 2932, 1460, 1380, 1370, 1258, 1224, 1206, 1054, 1027, 878, 714 cm^{-1}; MS, *m/z* (EI, 70 eV) 368 ($M^{+\cdot}$, <1%), 353 (11), 260 (71), 231 (35), 203 (72), 202 (62), 187 (65), 160 (71), 95 (100).

3.2.11.2. Step (ii). A magnetically stirred solution of the relevant xanthate (538 mg, 1.46 mmol), formed as described above, and AIBN (3.4 mg, 0.02 mmol) in toluene (20 mL) was treated with tri-*n*-butyltin hydride (1.20 mL, 4.46 mmol) and the resulting mixture heated at 100 °C for 17 h. The cooled reaction mixture was treated with additional tri-*n*-butyltin hydride (0.80 mL, 2.97 mmol) and AIBN (5.0 mg) and the resulting mixture heated at reflux for 1 h. The cooled reaction mixture was then concentrated under reduced pressure and the residue subjected to flash chromatography (silica, 0:100→5:95 v/v ethyl acetate–hexane gradient elution). Concentration of the relevant fractions (R_f 0.7 in 3:7 v/v ethyl acetate–hexane) afforded a solid. Recrystallization (ethyl acetate) of this material afforded the title *acetonide* **25** (218 mg, 57%) as a white, crystalline solid, mp 65–66 °C, $[\alpha]_D$=+39 (*c* 0.4, $CHCl_3$) [Found: $(M-CH_3\cdot)^+$, 247.1697. C, 77.5; H, 10.0. $C_{17}H_{26}O_2$ requires $(M-CH_3\cdot)^+$, 247.1698. C, 77.8; H, 10.0%]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.07 (broad t, *J*=7.2 Hz, 1H), 5.79 (dt, *J*=8.4, 0.9 Hz, 1H), 4.13 (dd, *J*=8.4, 3.9 Hz, 1H), 3.79 (d, *J*=8.4 Hz, 1H), 2.85–2.66 (complex m, 2H), 2.57–2.48 (m, 1H), 1.50 (s, 3H), 1.44–1.32 (complex m, partially obscured, 2H), 1.34 (s, 3H), 1.15 (s, 3H), 1.01–0.93 (m, partially obscured, 2H), 0.96 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 139.0, 132.9, 111.8, 81.0, 76.9, 45.3, 44.1, 42.1, 41.0, 39.7, 38.5, 36.7, 29.0, 28.1, 26.7, 24.9, 20.7; IR, ν_{max} 2951, 2934, 1457, 1380, 1370, 1262, 1206, 1064, 1053, 881, 714 cm^{-1}; MS, *m/z* (EI, 70 eV) 247 [$(M-CH_3\cdot)^+$, 10%], 204 (58), 162 (100), 105 (36), 91 (42).

3.2.12. (3a*S*,4*S*,7*R*,7a*R*,8*S*,9*R*)-2,3,3a,4,7,7a-Hexahydro-2,2,4-trimethyl-4,7-ethano-1*H*-indene-8,9-diol (26). A magnetically stirred solution of acetonide **25** (1.37 g, 5.2 mmol) in acetic acid (20 mL of a 60% v/v solution in water)–THF (5 mL) was heated at 60 °C for 48 h. The cooled reaction mixture was treated with $NaHCO_3$ (18 g, 214 mmol) and water (20 mL). After carbon dioxide evolution had ceased the separated aqueous phase was extracted with ethyl acetate (5×50 mL) and the combined organic phases then dried ($MgSO_4$), filtered and concentrated under reduced pressure. Subjection of the ensuing light-yellow oil to flash chromatography (silica, 5:95→30:70 v/v ethyl acetate–hexane gradient elution) afforded two fractions, A and B.

Concentration of fraction A (R_f 0.7 in 3:7 v/v ethyl acetate–hexane) afforded the starting acetonide **25** (772 mg, 56% recovery) which proved identical, in all respects, with authentic material.

Concentration of fraction B (R_f 0.3 in 3:7 v/v ethyl acetate–hexane) afforded a light-yellow solid. Recrystallization (ethyl acetate) of this material afforded the title *diol* **26** (483 mg, 95% at 44% conversion) as a white crystalline solid, mp 91–92 °C, $[\alpha]_D$=+56 (*c* 0.4, $CHCl_3$) [Found: $(M-C_2H_4O_2)^{+\cdot}$, 162.1410. C, 75.4; H, 9.8. $C_{14}H_{22}O_2$ requires $(M-C_2H_4O_2)^{+\cdot}$, 162.1409, C, 75.6; H, 10.0%]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.06 (broad t, *J*=7.8 Hz, 1H), 5.77 (broad d, *J*=8.4 Hz, 1H), 3.76–3.70 (m, 1H), 3.34 (dd, *J*=8.7, 5.4 Hz, 1H), 2.97 (d, *J*=5.1 Hz, 1H), 2.79 (d, *J*=5.7 Hz, 1H), 2.72–2.57 (complex m, 2H), 2.46–2.36 (m, 1H), 1.45–1.33 (complex m, 2H), 1.14 (s, 3H), 1.05–0.94 (m, partially obscured, 2H), 0.96 (s, 3H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 138.7, 132.5, 70.2, 66.5, 45.6, 44.2, 42.7, 41.9, 40.5, 38.6, 36.1, 29.0, 28.1, 20.4; IR, ν_{max} 3344, 2949, 2930, 1456, 1365, 1053, 1012, 711 cm^{-1}; MS, *m/z* (EI, 70 eV) 162 [$(M-C_2H_4O_2)^{+\cdot}$, 100], 147 (40), 106 (35), 91 (33).

3.2.13. (3a*S*,4*S*,7*R*,7a*R*,9*R*)-2,3,3a,4,7,7a-Hexahydro-9-hydroxy-2,2,4-trimethyl-4,7-ethano-1*H*-indene-8-one (27). A magnetically stirred suspension of diol **26** (457 mg, 2.06 mmol) and *p*-TsOH·H_2O (822 mg, 4.32 mmol) in dichloromethane (40 mL) was cooled to 0 °C and 4-acetamido-TEMPO (922 mg, 4.32 mmol) was added in portions over 1 h. The resulting pale-orange mixture was stirred at 0 °C for 6 h then warmed to 18 °C and stirred at this temperature for 16 h. After this time the reaction mixture was treated with $NaHCO_3$ (20 mL of a saturated aqueous solution) and extracted with dichloromethane (4×20 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated under reduced pressure. Subjection of the ensuing orange oil to flash chromatography (silica, 0:1→4:6 v/v ethyl acetate–hexane gradient elution) afforded two fractions, A and B.

Concentration of fraction A (R_f 0.4 in 3:7 v/v ethyl acetate–hexane) afforded the title *acyloin* **27** (395 mg, 91% at 96%

conversion) as a clear, colourless oil, $[\alpha]_D=-34$ (*c* 0.4, $CHCl_3$) ($M^{+\cdot}$, 220.1464. C, 76.1; H, 9.0. $C_{14}H_{20}O_2$ requires $M^{+\cdot}$, 220.1463. C, 76.3; H, 9.2%). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.14–6.05 (complex m, 1H), 3.39 (d, *J*=1.8 Hz, 1H), 3.10–3.07 (m, 1H), 2.70–2.50 (complex m, 3H), 1.68–1.67 (broad m, 1H), 1.54–1.45 (complex m, 2H), 1.25 (s, 3H), 1.17 (dd, *J*=11.7, 9.9 Hz, 1H), 1.10 (dd, *J*=12.6, 10.5 Hz, 1H), 0.99 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 213.7, 140.3, 127.8, 74.7, 51.3, 45.9, 45.5, 44.8, 44.2, 41.3, 39.9, 28.8, 27.9, 19.1; IR, ν_{max} 3442, 2952, 2931, 1734, 1722, 1456, 1366, 1074, 788, 767, 707 cm^{-1}; MS, *m/z* (EI, 70 eV) 220 ($M^{+\cdot}$, 100%), 205 (43), 163 (64), 161 (70), 105 (47), 91 (61).

Concentration of fraction B (R_f 0.3 in 3:7 v/v ethyl acetate–hexane) afforded the starting diol **26** (18 mg, 4% recovery) which was identical, in all respects, with authentic material.

3.2.14. (3a*S*,4*S*,7*R*,7a*R*,9*R*)-2,3,4,4a,7,7a-Hexahydro-9-[(2-methoxyethoxy)methoxy]-2,2,4-trimethyl-4,7-ethano-1*H*-indene-8-one (28). A solution of acyloin **27** (347 mg, 1.57 mmol) and Hünig's base (690 μL, 3.96 mmol) in dichloromethane (3.5 mL) maintained at 18 °C was treated in a dropwise fashion with MEM-Cl (360 μL, 3.15 mmol) and the resulting mixture stirred at 18 °C for 16 h then treated with $NaHCO_3$ (2 mL of a saturated aqueous solution). The separated aqueous phase was extracted with dichloromethane (5×10 mL) and the combined organic phases then washed with water (1×50 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. Subjection of the ensuing light-brown oil to flash chromatography (silica, 0:99:1→20:79:1 v/v/v ethyl acetate–hexane–triethylamine gradient elution) afforded, after concentration of the appropriate fractions (R_f 0.4 in 3:7 v/v ethyl acetate–hexane), the title *ether* **28** (444 mg, 91%) as a clear, colourless oil, $[\alpha]_D=+28$ (*c* 0.4, $CHCl_3$) (Found: $M^{+\cdot}$, 308.1986. C, 69.8; H, 8.8. $C_{18}H_{28}O_4$ requires $M^{+\cdot}$, 308.1988. C, 70.1; H, 9.2%). ^1H NMR ($CDCl_3$, 300 MHz) δ 6.10 (dd, *J*=8.4, 6.0 Hz, 1H), 6.03 (broad d, *J*=8.1 Hz, 1H), 5.11 (d, *J*=6.8 Hz, 1H), 4.81 (d, *J*=6.8 Hz, 1H), 3.88–3.75 (m, 2H), 3.59–3.56 (m, 2H), 3.46 (s, 1H), 3.39 (s, 3H), 2.98 (dm, *J*=6 Hz, 1H), 2.71–2.58 (m, 2H), 1.54–1.43 (m, 2H), 1.21 (s, 3H), 1.16–1.08 (m, 1H), 1.04–0.97 (m, partially obscured, 1H), 0.99 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 210.0, 140.1, 128.4, 96.4, 76.9, 72.0, 67.7, 59.4, 52.5, 45.5, 44.9, 44.2, 43.4, 42.1, 39.6, 28.8, 27.9, 19.6; IR, ν_{max} 2951, 2931, 1736, 1457, 1366, 1110, 1036, 708 cm^{-1}; MS, *m/z* (EI, 70 eV) 308 ($M^{+\cdot}$, 6%), 279 (5), 175 (47), 108 (50), 89 (100), 59 (93).

3.2.15. (1*R*,2a*S*,2b*S*,2c*S*,5a*S*,5b*R*,5c*R*)-Decahydro-1-[(2-methoxyethoxy)methoxy]-4,4,5d-trimethyl-2*H*-cyclopenta[*a*]cyclopropa[*cd*]pentalen-2-one (29). A deoxygenated solution of compound **28** (254 mg, 0.82 mmol) and acetophenone (240 μL, 2.06 mmol) in acetone (120 mL) and contained in a Pyrex™ vessel jacketed by a water-cooled solution of sodium bromide (750 g) and lead(II) nitrate (8 g) in water (1 L) was subject to irradiation from a Philips 125 W HPL-N lamp for 32 h. The reaction mixture was then concentrated under reduced pressure and the resulting clear, colourless oil subject to flash chromatography (silica, 0:1→3:7 v/v ethyl acetate–hexane gradient elution) and thereby yielding two major fractions, A and B.

Concentration of fraction A (R_f 0.2 in 3:7 v/v ethyl acetate–hexane) afforded a white crystalline solid which was recrystallized (ethyl acetate) thus affording *cyclopropane* **29** (145 mg, 80% at 71% conversion) as a white crystalline solid, mp 78–79 °C, $[\alpha]_D=+102$ (*c* 0.2, $CHCl_3$) (Found: $M^{+\cdot}$, 308.1988. C, 70.1; H, 8.9. $C_{18}H_{28}O_4$ requires $M^{+\cdot}$, 308.1988. C, 70.1; H, 9.2%). ^1H NMR ($CDCl_3$, 300 MHz) δ 4.99 (d, *J*=6.9 Hz, 1H), 4.81 (d, *J*=6.9 Hz, 1H), 3.88-3.74 (complex m, 3H), 3.58-3.55 (m, 2H), 3.39 (s, 3H), 2.68 (dt, *J*=12.0 and 7.0 Hz, 1H), 2.38-2.31 (m, 1H), 2.10 (t, *J*=5.4 Hz, 1H), 1.90-1.78 (complex m, 3H), 1.64 (dd, *J*=10.3, 5.6 Hz, 1H), 1.45 (t, *J*=11.9 Hz, 1H), 1.36-1.22 (complex m, partially obscured, 1H), 1.34 (s, 3H), 1.08 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 210.8, 95.6, 88.1, 72.0, 67.5, 59.4, 53.4, 49.9, 48.9, 43.5, 43.3, 40.8, 36.0, 33.7, 32.1, 29.8, 27.7, 21.4; IR, ν_{max} 2971, 2956, 2933, 2869, 1731, 1110, 1055, 1010 cm^{-1}; MS, (EI, 70eV) *m/z* 308 ($M^{+\cdot}$, 2%), 279 (9), 219 (53), 108 (70), 89 (88), 59 (100).

Concentration of fraction B (R_f 0.4 in 3:7 v/v ethyl acetate–hexane) afforded the starting ether **28** (73 mg, 29% recovery) which proved identical, in all respects, with authentic material.

3.2.16. (3*R*,3a*S*,3b*S*,6a*R*,7a*R*)-Decahydro-3-[(2-methoxyethoxy)methoxy]-3a,5,5-trimethyl-2*H*-cyclopenta[*a*]pentalen-2-one (30). A magnetically stirred solution of compound **29** (145 mg, 0.47 mmol) and AIBN (3.4 mg, 0.021 mmol) in benzene (15 mL) maintained at 18 °C was treated, dropwise, with tri-*n*-butyltin hydride (254 μL, 0.94 mmol) and the resulting mixture allowed to stand for 1 h, then heated at reflux for the same period. The cooled reaction mixture was treated with further aliquots of AIBN (5.9 mg, 0.036 mmol) and tri-*n*-butyltin hydride, then heated at reflux for a further 2 h. This process was repeated twice more and such that the total heating time was 8 h. The cooled reaction mixture was then concentrated under reduced pressure and the ensuing light-yellow oil subject to flash chromatography (silica, 0:1→3:7 v/v ethyl acetate–hexane elution) thus affording two fractions, A and B.

Concentration of fraction A (R_f 0.3 in 3:7 v/v ethyl acetate–hexane) afforded the title *triquinane* **30** (104 mg, 87% at 81% conversion) as a clear, colourless oil, $[\alpha]_D=+20$ (*c* 0.4, $CHCl_3$) (Found: $M^{+\cdot}$, 310.2141. C, 69.6; H, 9.7. $C_{18}H_{30}O_4$ requires $M^{+\cdot}$, 310.2144. C, 69.6; H, 9.7). ^1H NMR ($CDCl_3$, 300 MHz) δ 4.95 (d, *J*=6.9 Hz, 1H), 4.80 (d, *J*=6.9 Hz, 1H), 4.05 (d, *J*=1.8 Hz, 1H), 3.80–3.76 (m, 2H), 3.57–3.53 (m, 2H), 3.38 (s, 3H), 2.78–2.62 (m, 1H), 2.54–2.37 (complex m, 3H), 1.88 (dd, *J*=17.7, 5.4 Hz, 1H), 1.78–1.70 (m, partially obscured, 2H), 1.66–1.50 (complex m, 1H), 1.40–1.24 (complex m, 2H), 1.16 (s, 3H), 1.07–1.00 (m, partially obscured, 1H), 1.04 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.6, 95.6, 88.4, 72.0, 67.3, 59.3, 50.6, 49.2, 47.1, 46.6, 44.8, 43.9, 42.2, 39.4, 37.1, 29.6, 27.4, 22.9; IR, ν_{max} 2949, 2867, 1752, 1466, 1134, 1111, 1098, 1044 cm^{-1}; MS, *m/z* (EI, 70 eV) 310 ($M^{+\cdot}$, 1%), 221 (35), 161 (35), 149 (25), 109 (29), 96 (80), 89 (84), 59 (100).

Concentration of fraction B (R_f 0.2 in 3:7 v/v ethyl acetate–hexane) afforded the starting cyclopropane **29** (27 mg, 19% recovery) which was identical, in all respects, with authentic material.

3.2.17. (3a*S*,3b*S*,4*S*,6a*S*,7a*R*)-Decahydro-4-[(2-methoxyethoxy)methoxy]-2,2,3b-trimethyl-1*H*-cyclopenta[*a*]pentalene (31)

3.2.17.1. Step (i). A magnetically stirred solution of ketone **30** (100 mg, 0.33 mmol) in methanol (15 mL) maintained at 18 °C was treated with $NaBH_4$ (28 mg, 0.73 mmol). After 4 h the reaction mixture was diluted with ethyl acetate (2 mL) then treated with water (15 mL). The separated aqueous phase was extracted with ethyl acetate (5×15 mL) and the combined organic phases were then dried (Na_2SO_4), filtered and concentrated under reduced pressure. Subjection of the ensuing clear, colourless oil to flash chromatography (silica, 1:9 v/v ethyl acetate-hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.2 in 3:7 v/v ethyl acetate–hexane), *(2S,3*R*,3aS,3bS,6a*R*,7a*R*)-decahydro-3-[(2-methoxyethoxy)-methoxy]-3a,5,5-trimethyl-2*H*-cyclopenta[a]pentalen-2-ol* (99.6 mg, 98%) as a clear, colourless oil, $[\alpha]_D$=+12 (*c* 0.6, $CHCl_3$) (Found: $M^{+\cdot}$, 312.2298. C, 69.0; H, 10.1. $C_{18}H_{32}O_4$ requires $M^{+\cdot}$, 312.2301 C, 69.2; 10.3%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.87 (d, *J*=6.9 Hz, 1H), 4.77 (d, *J*=6.9 Hz, 1H), 4.16–4.08 (m, 1H), 3.88–3.81 (complex m, 1H), 3.78–3.71 (complex m, 1H), 3.59–3.52 (complex m, 3H), 3.40 (s, 3H), 3.01 (d, *J*=5.7 Hz, 1H), 2.82–2.66 (complex m, 2H), 2.18–2.08 (m, 1H), 2.01–1.92 (m, 1H), 1.69–1.58 (m, partially obscured, 1H), 1.52–1.04 (complex m, partially obscured, 6H), 1.06 (s, 3H), 1.01 (s, 3H), 0.94 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 97.4, 91.3, 72.9, 72.0, 68.1, 59.4, 53.4, 50.0, 48.4, 47.7, 44.3, 42.8, 42.3, 39.9, 39.2, 30.9, 29.1, 24.9; IR, ν_{max} 3479, 2930, 2865, 1462, 1364, 1170, 1097, 1036, 849 cm^{-1}; MS, *m/z* (EI, 70 eV) 313 [(M+H)$^+$, 1%], 312 ($M^{+\cdot}$, <1), 236 (24), 206 (22), 179 (39), 161 (55), 149 (53), 89 (78), 59 (100).

3.2.17.2. Step (ii). Following the same protocol as employed for the conversion of compound **24** into the corresponding xanthate ester, the abovementioned alcohol was converted into *(2S,3*R*,3aS,3bS,6a*R*,7a*R*)-decahydro-3-[(2-methoxyethoxy)methoxy]-3a,5,5-trimethyl-2*H*-cyclopenta[a]pentalen-2-ol* S-*methyl xanthate* (92%) which was obtained as a clear, colourless oil, $[\alpha]_D$=+45 (*c* 0.4, $CHCl_3$) [Found: $(M-HS\cdot)^+$, 369.2100. C, 59.8; H, 8.7; S, 15.6. $C_{20}H_{34}O_4S_2$ requires $(M-HS\cdot)^+$, 369.2100. C, 59.7; 8.5; S, 15.9%]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.87–5.82 (m, 1H), 4.73 (dd, *J*=11.4, 6.9 Hz, 2H), 3.83 (d, *J*=5.1 Hz, 1H), 3.80–3.65 (complex m, 2H), 3.53 (t, *J*=5.1 Hz, 2H), 3.37 (s, 3H), 3.01–2.91 (m, 1H), 2.79–2.65 (m, 1H), 2.56 (s, 3H), 2.38–2.28 (m, 1H), 2.09–2.02 (m, 1H), 1.72–1.58 (complex m, 3H), 1.55–1.45 (m, 1H), 1.37–1.25 (m, 2H), 1.15–1.10 (m, 1H), 1.08 (s, 3H), 1.06 (s, 3H), 0.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 215.4, 96.2, 86.7, 84.3, 72.0, 67.6, 59.3, 53.4, 49.7, 47.7, 44.2, 43.0, 42.8, 40.4, 36.0, 30.6, 28.7, 24.5, 19.3 (one signal obscured or overlapping); IR, ν_{max} 2929, 1730, 1464, 1220, 1062 cm^{-1}; MS, *m/z* (EI, 70 eV) 369 [$(M-HS\cdot)^+$, 7%], 355 (5), 190 (8), 149 (12), 105 (10), 89 (100), 59 (95).

3.2.17.3. Step (iii). Following the same protocol as employed for the conversion of compound **24** into the corresponding hydrocarbon, the abovementioned xanthate was converted into compound **31** (92%) which was obtained as a clear, colourless oil, $[\alpha]_D$=+32 (*c* 0.4, $CHCl_3$) (Found: $M^{+\cdot}$, 296.2337. C, 72.8; H, 11.3. $C_{18}H_{32}O_3$ requires $M^{+\cdot}$, 296.2351. C, 72.9; 10.9%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.75 (d, *J*=6.9 Hz, 1H), 4.70 (d, *J*=6.9 Hz, 1H), 3.72–3.65 (complex m, 3H), 3.57–3.54 (complex m, 2H), 3.40 (s, 3H), 2.69 (q, *J*=9.6 Hz, 1H), 2.62–2.48 (m, 1H), 2.07–2.00 (m, 1H), 1.98–1.86 (m, 1H), 1.76–1.56 (complex m, 4H), 1.49–1.25 (complex m, 4H), 1.10–1.03 (m, partially obscured, 1H), 1.05 (s, 3H), 0.99 (s, 3H), 0.94 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 95.3, 88.1, 72.1, 66.9, 59.3, 54.2, 51.7, 48.5, 47.9, 44.8, 43.5, 42.5, 40.9, 31.2, 30.5, 28.5, 28.3, 23.8; IR, ν_{max} 2949, 2869, 1464, 1364, 1136, 1111, 1045 cm^{-1}; MS, *m/z* (EI, 70 eV) 296 ($M^{+\cdot}$, <1%), 220 (60), 207 (50), 190 (44), 189 (49), 163 (37), 107 (46), 89 (100).

3.2.18. (3*S*,3a*S*,3b*S*,6a*R*,7a*S*)-Decahydro-3a,5,5-trimethyl-1*H*-cyclopenta[*a*]pentalen-3-ol (32).

A magnetically stirred solution of the MEM-ether **31** (9.5 mg, 0.032 mmol) and PPTS (17 mg, 0.068 mmol) in *t*-butanol (2 mL) was heated at reflux for 4 h. TLC analysis after this time indicated that starting material remained so additional PPTS (4.1 mg, 0.016 mmol) was added and the reaction mixture heated at reflux for a further 4 h. The cooled reaction mixture was then concentrated under reduced pressure. Subjection of the ensuing light-brown oil to flash chromatography (silica, 0:1→1:4 v/v ethyl acetate–hexane elution) afforded, after concentration of the appropriate fractions (R_f 0.2 in 1:9 v/v ethyl acetate–hexane), the title *alcohol* **32** (5.1 mg, 76%) as a white, crystalline solid, mp 44–46 °C, $[\alpha]_D$=+36 (*c* 0.1, $CHCl_3$) (Found: $M^{+\cdot}$, 208.1830. C, 80.7; H, 11.4. $C_{14}H_{24}O$ requires $M^{+\cdot}$, 208.1827. C, 80.7; H, 11.6%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.78 (t, *J*=6.6 Hz, 1H), 2.66–2.51 (complex m, 2H), 2.12–2.04 (m, 1H), 2.01–1.91 (complex m, 1H), 1.80–1.25 (complex m, 9H), 1.12–1.05 (m, partially obscured, 1H), 1.06 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 83.2, 55.0, 51.7, 48.5, 47.6, 44.9, 43.6, 42.5, 40.9, 33.8, 30.4, 28.3, 28.2, 23.3; IR, ν_{max} 3376, 2949, 2932, 2864, 1464, 1364, 1075 cm^{-1}; MS, *m/z* (EI, 70 eV) 208 ($M^{+\cdot}$, 50%), 190 (14), 149 (100), 123 (30), 107 (54), 93 (38).

3.2.19. (3a*S*,3b*S*,6a*R*,7a*S*)-Decahydro-3a,5,5-trimethyl-3*H*-cyclopenta[*a*]pentalen-3-one (33).

A magnetically stirred solution of alcohol **32** (33.2 mg, 0.159 mmol) in dichloromethane (10 mL) was treated with PCC (68.7 mg, 0.32 mmol). The resulting orange-yellow mixture was stirred at 18 °C for 16 h by which time it had turned red-brown in color. The solvent was removed under a stream of nitrogen and the residue subject to flash chromatography (silica, 5:95 v/v ethyl acetate–pentane elution). Concentration of the appropriate fractions (R_f 0.5) afforded the title ketone **33**[7] (23 mg, 71%) as a white crystalline solid, mp 23–24 °C, $[\alpha]_D$=−56 (*c* 0.4, $CHCl_3$) (Found: $M^{+\cdot}$, 206.1671. C, 81.7; H, 11.1. $C_{14}H_{22}O$ requires $M^{+\cdot}$, 206.1671. C, 81.5; H, 10.8%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.84–2.75 (m, 1H), 2.58–2.45 (m, 1H), 2.44–2.21 (complex m, 3H), 2.06–1.93 (complex m, 1H), 1.77–1.55 (complex m, 3H), 1.48–1.34 (complex m, 2H), 1.17 (t, *J*=11.1 Hz, 1H), 1.04 (s, 3H), 1.04–0.94 (m, partially obscured, 1H), 0.94 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 224.8, 59.7, 49.2 (0), 49.1 (8), 47.0,

43.7, 42.2, 41.5, 37.9, 34.6, 29.6, 26.9, 22.7, 17.7; IR, ν_{max} 2933, 2866, 1739, 1464, 1365, 1008 cm^{-1}; MS, *m/z* (EI, 70 eV) 206 ($M^{+\cdot}$, 100%), 191 (10), 162 (46), 150 (39), 149 (36), 107 (56).

3.2.20. (3a*S*,3b*S*,6a*S*,7a*R*)-Decahydro-2,2,3b-trimethyl-4-methylene-1*H*-cyclopenta[*a*]pentalene [(−)-hirsutene, (−)-1]. A magnetically stirred solution of methyl triphenylphosphonium bromide (68.9 mg, 0.19 mmol) in freshly distilled toluene (10 mL) maintained at 0 °C was treated, dropwise, with KHMDS (297 μL of a 15% w/v solution in toluene, 0.197 mmol). The resulting intensely yellow-colored solution was stirred at 0 °C for 1 h then brought to 18 °C over 1 h and immediately re-cooled to 0 °C. A degassed solution of ketone **33** (19.8 mg, 0.096 mmol) in toluene (5 mL) was added, dropwise, to the reaction mixture which was then heated at reflux for 1.5 h. The solvent was removed from the cooled reaction mixture under a stream of nitrogen. Subjection of the ensuing light-yellow oil to flash chromatography (silica, pentane elution) afforded two fractions, A and B.

Concentration of fraction A (R_f 0.6) afforded (−)-hirsutene (−)-**1**] (6.3 mg, 100% at 32% conversion) as a clear, colourless oil, $[\alpha]_D$=−26 (*c* 0.2, $CDCl_3$) (Found: $M^{+\cdot}$, 204.1876. $C_{15}H_{24}$ requires $M^{+\cdot}$, 204.1878). ^1H NMR ($CDCl_3$, 600 MHz) δ 4.83–4.81 (m, 1H), 4.78–4.76 (m, 1H), 2.63–2.58 (m, 1H), 2.53–2.47 (m, 1H), 2.48–2.44 (complex m, 2H), 2.17–2.13 (m, 1H), 1.76–1.70 (m, 1H), 1.63 (ddd, *J*=10.2, 8.4, 1.8 Hz, 1H), 1.48–1.43 (m, 1H), 1.43–1.40 (m, 2H), 1.25 (broad s, 1H), 1.20 (t, *J*=11.4 Hz, 1H), 1.05 (s, 3H), 1.02 (dd, *J*=12.6, 7.8 Hz, 1H), 0.94 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 163.0, 103.8, 56.3, 53.8, 50.3, 49.3, 44.6, 42.2, 41.3, 39.0, 31.3, 30.1, 27.6, 27.2, 23.6; IR, ν_{max} 2931, 2865, 1649, 1464, 1364, 876 cm^{-1}; MS, *m/z* (EI, 70 eV) 204 ($M^{+\cdot}$, 9%), 189 (3), 94 (100), 79 (34).

Concentration of fraction B (R_f 0.1) afforded ketone **33** (13.5 mg, 68% recovery) which was identical, in all respects, with authentic material.

3.3. Crystallographic studies

3.3.1. Crystal data for 11. $C_{17}H_{26}O_4$, *M*=294.391, *T*=200(1) K, monoclinic, space group $P2_1$, *Z*=2, *a*=9.2853(2), *b*=8.2538(2), *c*=10.7776(3) Å, β=107.9399(10)°, *V*=785.83(3) $Å^3$, *Dx*=1.244 Mgm^{-3}, 1926 unique data ($2\Theta_{max}$=55.06°), 1608 with $I \geq 3.00\sigma(I)$; *R*=0.032, *Rw*=0.030, *S*=1.074.

Images were measured on a Nonius Kappa CCD diffractometer (Mo Kα, graphite monochromator, λ=0.71073 Å) and data extracted using the DENZO package.[26] Structure solution was by direct methods (SIR97)[27] and refinement was by full matrix least-squares on F using the CRYSTALS program package.[28] Atomic coordinates, bond lengths and angles, and displacement parameters have been deposited at the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 213833. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax:+44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

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Dioxygenase-catalysed sulfoxidation of bicyclic alkylaryl sulfides and chemoenzymatic synthesis of acyclic disulfoxides

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Abstract—Toluene- and naphthalene-dioxygenase-catalysed oxidation of six bicyclic disulfide substrates, using whole cells of *Pseudomonas putida*, gave the corresponding monosulfoxides with high ee values and enantiocomplementarity, in most cases. Two alcohol-sulfoxide diastereoisomers, formed from the reaction of the (*R*)-1,3-benzodithiole-1-oxide metabolite with *n*-butyllithium and benzaldehyde, were separated and stereochemically assigned. Treatment, of enantiopure (1*R*,3*R*)-benzo-1,3-dithiole-1,3-dioxide, obtained by chemoenzymatic synthesis, with alkyllithium reagents, resulted in a novel ring-opening reaction which proceeded with inversion of configuration to yield a series of acyclic disulfoxides.

1. Introduction

The potential of microbial enzymes in the biocatalytic oxidation of sulfides, to yield enantiopure sulfoxides, has been recognised for many years. *t*-Butyl *p*-tolyl sulfoxide, the first reported enantiopure example of a sulfoxide produced by enzyme-catalysed oxidation of an achiral sulphide,[1] was obtained using the fungus *Aspergillus niger* (*foetidus*), a source of monooxygenase enzymes. More recent biotransformation studies, from these[2–7] and other laboratories,[8,9] have shown that the ring hydroxylating dioxygenase enzymes (Rieske non-heme iron oxygenases), present in the soil bacterium *Pseudomonas putida*, are among the most stereoselective biocatalysts, currently available, for the production of enantiopure acyclic sulfoxides. Wild-type and mutant *P. putida* strains,[2–8] and *E. coli* recombinant strains,[3,5,8,9] containing toluene dioxygenase (TDO) and naphthalene-dioxygenase (NDO), have thus, to date, yielded >30 sulfoxides with high (>90%) enantiomeric excess (ee) values.[2–9] Routes to enantiopure sulfoxides are required for asymmetric synthesis studies (e.g. as chiral auxiliaries and ligands) and for the production of pharmaceuticals including anti-ulcer drugs containing a chiral sulfoxide group (e.g. the proton pump inhibitors omeprazole, and lanzaprazole). The results, presented in this article, provide a further demonstration of the value of enantiocomplementary dioxygenase enzymes for the production of enantiopure cyclic monosulfoxides from 1,3-, 1,4- and 1,5-disulfides. The enantiopure monosulfoxide metabolite **1B**, from benzo-1,3-dithiole **1A**, and the derived disulfoxide **1C**, have, in turn, been used to investigate their reactions with alkyllithium reagents.

2. Results and discussion

2.1. Dioxygenase-catalysed monosulfoxidation of sulfides 1A–8A to yield the corresponding sulfoxides 1B–8B

The initial phase of the study involved biotransformations of bicyclic disulfide substrates **1A**–**6A** using: (i) *P. putida* UV4 (a constitutive mutant strain containing TDO), (ii) *P. putida* NCIMB 8859 (a wild-type strain containing NDO) and, to a lesser extent, (iii), *P. putida* 9816/11 (an inducible mutant strain containing NDO). These whole-cell systems provide sources of the required dioxygenase biocatalysts, under biotransformation conditions similar to those reported for earlier sulfoxidation studies.[2–9] The isolated yields, enantiopurity values, and absolute configurations of monosulfoxides **1B**–**6B** are shown in Table 1. Sulfoxides **1B**,[10] $\mathbf{2B}_{cis}$,[10] $\mathbf{2B}_{trans}$,[10] $\mathbf{3B}_{cis}$,[10] $\mathbf{3B}_{trans}$[10] and **6B**[11] had earlier been reported as single enantiomers, while sulfoxides **4B** and **5B** were obtained for the first time as single enantiomers, during the present study.

Keywords: Dioxygenase-catalysed sulfoxidation; Enantiopure bicyclic sulfoxides; Alkylithium; Acyclic disulfoxides.
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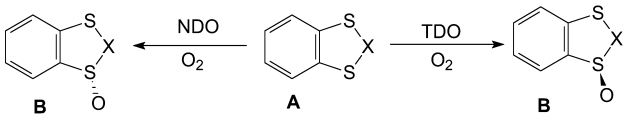
Table 1. Isolated yields (%Yld.), ee values (% ee) and absolute configurations (Ab.Con.) of sulfoxides **1B**–**8B** obtained from the corresponding sulfides **1A**–**8A** using TDO (*P. putida* UV4) and NDO (*P. putida* 8859) in whole-cell systems

Compound	TDO enzyme			NDO enzyme		
	%Yld.	% ee	Ab.Con.	%Yld.	% ee	Ab.Con.
1B	20	33	*S*	74, 72	>98	*R*
2B$_{cis}$	40, 26	>98	1*S*,2*R*	49	82	1*R*,2*S*
2B$_{trans}$	5	>98	1*S*,2S	7	38	1*R*,2*R*
3B$_{cis}$	26	>98	1*S*,2*R*	—	—	—
3B$_{trans}$	5	18	1*R*,2R	—	—	—
4B	1, 3	>98	*R*	78	>98	*S*[a]
5B	[b]	[b]	[b]	26	80 (93)[c]	*S*
6B	23	>98	*R*	60	>98	*S*
7B	5	26	*R*	21	38	*S*
8B	10	>98	*R*	2	43	*R*

[a] Predicted absolute configuration.
[b] No sulfoxide isolated; using *P. putida* 9816/11 as a source of NDO biocatalyst.
[c] Recrystallisation of bioproduct from *P. putida* 9816/11 gave an ee value of >98%.

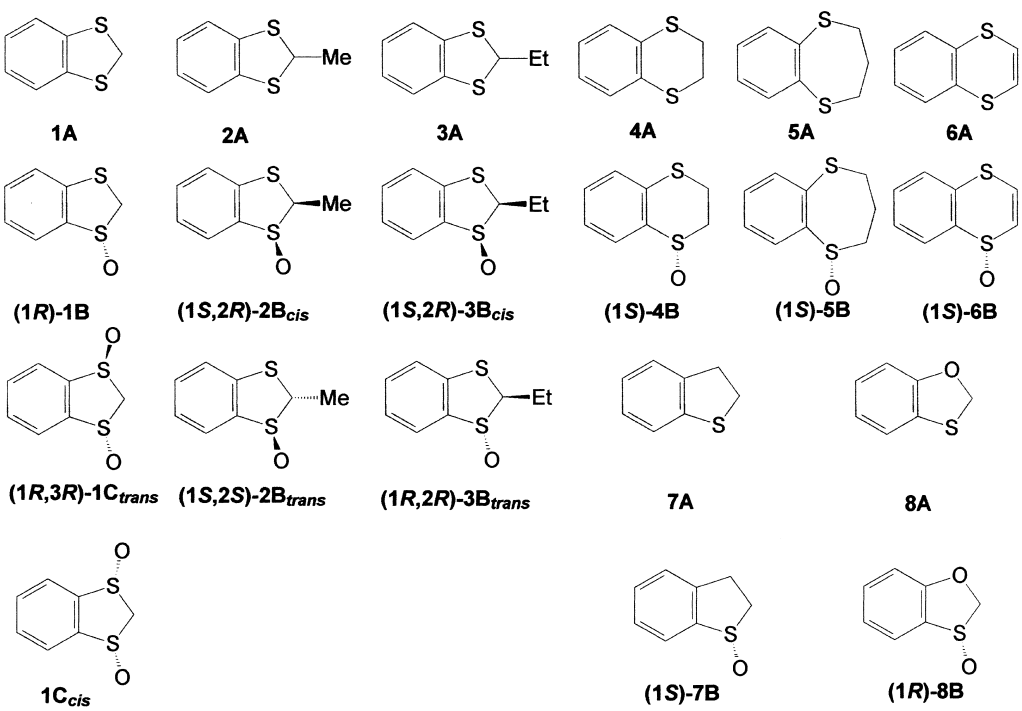
The circular dichroism (CD) spectra, of sulfoxide metabolites **1B**, **2B**$_{cis}$, **2B**$_{trans}$, **3B**$_{cis}$, and **3B**$_{trans}$, were found to have a strong absorption around 220 nm due to an n–π^* transition associated with the sulfoxide group.[10] This trend, of a positive CD absorption in the 220 nm region, was consistently associated with the rigorously established (*R*) configuration in sulfoxides **1B**, **2B**$_{cis}$, **2B**$_{trans}$, **3B**$_{cis}$, and **3B**$_{trans}$. The absolute configurations of sulfoxide enantiomers **4B**–**6B** have not been reported in the literature; no consistent CD spectral trend was evident that would allow reliable assignment of their absolute configurations. Hence, alternative approaches, to the configuration assignments for sulfoxide metabolites **4B**–**6B** were examined. Earlier studies, of the biotransformation of alkyl- and alkenylphenyl sulfides (RSPh, R=Me, Et, CH_2=CH_2, etc.), using *P. putida* UV4 (TDO), showed that an (*R*) configuration was observed in all cases.[5] This empirical method, of configuration assignment, was thus based on the assumption that the cyclic alkylaryl and alkenylaryl sulfides **4A**–**6A** would bind in a similar manner, at the active site of TDO, and would also show a preference for (1*R*) configurations for sulfoxide metabolites **4B**–**6B** (Scheme 1).

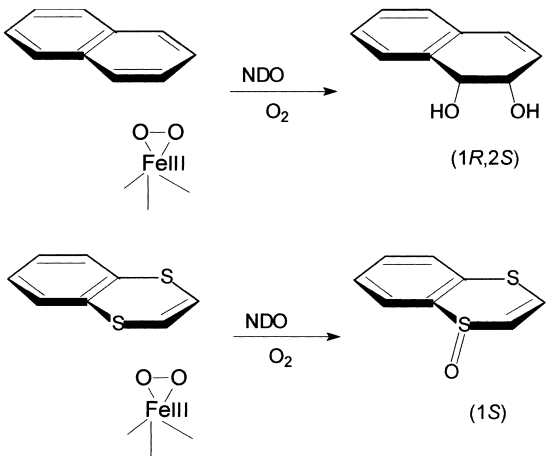


Scheme 1.

Conversely, a strong preference for the opposite (*S*) sulfoxide configuration was observed earlier, using NDO with some alkylphenyl and alkenylphenyl sulfides (RSPh, R=Me, Et, CH_2=CH_2, etc.).[5] X-ray crystallographic evidence, of the substrate binding and catalytic sites of NDO, that can account for the absolute stereochemistry of the *cis*-diol metabolites of bicyclic alkenes (e.g. indene) and arenes (e.g. naphthalene), has recently been reported.[12] In view of the similarity in shape between naphthalene and the bicyclic sulfide substrates **4A** and **6A** it is probable that substrate binding and catalysis, using NDO (Scheme 2), will yield bioproducts of similar configuration (but opposite to that obtained using TDO). Thus, absolute configurations of sulfoxide metabolites **4B**–**6B** were tentatively assigned by comparison with earlier results, using the TDO and NDO enzyme systems and acyclic alkylphenyl sulfides as substrates.

Recrystallization of the bioproduct, (−)-1,2,3,4-tetrahydro-1λ^4,5-benzodithiepin-1-oxide **5B** (80–93% ee) obtained by NDO-catalysed sulfoxidation, provided an enantiopure





Scheme 2.

sample whose absolute configuration was unequivocally established, as (1*S*) by X-ray crystallography, using the anomalous dispersion method (Fig. 1). The tetrahydrodithiepine ring adopts the chair conformation, with an equatorial oxygen atom. This result lends additional support to the tentative assignments of (1*R*) and (1*S*) configurations for alkylaryl sulfoxides **4B**–**6B** formed with TDO and NDO biocatalysts, respectively.

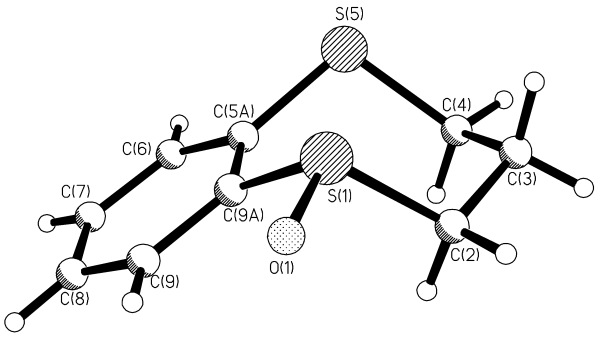


Figure 1. X-ray structure of **5B**.

As an extension to the dioxygenase-catalysed sulfoxidation study, the monosulfide isosteres **7A** and **8A**, of the parent disulfide substrate **1A**, were also examined. Thus, the corresponding enantioenriched sulfoxides, **7B** and **8B**, of known absolute configuration, were obtained with TDO and NDO as biocatalysts (Table 1). While sulfoxides **7B**, obtained using TDO and NDO, were of opposite absolute configuration, surprisingly, identical (1*R*) configurations were found for the sulfoxide metabolites **8B** using either enzyme. Sulfoxide **7B** was the most polar of the five chiral metabolites isolated with *P. putida* UV4 and 2,3-dihydrobenzo[*b*]thiophene **7A** as substrate. The structure and stereochemistry (absolute configurations and ee values) of the other metabolites, resulting from benzylic hydroxylation, desaturation and dihydroxylation of the heterocyclic and carbocyclic rings, will be discussed elsewhere.

The results, shown in Table 1, indicate that the cyclic sulfoxides were isolated in variable yields (1–78%) and that all but the sulfoxides $\mathbf{3B}_{trans}$ and **7B** could be obtained with high ee values (>90%), using either TDO or NDO as biocatalyst. The contrasting stereopreferences, previously observed for TDO- and NDO-catalysed oxidation of some acyclic alkylaryl sulfides[5] (Scheme 1), were even more common during the sulfoxidation of cyclic alkylaryl sulfides to yield sulfoxide metabolites, **1B**, $\mathbf{2B}_{cis}$, $\mathbf{2B}_{trans}$, **4B**, **6B** and **7B**. The most striking examples of enantiocomplementarity were found when TDO or NDO biocatalysts were used to produce enantiopure sulfoxides **4B** and **6B** of opposite configuration.

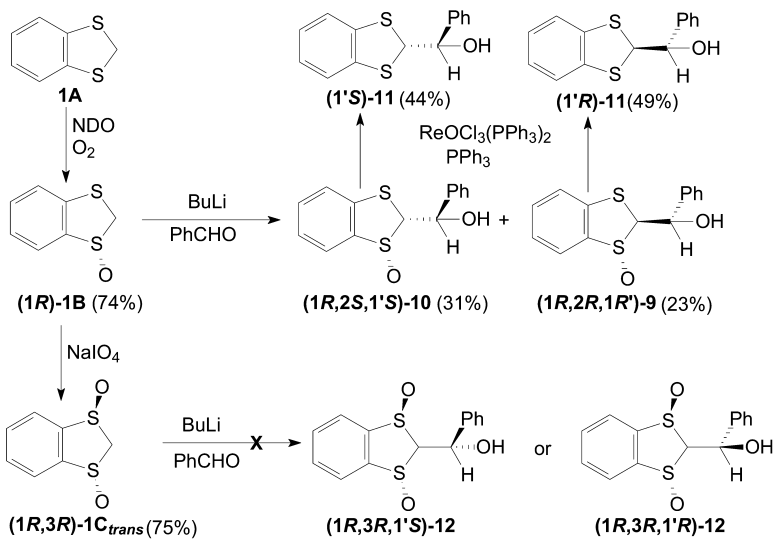
The Kagan modified Sharpless reagent, (Ti (*i*-PrO)$_4$/DET/*t*-BuOOH), had earlier[11] been used to obtain sulfoxide **6B**, as a single enantiomer (8% yield), after separation from a product mixture containing three other sulfoxidation products (92% yield). Biocatalysis now provides a complementary synthesis (asymmetric oxidation) of sulfoxide **6B** where either enantiomer is available in higher yield and state of purity. While the stereoselectivity values, found during sulfoxidations using TDO or NDO, were similar, the isolated yields were generally better, using the NDO system.

Results from earlier biotransformation studies, using both acyclic sulfides, and the corresponding racemic sulfoxides as substrates, for purified dioxygenases (TDO and NDO), or whole cell bacterial strains, containing TDO and NDO,[5,8] suggested that the enantiopure sulfoxide products were exclusively (or mainly) formed by asymmetric sulfoxidation. During the present study, the cyclic racemic sulfoxides **1B**–**8B** were also tested as substrates for the UV4 and 8859 strains of *P. putida*. With the exception of recovered sulfoxide **8B** (1*S*, 74% ee, 8859 strain), the other biotransformations showed only poor enantioenrichment of residual sulfoxides. On this basis, it would again appear that the high ee values, observed for the sulfoxide metabolites of cyclic sulfides, using TDO and NDO, were mainly the products of asymmetric sulfoxidation rather than kinetic resolution. However, during the course of these biotransformation studies of racemic sulfoxides with *P. putida* UV4, tentative evidence was found of other reactions occurring (<5% yield of bioproducts). This included evidence of a reductase-catalysed deoxygenation of sulfoxides which will be presented elsewhere.

2.2. Reaction of (*R*)-benzo-1,3-dithiole-1-oxide 1B with *n*-butyllithium and benzaldehyde

Small quantities, of enantiopure (+)- and (−)-sulfoxide **1B**, had earlier been obtained by chromatographic resolution of the racemate, using semi-preparative chiral stationary phase HPLC (CSPHPLC).[10] Treatment, of the sulfoxide enantiomers **1B** with strong base, potassium bis(trimethysilyl) acetamide, yielded a carbanion at C-2 that was used in the nucleophilic substitution of alkyl halides, to provide twelve enantiopure 2-substituted benzo-1,3-dithiole sulfoxides, including compounds $\mathbf{2B}_{cis}$, $\mathbf{2B}_{trans}$, $\mathbf{3B}_{cis}$, $\mathbf{3B}_{trans}$.[10] Absolute configuration determination, of the derived (1*S*,2*S*,2′*S*)-2-(2′-methylbutyl)-1,3-benzodithiole 1-oxide, by X-ray crystallography, allowed the configurations of the other 2-substituted benzo-1,3-dithiole sulfoxides **1B**, $\mathbf{2B}_{cis}$, $\mathbf{2B}_{trans}$, $\mathbf{3B}_{cis}$, $\mathbf{3B}_{trans}$ to be unequivocally assigned by stereochemical correlation and CD spectral comparison.

A major disadvantage of the CSPHPLC resolution procedure was that only small quantities, of each sulfoxide



Scheme 3.

enantiomer **1B** (ca: 40 mg), were obtained for reactivity studies.[10] With the availability of larger samples of the enantiopure benzo-1,3-dithiole sulfoxide **1B**, from the NDO-catalysed biotransformation route, it was possible to re-examine the reactivity of this compound and its derivatives. Thus, the second phase of the study involved the reactions of enantiopure monosulfoxide **1B** and disulfoxide $\mathbf{1C}_{trans}$ with alkyllithium reagents.

(*R*)-Monosulfoxide metabolite **1B** ($[\alpha]_D$=+505) was treated with 1 equiv. of *n*-butyllithium in dry THF (−78 °C) followed by the addition of benzaldehyde to the C-2 carbanion intermediate, to yield a diastereoisomeric mixture of 2-substituted benzo-1,3-dithiole sulfoxides **9**/**10** (Scheme 3). Separation, by PLC, gave *cis* isomer **10** as a crystalline solid (R_f 0.31, $[\alpha]_D$=+344, 31% yield) and *trans* isomer **9** as a viscous oil (R_f 0.19, $[\alpha]_D$=+116, 23% yield). X-ray crystallographic analysis (Fig. 2) confirmed the *cis* geometry of sulfoxide **10** and, using the anomalous dispersion method, allowed assignment of absolute configuration, (1*R*,2*S*,1′*S*), of the three chiral centres. The heterocyclic ring adopts an envelope conformation, with pseudoaxial oxygen and pseudoequatorial side chain at C(2).

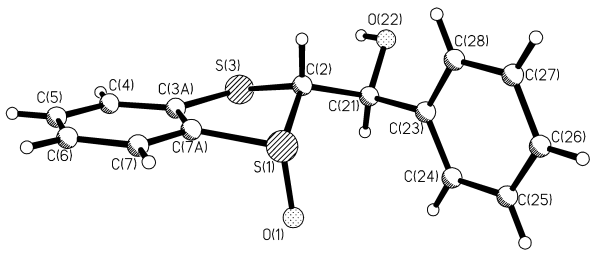
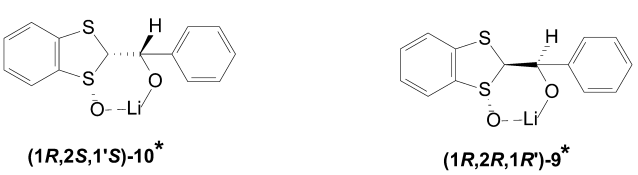


Figure 2. X-ray structure of **10**.

The X-ray crystallographic analysis, of the minor diastereoisomer **9**, was precluded, since it could not be obtained in crystalline form. Its absolute configuration was, however, assigned by a mild rhenium-catalysed deoxygenation process.[13] Thus, using the *trans* monosulfoxide **9** and a catalytic amount of $ReOCl_3(PPh_3)_2$ in the presence of triphenyl phosphine (ambient temperature, 1.5 h), the (−)-enantiomer of 2-substituted benzo-1,3-dithiole **11** ($[\alpha]_D$=−12, 49% yield) was obtained. Similar treatment (72 h), of the more hindered *cis* sulfoxide **10**, yielded the (+)-enantiomer of 2-substituted benzo-1,3-dithiole **11** ($[\alpha]_D$=+12, 44% yield). Since the absolute configuration, of the exocyclic chiral centre of sulfoxide **10**, was established as (1′*S*) by X-ray crystallography, sulfoxide **9** must have the (1*R*,2*R*,1′*R*) configuration. The formation, of either the (1′*R*) or (1′*S*) enantiomer of alcohol **11**, provides a good example, of the transfer of chirality from a (1*R*) cyclic sulfoxide stereogenic centre in metabolite **1B**, to a new exocyclic chiral centre in alcohol **11**. The converse process, i.e. chiral relay from the chiral alcohol centres in an arene *cis*-dihydrodiol metabolite, to a new chiral sulfoxide centre of either configuration (catechol sulfoxides), has recently been reported from these laboratories.[7]

The marked degree of diastereoselectivity, found during substitution at the C-2 position for the (*R*) enantiomer of monosulfoxide **1B**, was noteworthy; thus, only two diastereoisomers, (1*R*,2*S*,1′*S*)-**10** and (1*R*,2*R*,1′*R*)-**9**, of the possible four were detected. Examination of molecular models suggests that this could be due to the preferential attractive interactions between the sulfoxide oxygen atom and the lithium alkoxide, favouring chelate formations (1*R*,2*R*,1′*R*)-**9*** and (1*R*,2*S*,1′*S*)-**10*** (Scheme 4). Similar types of chelate interactions have been suggested, to account for the diastereoselectivity associated with carbanion reactions of *trans*-1,3-dithiane 1,3-dioxide with benzaldehyde.[14]

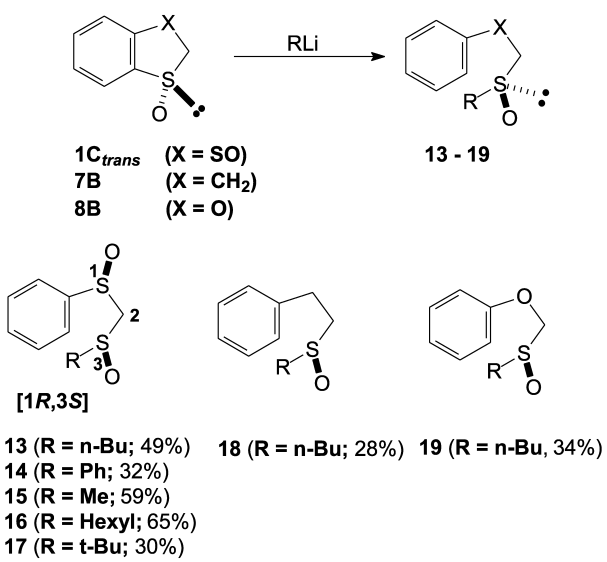


Scheme 4.

2.3. Ring-opening reactions of (1*R*,3*R*)-disulfoxide $\mathbf{1C}_{trans}$ and monosulfoxides 7B and 8B

Chemical oxidation of (+)-(*R*)-benzo-1,3-dithiole 1-oxide

1B ($[\alpha]_D$=+505), using $NaIO_4$ as oxidant, yielded enantiopure (+)-(1*R*,3*R*)-disulfoxide $\mathbf{1C}_{trans}$ ($[\alpha]_D$=+646, 75% yield, Scheme 3) as a major product. Compound $\mathbf{1C}_{trans}$ was readily separated from achiral (1*R*,3*S*) isomer $\mathbf{1C}_{cis}$ (19% yield) by flash chromatography. It was anticipated that treatment of (+)-disulfoxide $\mathbf{1C}_{trans}$ in a manner similar to (+) sulfoxide **1B**, i.e. reaction with *n*-butyllithium in dry THF (−78 °C), followed by addition of benzaldehyde, would give the two diastereoisomers of alcohol sulfoxide **12** (Scheme 3). In an earlier study,[14] on the synthesis and reaction of (±)-disulfoxide $\mathbf{1C}_{trans}$ with *n*-butyllithium and pivalaldehyde, alcohol disulfoxide diastereoisomers of similar structure to compound **12** (Ph replaced by *t*-Bu) were reported. However, spectral analysis and PLC purification indicated that only one product was formed from the reaction of *n*-butyllithum/benzaldehyde (−78 °C) with the (1*R*,3*R*) enantiomer of cyclic disulfoxide $\mathbf{1C}_{trans}$; it was identified as the acyclic disulfoxide **13** ($[\alpha]_D$=−180, 49% yield, Scheme 5). When this reaction was repeated under identical conditions, but without addition of benzaldehyde, the acyclic (−)-disulfoxide **13** was again formed (Scheme 5).



Scheme 5.

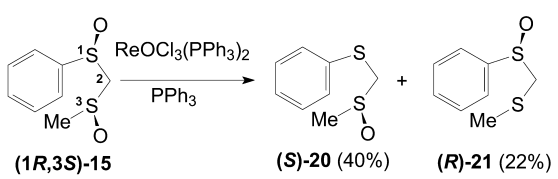
Since only a few methods are available for the synthesis of enantiopure acyclic disulfoxides, using either chemical or enzymatic approaches,[15–18] the unusual ring-opening reaction of (1*R*,3*R*)-disulfoxide $\mathbf{1C}_{trans}$ was examined further (Scheme 5). Reactions of $\mathbf{1C}_{trans}$ with other alkyllithium reagents (RLi, R=Ph, Me, $(CH_2)_5Me$, *t*-Bu) were conducted under identical conditions. ^{1}H NMR analysis, of the resulting acyclic disulfoxide products **13**–**17** indicated that in all cases single diastereoisomers were formed, albeit in relatively modest yields (28–65%, Table 2). Compound **14** was the only disulfoxide obtained without either a measurable optical rotation or CD absorption. This observation was consistent with total inversion of configuration, occurring during the ring opening process, to yield achiral *meso* isomer **14**. Comparison of the melting points and ^{1}H NMR data for disulfoxide **14** (mp 124–125 °C, $[\alpha]_D$=0), isolated during the present study, with that reported for the *meso* (mp 123 °C, $[\alpha]_D$=0) and racemic (*R*/*S* or *S*/*R*) (mp 136 °C) isomeric forms,[15,16] confirmed that it was, indeed, the *meso* compound.

Table 2. Reactions of bicyclic sulfoxides $\mathbf{1C}_{trans}$, **7B** and **8B** with alkyllithium reagents to yield the acyclic disulfoxides **13**–**17** and monosulfoxides **18** and **19**

Reactant sulfoxide		Alkyl lithium	Di- and mono-sulfoxide products			
Structure	Ab.Con.		Number	Yield	$[\alpha]_D$[a]	Ab.Con.
$\mathbf{1C}_{trans}$	1*R*,3*R*	BuLi	**13**	49	−180	1*R*,3*S*
$\mathbf{1C}_{trans}$	1*R*,3*R*	PhLi	**14**	32	[b]	[b]
$\mathbf{1C}_{trans}$	1*R*,3*R*	MeLi	**15**	59	−234	1*R*,3*S*
$\mathbf{1C}_{trans}$	1*R*,3*R*	HexylLi	**16**	65	−247	1*R*,3*S*
$\mathbf{1C}_{trans}$	1*R*,3*R*	*t*-BuLi	**17**	30	+75	1*R*,3*S*
7B	*R*	BuLi	**18**	28	−13[c]	*R*
8B	*S*	BuLi	**19**	34	+74[d]	*R*

[a] $CHCl_3$ solvent.
[b] Achiral compound.
[c] Formed from **7B** of 90% ee.
[d] Formed from **8B** of 73% ee.

The characteristic ^{1}H NMR spectral data, reported for the racemic (1*R*,3*R*/1*S*,3*S*) and (1*R*,3*S*/1*S*,3*R*) diastereoisomeric pairs of sulfoxide **15**,[15] suggested that the ring-opening product **15** ($[\alpha]_D$=−234, 59% yield) was either (1*R*,3*S*) or (1*S*,3*R*) diastereoisomer. Unfortunately an $[\alpha]_D$ value for either enantiomer of compound **15** has not been reported. The (1*R*,3*S*) configuration for (−)-sulfoxide **15** was confirmed by partial deoxygenation, using the rhenium complex-catalysed procedure (Scheme 6).[13] As expected from earlier studies,[13] the dialkyl sulfoxide group was deoxygenated more slowly compared with the alkylaryl sulfoxide group, leading to a 2:1 ratio of dialkyl sulfoxide **20** and alkylaryl sulfoxide **21**. Partial racemisation, of monosulfoxides **20** ($[\alpha]_D$=−27, 24% ee) and **21** ($[\alpha]_D$=−110, 87% ee), occurring to different degrees during the deoxygenation process, was also observed. Despite the partial racemisation, it was possible to assign (*S*) and (*R*) configurations, respectively, to monosulfoxides (−)-**20** and (−)-**21**, by comparison of the $[\alpha]_D$ values with authentic samples;[18] thus, a (1*R*,3*S*) configuration was assigned to disulfoxide **15**. The CD spectra of single diastereoisomers **13**, **15**–**17**, obtained by the ring-opening reaction of disulfoxide $\mathbf{1C}_{trans}$ (Scheme 5), displayed a similar pattern, i.e. a strong positive absorption at 218–220 nm and a strong negative absorption at 246–256 nm. The similarity in CD spectra of compounds **13**, **15**–**17** and unequivocal assignment of absolute configuration to disulfoxide **15** led us to the conclusion that in the ring-opening reactions inversion of configuration had occurred. This premise was supported by two reports in the literature,[19,20] where enantiopure acyclic alkylaryl monosulfoxides were found to react with alkyllithium reagents, to yield dialkyl sulfoxides via an S_N2 exchange process occurring with inversion of configuration. As found for acyclic alkylaryl sulfoxides,[19,20] the aryl–SO bond in the



Scheme 6.

cyclic disulfoxide **1C**$_{trans}$ was cleaved in preference to the alkyl–SO bond.

It was reported earlier[20] that alternative reactions of acyclic monosulfoxides with alkyl lithium reagents result in (a) abstraction of an α-hydrogen atom to give an α-lithiosulfoxide (carbanion formation), and (b) replacement of an aryl sulfoxide group with the alkyllithium group (group exchange). The relative proportion of either reaction was found to depend on the structure of sulfoxide (number and acidity of α-hydrogen atoms, substituent size and leaving group ability) and the type of alkyllithium reagent (size and basicity). Our observation of ring-opening reactions (group exchange), of disulfoxide **1C**$_{trans}$ with alkyllithium reagents (−78 °C), suggests that the temperature may also be an important factor. The reported[14] substitution reaction of a racemic sample of compound **1C**$_{trans}$, using *n*-butyllithium and pivalaldehyde to form *t*-butyl analogues of compounds (1*R*,3*R*,1′*S*)-**12** and (1*R*,3*R*,1′*R*)-**12** (Scheme 3), was carried out at higher temperatures (0 to −45 °C). Furthermore, ^{1}H NMR spectral analysis of the crude product mixture, obtained from the reaction of compound **1C**$_{trans}$ with *n*-butyllithium and benzaldehyde at a relatively higher temperature (−45 °C) indicated the formation of compounds (1*R*,3*R*,1′*S*)-**12** and (1*R*,3*R*,1′*R*)-**12**.

When the enantiopure monosulfoxide metabolites **4B**–**6B** containing six and seven-membered rings, were converted to the corresponding disulfoxides, and subsequently treated with alkyllithium reagents, no evidence of comparable ring-opening reactions was observed. However, under the standard reaction conditions monosulfoxide metabolites **7B** and **8B**, containing a five-membered ring, were also found to undergo a similar ring opening reaction to give the corresponding acyclic dialkyl monosulfoxides **18** and **19** in low yields (28 and 34%, Scheme 5, Table 2). Since only limited amounts of enantioenriched/enantiopure sulfoxides **7B** and **8B** were available from the small-scale biotransformations, additional samples of enantioenriched sulfoxides (−)-(*R*)-**7B** (90% ee) and (−)-(*S*)-**8B** (73% ee) were synthesised by chemical asymmetric sulfoxidation using the Kagan modified Sharpless reagent (Ti(*i*-PrO)$_4$/DET/*t*-BuOOH).[11]

It was assumed that inversion of configuration had occurred, again, during the ring-opening reaction of compound (−)-(*R*)-**7B** to yield monosulfoxide **18** ([α]$_D$=−13, 28% yield) and of compound (−)-(*S*)-**8B** to give the hemiacetal derivative **19** ([α]$_D$=+74, 34% yield). Chiral stationary phase HPLC (CSPHPLC) analysis of the sulfoxide **19** (49% ee), obtained from sulfoxide **8B** (73% ee), showed that partial racemization had occurred. The ee value for sulfoxide **18** could not be obtained using the latter CSPHPLC method.

3. Conclusions

The dioxygenase enzyme system has been shown to catalyse the enantioselective heteroatom oxidation of bicyclic sulfides (**7A**, **8A**) and disulfides (**1A**–**6A**). In many cases, the corresponding enantiopure monosulfoxides (e.g. **1B**, **2B**$_{cis}$, **2B**$_{trans}$, **3B**$_{cis}$, **4B**–**6B**) were obtained with opposite absolute configurations, using either TDO or NDO as biocatalyst. The monosulfoxide **1B** was found to undergo an α-hydrogen atom abstraction process with butyllithium followed by reaction, at low temperatures, with benzaldehyde in a diastereoselective manner, to form alcohols **9** and **10**. Under these conditions, disulfoxide **1C**$_{trans}$ was found to undergo a ring-opening reaction to yield the acyclic disulfoxides. A new chemoenzymatic route to enantiopure/enantioenriched disulfoxides (**13**, **15**–**17**) and monosulfoxides (**18** and **19**) was revealed, by the reactions of sulfoxides **1C**, **7B** and **8B**, with alkyllithium reagents.

4. Experimental

4.1. General

^{1}H NMR spectra were recorded at 300 MHz (Bruker Avance DPX-300) and 500 MHz (Bruker Avance DRX-500) in $CDCl_3$ solvent, unless stated otherwise. Chemical shifts (δ) are reported in ppm relative to $SiMe_4$; coupling constants (J) are given in Hz. Mass spectra were recorded at 70 eV on a VG Autospec Mass Spectrometer, using a heated inlet system. Accurate molecular weights were determined by the peak matching method with perfluorokerosene as standard. Elemental microanalyses were obtained on a Perkin–Elmer 2400 CHN microanalyser. HPLC analyses were carried out using a Perkin–Elmer Series 3B liquid chromatograph coupled to a Perkin–Elmer LC1-100 computing integrator. Analytical TLC was performed on Merck Kieselgel 60_{254} plastic sheets and preparative TLC (PLC) on glass plates (20 cm×20 cm) coated with Merck Kieselgel $PF_{254+366}$. Optical rotation ([α]$_D$) measurements were carried out with a Perkin–Elmer 214 polarimeter at ambient temperature (ca. 20 °C) at a concentration of ~0.01 g cm^{-3} and are given in units of 10^{-1} deg cm^2 g^{-1}. Circular dichroism spectra were recorded on a JASCO J-720 instrument, using spectroscopic grade acetonitrile as solvent. Enantiopurity of sulfoxides was determined by CSPHPLC analysis, using Chiralcel OB, OD and OJ columns and hexane/2-propanol (9/1) as eluent. The NCIMB 8859 strain of *P. putida* was acquired from the National Cultures of Industrial and marine Bacteria, Aberdeen. The 9816/11 strain of *P. putida* originated from the laboratories of Professor D. T. Gibson (University of Iowa) and the UV4 strain of *P. putida* was originally provided by Zeneca (now Avecia Lifescience Molecules, Billingham).

4.2. Synthesis and characterization of compounds 1A–8A

The di- and mono-sulfide substrates **1A**–**3A**,[10] **4A**,[21] **5A**,[22] **6A**,[23] **7A**,[24] **8A**[25] were synthesised using the reported methods.

4.2.1. 1,3-Benzodithiole 1A. Colourless oil; bp 113–114 °C/3.5 mmHg; lit.[10] 105 °C/3 mmHg; δ_H (500 MHz, $CDCl_3$) 4.48 (2H, s, CH_2), 7.00–7.02 (2H, dd, J=5.7, 3.3 Hz, 2×Ar-H), 7.19–7.22 (2H, dd, J=5.7, 3.3 Hz, 2×Ar-H).

4.2.2. 2-Methyl-1,3-benzodithiole 2A. Colourless oil; bp 98 °C/0.05 mmHg; lit.[10] 62–64 °C/0.02 mmHg; δ_H

(500 MHz, $CDCl_3$) 1.70 (3H, d, $J_{Me,H}$=6.8 Hz, Me), 4.98 (1H, q, $J_{H,Me}$=6.8 Hz, C*H*Me), 7.01–7.03 (2H, dd, *J*=5.8, 3.2 Hz, 2×Ar-H), 7.21–7.23 (2H, dd, *J*=5.8, 3.2 Hz, 2×Ar-H).

4.2.3. 2-Ethyl-1,3-benzodithiole 3A. Colourless oil; bp 70 °C/0.02 mmHg; lit.[10] 72–76 °C/0.01 mmHg; δ_H (300 MHz, $CDCl_3$) 1.02 (3H, t, J_{Me,CH_2}=7.0 Hz, CH_2Me), 1.90–1.97 (2H, m, CH_2Me), 4.76 (1H, t, $J_{CH_2,Me}$=7.0 Hz, 2-H), 6.98–7.03 (2H, m, Ar-H), 7.18–7.22 (2H, m, Ar-H).

4.2.4. 2,3-Dihydro-1,4-benzo[*d*]dithiine 4A. Yellow oil; (R_f 0.4, hexane); lit.[21] 82.5–85 °C/0.18 mmHg; δ_H (500 MHz, $CDCl_3$) 3.26 (4H, s, 2×CH_2S), 6.97–7.00 (2H, dd, *J*=5.9, 3.4 Hz, 2×Ar-H), 7.13–7.17 (2H, dd, *J*=5.9, 3.4 Hz, 2×Ar-H).

4.2.5. 3,4-Dihydro-2*H*-1λ^4,5-benzodithiepin 5A. White solid; mp 60–61 °C (from $CHCl_3$); lit.[22] 59–60 °C; δ_H (500 MHz; $CDCl_3$) 2.30 (2H, m, $CH_2CH_2CH_2$), 2.86 (4H, m, $CH_2CH_2CH_2$), 7.15–7.18 (2H, dd, *J*=5.7, 3.5 Hz, 2×Ar-H), 7.62–7.64 (2H, dd, *J*=5.6, 3.5 Hz, 2×Ar-H).

4.2.6. 1,4-Benzo[*d*]dithiine 6A. Light pink coloured oil; bp 58–60 °C/0.03 mmHg; lit.[23] 67–70 °C/0.1 mmHg; δ_H (500 MHz, $CDCl_3$) 6.52 (2H, s, 2×CHS), 7.19–7.23 (2H, dd, *J*=5.8, 3.4 Hz, 2×Ar-H), 7.26–7.29 (2H, dd, *J*=5.8, 3.4 Hz, 2×Ar-H).

4.2.7. 2,3-Dihydrobenzo[*b*]thiophene 7A. Colourless oil; bp 44–46 °C/0.15 mmHg; lit.[24] 67–69 °C/2 mmHg; δ_H (500 MHz, $CDCl_3$) 3.23–3.27 (2H, m, SCH_2), 3.30–3.34 (2H, m, CH_2Ar), 6.90–6.93 (1H, ddd, $J_{5,4}$=$J_{5,6}$=7.4 Hz, $J_{5,7}$=1.0 Hz, 5-H), 7.00–7.04 (1H, ddd, $J_{6,5}$=$J_{6,7}$=7.4 Hz, $J_{6,4}$=0.6 Hz, 6-H), 7.08–7.10 (1H, ddd, $J_{4,5}$=7.4 Hz, $J_{4,6}$=0.6 Hz, 4-H), 7.12–7.13 (1H, dd, $J_{7,6}$=7.4 Hz, $J_{7,5}$=1.0 Hz, 7-H).

4.2.8. 1,3-Benzoxathiole 8A. Colourless liquid; bp 36 °C/0.05 mmHg; lit.[25] 93–95 °C/5 mmHg; δ_H (500 MHz, $CDCl_3$) 5.66 (2H, s, CH_2), 6.81–6.83 (1H, ddd, $J_{7,6}$=8.0 Hz, $J_{7,5}$=1.2 Hz, $J_{7,4}$=0.4 Hz, 7-H), 6.85–6.89 (1H, ddd, $J_{6,7}$=8.0 Hz, $J_{6,5}$=7.0 Hz, $J_{6,4}$=1.2 Hz, 6-H), 6.97–7.00 (1H, ddd, $J_{5,4}$=7.7 Hz, $J_{5,6}$=7.6 Hz, $J_{5,7}$=1.2 Hz, 5-H), 7.15–7.17 (1H, ddd, $J_{4,5}$=7.7 Hz, $J_{4,6}$=1.2 Hz, $J_{4,7}$=0.4 Hz, 4-H).

Sulfoxides **1B**,[10] **2B**$_{cis}$,[10] **2B**$_{trans}$,[10] **3B**$_{cis}$,[10] **3B**$_{trans}$,[10] **6B**,[11] **7B**,[26,27] and **8B**,[25] synthesised by the literature methods, were found to be spectrally identical to the metabolites isolated during the study.

4.3. Biotransformation of substrates 1A–8A using *P. putida* UV4, *P. putida* 8859 and *P. putida* 9816/11 to yield sulfoxides 1B–8B

Biotransformations of the bicyclic substrates **1A**–**8A** were carried out with whole cell preparations of *P. putida* UV4 (TDO) and *P. putida* 8859 (NDO) and *P. putida* 9816/11 (NDO), using both small-scale (<10 g) shake-flask and 10 l fermenter (>10 g) conditions reported earlier for the sulfoxidation of both acyclic[5] and cyclic sulfides.[2] The workup procedure involved extraction of the culture medium using ethyl acetate.[2,5] Yields, chiroptical and ^{1}H NMR spectral data, obtained for each biotransformation, are summarised below.

4.3.1. 1,3-Benzodithiole 1A. (i) TDO product: (*S*)-1,3-benzodithiole-1-oxide **1B**; white crystalline solid, 22 mg, 20% yield; $[\alpha]_D$=−170 (*c* 1.0, $CHCl_3$); lit.[10] $[\alpha]_D$=−505 (EtOH); 33% ee (CSPHPLC, Chiralcel OB, α=1.4); δ_H (500 MHz, $CDCl_3$) 4.18 (1H, d, $J_{A,B}$=13.0 Hz, CH_AH_B), 4.33 (1H, d, $J_{B,A}$=13.0 Hz, CH_AH_B), 7.30–7.33 (1H, m, Ar-H), 7.48–7.54 (2H, m, 2×Ar-H), 7.89–7.90 (1H, m, Ar-H).

(ii) NDO (8859) product: (*R*)-1,3-benzodithiole-1-oxide **1B**; 400 mg, 74% yield; $[\alpha]_D$=+504 (*c* 1.9, $CHCl_3$); >98% ee (CSPHPLC). A 10 litre fermenter (15 g substrate) gave sulfoxide **1B**, 12 g, 72% yield; $[\alpha]_D$=+498 (*c* 1.9, $CHCl_3$) >98% ee (CSPHPLC).

(iii) NDO (9816/11) product: (*R*)-1,3-benzodithiole-1-oxide **1B**; 60 mg, 54% yield; $[\alpha]_D$=+401 (*c* 1.3, $CHCl_3$); 81% ee (CSPHPLC).

4.3.2. 2-Methyl-1,3-benzodithiole 2A. (i) TDO products: (1*S*,2*R*)-2-methyl-1,3-benzodithiole-1-oxide **2B**$_{cis}$; white crystalline solid, 2.21 g, 40% yield; $[\alpha]_D$=−319 (*c* 0.7 $CHCl_3$); >98% ee (CSPHPLC, Chiralcel OD, α=1.33); lit.[10] $[\alpha]_D$=−68 (EtOH) for 25% ee; δ_H (500 MHz, $CDCl_3$) 1.83 (3H, d, $J_{Me,H}$=6.9 Hz, Me), 4.44 (1H, q, $J_{H,Me}$=6.9 Hz, C*H*Me), 7.26–7.30 (1H, ddd, $J_{5,4}$=7.8 Hz, $J_{5,6}$=7.0 Hz, $J_{5,7}$=1.0 Hz, 5-H), 7.43–7.46 (2H, m, 4-H and 6-H), 7.84–7.86 (1H, dd, $J_{7,6}$=7.2 Hz, $J_{7,5}$=1.0 Hz, 7-H) and (1*S*,2*S*)-2-methyl-1,3-benzodithiole-1-oxide **2B**$_{trans}$; 290 mg, 5% yield; $[\alpha]_D$=−57 (*c* 0.5, $CHCl_3$); >98% ee (CSPHPLC, Chiralcel OD, α=1.14); lit.[10] $[\alpha]_D$=−29 (EtOH) for 25% ee; δ_H (500 MHz, $CDCl_3$) 1.60 (3H, d, $J_{Me,H}$=7.4 Hz, Me), 4.59 (1H, q, $J_{H,Me}$=7.4 Hz, C*H*Me), 7.28–7.33 (1H, m, 5-H), 7.47–7.48 (2H, m, 4-H and 6-H), 7.83–7.85 (1H, dd, $J_{7,6}$=7.7 Hz, $J_{7,5}$=0.7 Hz, 7-H). When repeated on a 10 litre fermenter-scale (10.0 g of substrate **2A**), 1*S*,2*R*)-2-methyl-1,3- benzodithiole-1-oxide **2B**$_{cis}$; 2.9 g, 26% yield; $[\alpha]_D$=−316 (*c* 0.7 $CHCl_3$); >98% ee (CSPHPLC) and (1*S*,2*S*)-2-methyl-1,3-benzodithiole-1-oxide **2B**$_{trans}$; 66 mg, 1% yield; $[\alpha]_D$=−60 (*c* 0.7, $CHCl_3$); >98% ee (CSPHPLC) were obtained.

(ii) NDO (8859) products: (1*R*,2*S*)-2-methyl-1,3-benzodithiole-1-oxide **2B**$_{cis}$; 2.21 g, 40% yield; $[\alpha]_D$=+256 (*c* 1.8, $CHCl_3$); 82% ee (CSPHPLC) and (1*R*,2*R*)-2-methyl-1,3-benzodithiole-1-oxide **2B**$_{trans}$; 390 mg, 7% yield; $[\alpha]_D$=+21 (*c* 0.7, $CHCl_3$); >38% ee (CSPHPLC).

4.3.3. 2-Ethyl-1,3-benzodithiole 3A. (i) TDO products: (1*S*,2*R*)-2-ethyl-1,3- benzodithiole-1-oxide **3B**$_{cis}$; white crystalline solid, 165 mg, 55% yield; $[\alpha]_D$=−189 (*c* 0.5, $CHCl_3$); >98% ee (CSPHPLC, Chiralcel OD, α=1.36); δ_H (300 MHz, $CDCl_3$) 1.30 (3H, t, J_{Me,CH_2}=7.4 Hz, CH_2Me), 2.05–2.16 (1H, m, CH_2Me), 2.33–2.44 (1H, m, CH_2Me), 4.26 (1H, dd, $J_{H,CH2}$=7.6 Hz, 2-H), 7.25–7.28 (1H, m, Ar-H), 7.45–7.46 (2H, m, Ar-H), 7.84–7.86 (1H, d, *J*=7.7 Hz, Ar-H) and (1*R*,2*R*)-2-ethyl-1,3-benzodithiole-1-oxide **3B**$_{trans}$;white crystalline solid, 15 mg, 5% yield; $[\alpha]_D$=+24 (*c* 0.4, $CHCl_3$); 18% ee (CSPHPLC, Chiralcel OD, α=1.11); δ_H (300 MHz, $CDCl_3$) 1.18 (3H, t,

J_{Me,CH_2}=7.4 Hz, CH_2Me), 1.65 (1H, m, CH_2Me), 2.00 (1H, m, CH_2Me), 4.48 (1H, dd, $J_{2,1'A}$=8.7 Hz, $J_{2,1'B}$=6.2 Hz, H-2), 7.26–7.32 (1H, m, Ar-H), 7.47 (2H, m, Ar-H), 7.87 (1H, d, J=7.7 Hz, Ar-H).

4.3.4. 2,3-Dihydro-1,4-benzo[*d*]dithiine 4A. (i) TDO product: (*R*)-2,3-dihydrobenzo[*d*]dithiin-1-oxide **4B**; white solid, 14 mg, 13% yield; mp 104–105 °C (from $CHCl_3$); $[\alpha]_D$=+37 (*c* 1.3, $CHCl_3$); HRMS found: M^+, 184.0016, $C_8H_8OS_2$ requires 184.0017; δ_H (500 MHz, $CDCl_3$) 3.05 (1H, ddd, $J_{3A,3B}$=13.4 Hz, $J_{3A,2A}$=6.6 Hz, $J_{3A,2B}$=3.8 Hz, SCH_AH$_B$), 3.13 (1H, ddd, $J_{3B,3A}$=13.4 Hz, $J_{3B,2A}$=10.8 Hz, $J_{3B,2B}$=3.6 Hz, SCH$_A$$H_B$), 3.40 (1H, ddd, $J_{2A,2B}$=13.4 Hz, $J_{2A,3A}$=6.6 Hz, $J_{2A,3B}$=3.6 Hz, SOCH_AH$_B$), 3.76 (1H, ddd, $J_{2B,2A}$=13.4 Hz, $J_{2B,3A}$=10.8 Hz, $J_{2B,3B}$=3.4 Hz, SOCH$_A$$H_B$), 7.25–7.28 (1H, ddd, $J_{6,5}$=8.2 Hz, $J_{6,7}$=7.5 Hz, $J_{6,8}$=1.2 Hz, 6-H), 7.31–7.32 (1H, dd, $J_{5,6}$=8.2 Hz, $J_{5,7}$=1.2 Hz, 5-H), 7.37–7.40 (1H, ddd, $J_{7,8}$=7.8 Hz, $J_{7,6}$=7.5 Hz, $J_{7,5}$=1.2 Hz, 7-H), 7.73–7.75 (1H, dd, $J_{8,7}$=7.8 Hz, $J_{8,6}$=1.2 Hz, 8-H); >98% ee (CSPHPLC, Chiralcel OB, α=1.4).

(ii) NDO (8859) product: (*S*)-2,3-dihydrobenzo[*d*]dithiin-1-oxide **4B**; 85 mg, 78% yield; $[\alpha]_D$=−35 (*c* 1.1, $CHCl_3$); >98% ee (CSPHPLC).

(iii) NDO (9816/11) product: (*S*)-2,3-dihydrobenzo[*d*]-dithiin-1-oxide **4B**; 67 mg, 62% yield; $[\alpha]_D$=−37 (*c* 1.3, $CHCl_3$); >98% ee (CSPHPLC); CD (λ, nm) 313 (Δε −0.375), 271 (Δε 0.240), 256 (Δε −2.27), 242 (Δε 0.561), 229 (Δε −2.815), 213 (Δε −5.865), 194.2 (Δε 2.100).

4.3.5. 3,4-Dihydro-2*H*-1λ^4,5-benzodithiepin 5A. (i) NDO (8859) product: (*S*)-1,2,3,4-tetrahydro-1λ^4,5-benzodithiepin-1-oxide **5B**; colourless crystalline solid, 30 mg, 26% yield; mp 120–121 °C (from $CHCl_3$/hexane); $[\alpha]_D$=−71 (*c* 1.4, $CHCl_3$); Found: C, 54.7; H, 5.0; $C_9H_{10}OS_2$ requires C, 54.5; H, 5.1%; δ_H (500 MHz, $CDCl_3$) 2.41–2.49 (2H, m, 4-H), 2.61–2.63 (1H, m, 3-H$_A$), 2.87–2.88 (1H, m, 3-H$_B$), 2.97–3.00 (1H, m, 2-H$_A$), 3.25–3.27 (1H, m, 2-H$_B$), 7.38–7.41 (1H, td, $J_{7,6}$=$J_{7,8}$=7.5 Hz, $J_{7,9}$=1.4 Hz, 7-H), 7.58–7.59 (2H, m, 6-H and 8-H), 7.87–7.89 (1H, dd, $J_{9,8}$=7.7 Hz, $J_{9,7}$=1.4 Hz, 9-H); 80% ee (CSPHPLC, Chiralcel OB, α=1.25).

(ii) NDO (9816/11) product: (*S*)-1,2,3,4-tetrahydro-1λ^4,5-benzodithiepin-1-oxide **5B**; 20 mg, 10% yield; $[\alpha]_D$=−82 (*c* 0.8, $CHCl_3$); 93% ee (CSHPLC, Chiralcel OB, α=1.25); CD (λ, nm) 265 (Δε −2.162), 250 (Δε 0.439), 236 (Δε −1.243), 228 (Δε 0.448), 220 (Δε −1.423), 215 (Δε −0.589), 198 (Δε −7.96).

4.3.6. X-ray crystal structure analysis of (*S*)-1,2,3,4-tetrahydro-1λ^4,5-benzodithiepin-1-oxide 5B. Recrystallization of compound **5B** (93% ee) gave a sample whose X-ray crystal structure analysis showed it to be of >98% ee and of the (*S*) configuration.

Crystal data for **5B**: $C_9H_{10}OS_2$, M_r=198.3, orthorhombic, a=8.106(4), b=9.043(3), c=13.042(6) Å, V=956.0(7) Å^3, T=293 K, Cu Kα radiation, λ=1.54178 Å, space group $P2_12_12_1$, Z=4, D_x=1.378 g cm^{-3}, 0.50×0.40×0.34 mm^3, μ=4.63 mm^{-1}, F(000)=416, Siemens P3/V2000 diffractometer, ωscan, 10<2θ<110°, measured/independent reflections: 5093/1195, direct methods solution, full matrix least squares refinement on F_0^2, anisotropic displacement parameters for non-hydrogen atoms, hydrogens located in difference Fourier but included at positions calculated from the geometry of the molecule using the riding model, $R1$=0.044 for 1115 data with $F_0 > 4\sigma(F_0)$, 111 parameters, $wR2$=0.113 (all data), GoF=1.07, Flack absolute structure parameter x=−0.09(4) establishes the absolute configuration as (1*S*), $\Delta\rho_{min,max}$=−0.30/0.23 e Å^{-3}. CCDC reference number 212386, Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax:+44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

4.3.7. 1,4-Benzo[*d*]dithiine 6A. (i) TDO product: (*S*)-1,4-benzo[*d*]dithiin-1-oxide **6B**; white solid, 25 mg, 23% yield; $[\alpha]_D$=−376 (*c* 0.5, $CHCl_3$); lit.[11] $[\alpha]_D$=−383 ($CHCl_3$); >98% ee (CSPHPLC, Chiralcel OB, α=1.17); δ_H (500 MHz, $CDCl_3$) 7.05 (1H, d, $J_{3,2}$=9.4 Hz, SCH), 7.31 (1H, d, $J_{2,3}$=9.4 Hz, S(O)CH), 7.51–7.54 (1H, ddd, $J_{6,5}$=$J_{6,7}$=7.6 Hz, $J_{6,8}$=1.4 Hz, 6-H), 7.56–7.60 (1H, ddd, $J_{7,6}$=$J_{7,8}$=7.6 Hz, $J_{7,5}$=1.3 Hz, 7-H), 7.60–7.62 (1H, dd, $J_{5,6}$=7.6 Hz, $J_{5,7}$=1.3 Hz, 5-H), 7.94–7.96 (1H, dd, $J_{8,7}$=7.6 Hz, $J_{8,6}$=1.4 Hz, 8-H); CD (λ, nm) 311 (Δε 0.522), 272 (Δε −0.444), 255 (Δε 3.357), 243 (Δε −0.675), 229 (Δε+3.657), 213 (Δε 7.470).

(ii) NDO (8859) product: (*R*)-benzo[*d*]dithiin-1-oxide **6B**; 66 mg, 60% yield; $[\alpha]_D$=+373 (*c* 1.0, $CHCl_3$); >98% ee (CSPHPLC).

(iii) NDO (9816/11) product: (*R*)-benzo[*d*]dithiin-1-oxide **6B**; 47 mg, 43% yield; $[\alpha]_D$=+373 (*c* 1.0, $CHCl_3$); >98% ee (CSPHPLC).

4.3.8. 2,3-Dihydrobenzo[*b*]thiophene 7A. (i) TDO product: (*R*)-2,3-dihydrobenzo[*b*]thiophene-1-oxide **7B**; white solid, 40 mg, 5% yield; $[\alpha]_D$=−59 (*c* 1.0, $CHCl_3$); lit.[27] $[\alpha]_D$=−285 (Me_2CO); 26% ee (CSPHPLC, Chiralcel OB, α=1.7); δ_H (500 MHz, $CDCl_3$) 3.23–3.26 (1H, m, 3-H$_A$), 3.28–3.34 (1H, m, 3-H$_B$), 3.39–3.40 (1H, m, 2-H$_B$), 3.83–3.91 (1H, m, 2-H$_A$), 7.41–7.44 (1H, ddd, $J_{5,4}$=$J_{5,6}$=7.4 Hz, $J_{5,7}$=0.5 Hz, 5-H), 7.46–7.47 (1H, dd, $J_{4,5}$=7.4 Hz, $J_{4,6}$=1.2 Hz, 4-H), 7.50–7.53 (1H, ddd, $J_{6,5}$=$J_{6,7}$=7.4 Hz, $J_{6,4}$=1.2 Hz, 6-H), 7.83–7.85 (1H, dd, $J_{7,6}$=7.4 Hz, $J_{7,5}$ 0.5, 7-H).

(ii) NDO (8859) product: (*S*)-2,3-dihydrobenzo[*b*]thiophene-1-oxide **7B**; 48 mg, 21% yield; $[\alpha]_D$=+95 (*c* 1.1, $CHCl_3$); 38% ee (CSPHPLC); CD (λ, nm) 263 (Δε 0.882), 231 (Δε 0.733), 217 (Δε −2.586), 198 (Δε 5.447).

(iii) NDO (9816/11) product: (*S*)-2,3-dihydrobenzo[*b*]thiophene-1-oxide **7B**; 19 mg, 17% yield; $[\alpha]_D$=+27 (*c* 1.0, $CHCl_3$), 11% ee (CSPHPLC).

4.3.9. 1,3-Benzoxathiole 8A. (i) TDO product: (*R*)-benzo[1,3]oxathiol-3-oxide **8B**; white solid, 11 mg, 11% yield; $[\alpha]_D$=+194 (*c* 1.0, $CHCl_3$); >98% ee (CSPHPLC, Chiralcel OB, α=1.3); δ_H (500 MHz, $CDCl_3$) 4.98 (1H, d,

$J_{A,B}$=11.0 Hz, CH_AH$_B$), 5.49 (1H, d, $J_{B,A}$=11.0 Hz, CH$_A$$H_B$), 7.18–7.20 (1H, ddd, $J_{4,5}$=7.6 Hz, $J_{4,6}$=1.2 Hz, $J_{4,7}$=0.5 Hz, 4-H), 7.22–7.24 (1H, ddd, $J_{5,4}$=7.6 Hz, $J_{5,6}$=7.2 Hz, $J_{5,7}$=1.2 Hz, 5-H), 7.56–7.59 (1H, ddd, $J_{6,7}$=7.7 Hz, $J_{6,5}$=7.2 Hz, $J_{6,4}$=1.2 Hz, 6-H), 7.88–7.90 (1H, ddd, $J_{7,6}$=7.7 Hz, $J_{7,5}$=1.2 Hz, $J_{7,4}$=0.5 Hz, 7-H); CD (λ, nm) 285 ($\Delta\varepsilon$ −2.831), 215 ($\Delta\varepsilon$ 6.55), 208 ($\Delta\varepsilon$ 5.259), 199 ($\Delta\varepsilon$ 15.600).

(ii) NDO (8859) product: (*R*)-benzo[1,3]oxathiol-3-oxide **8B**; 50 mg, 2% yield; $[\alpha]_D$=+87 (*c* 0.5, $CHCl_3$); 43% ee (CSPHPLC)

(iii) NDO (9816/11) product: (*R*/*S*)-benzo[1,3]oxathiol-3-oxide **8B**; 200 mg, 70% yield; $[\alpha]_D$=0 (*c* 0.5, $CHCl_3$); racemic (CSPHPLC).

4.4. Reaction of (+)-(*R*)-benzo-1,3-dithiole-1-oxide 1B with *n*-butyllithium and benzaldehyde

n-Butyllithium solution in hexane (1.6 M, 1.6 ml) was added to a stirring solution of (+)-(*R*)-benzo-1,3-dithiole-1-oxide **1B** (300 mg, 1.7 mmol in 10 ml of dry THF) kept under nitrogen at −78 °C. After 0.5 h, benzaldehyde (186 mg, 1.7 mmol) was added to the reaction mixture; it was allowed to warm to room temperature and stirred for another 0.5 h. The reaction mixture was then carefully poured into water (50 ml), the products extracted with ether (3×25 ml), the extract washed with water (30 ml) and dried ($MgSO_4$). Removal of the solvent yielded the crude product mixture which on separation by PLC (1% MeOH in $CHCl_3$) afforded pure samples of *cis*-(1*R*,2*S*,1′*S*)-2-(1′-phenylmethanol)-benzo-1,3-dithiole-1-oxide **10** and the more polar *trans*-(1*R*,2*R*,1′R)-2-(1′-phenylmethanol)-benzo-1,3-dithiole-1-oxide **9**.

4.4.1. (1*R*,2*S*,1′*S*)-2-(1′-Phenylmethanol)-benzo-1,3-dithiole-1-oxide 10. Colourless crystals, 147 mg, 31% yield; R_f 0.31 (1% MeOH in $CHCl_3$); mp 163–164 °C (from $CHCl_3$/hexane); $[\alpha]_D$=+344 (*c* 0.49, $CHCl_3$); HRMS found: M^+276.0278, $C_{14}H_{12}O_2S_2$ requires 276.0278; δ_H (300 MHz, $CDCl_3$) 4.23 (1H, d, $J_{OH,1'}$ 1.7, OH), 4.65 (1H, d, $J_{2,1}$=4.8 Hz, 2-H), 5.77 (1H, d, $J_{2,1'}$=4.8 Hz, 1′-H), 7.25–7.28 (1H, m, Ar-H), 7.36–7.46 (5H, m, Ar-H), 7.54 (2H, d, *J*=7.1 Hz, Ar-H), 7.77 (1H, d, *J*=7.6 Hz, Ar-H); *m*/*z* 276 (M^+, 7%), 259 (13), 229 (30) and 153 (100).

4.4.2. X-ray crystal structure analysis of (1*R*,2*S*,1′*S*)-2-(1′-phenylmethanol)-benzo-1,3-dithiole-1-oxide 10. *Crystal data for* **10**: $C_{14}H_{12}O_2S_2$, M_r=276.4, orthorhombic, *a*=5.531(1), *b*=10.410(3), *c*=21.931(7) Å, *V*=1262.8(6) Å^3, *T*=293 K, Cu Kα radiation, λ=1.54178 Å, space group $P2_12_12_1$, *Z*=4, D_x=1.45 g cm^{-3}, 0.56×0.49×0.43 mm^3, μ=3.74 mm^{-1}, Siemens P3/V2000 diffractometer, ωscan, $8 < 2\theta < 150°$, measured/independent reflections: 2748/2303, direct methods solution, full matrix least squares refinement on F_0^2, anisotropic displacement parameters for non-hydrogen atoms, hydrogens located in difference Fourier but included at positions calculated from the geometry of the molecule using the riding model, *R*1=0.071 for 2181 data with $F_0 > 4\sigma(F_0)$, 165 parameters, *wR*2=0.188 (all data), *GoF*=1.05, Flack absolute structure parameter *x*=0.00(4) establishes the absolute configuration as (1*R*,2*S*,1′*S*), $\Delta\rho_{min,max}$=−0.47/0.54*e* Å^{-3}. CCDC reference number 212387.

4.4.3. (1*R*,2*R*,1′*R*)-2-(1′-Phenylmethanol)-benzo-1,3-dithiole-1-oxide 9. White solid, 108 mg, 23% yield; R_f 0.19 (1% MeOH in $CHCl_3$); $[\alpha]_D$=+116 (*c* 1.2, $CHCl_3$); HRMS found: M^+276.0291, $C_{14}H_{12}O_2S_2$ requires 276.0278; δ_H (300 MHz; $CDCl_3$) 4.68 (1H, d, $J_{2,1'}$= 10.0 Hz, 2-H), 4.93 (1H, br s, OH), 5.32 (1H, d, $J_{1',2}$=10.0 Hz, 1′-H), 7.22–7.44 (6H, m, Ar-*H*), 7.49–7.52 (2H, m, Ar-*H*), 7.83 (1H, d, *J*=7.8 Hz, Ar-*H*); *m*/*z* 276 (M^+, 4%), 259 (11), 229 (28) and 153 (100).

4.4.4. Deoxygenation of (1*R*,2*S*,1′*S*)-2-(1′-phenylmethanol)-benzo-1,3-dithiole-1-oxide (10) and (1*R*,2*R*,1′*R*)-2-(1′-phenylmethanol)-benzo-1,3-dithiole-1-oxide 9. To each sample of monosulfoxides **10** and **9** (20 mg, 0.72 mmol), dissolved separately in CH_2Cl_2 (3 ml), was added triphenyl phosphine (25 mg, 0.1 mmol) and a catalytic amount of trichlorooxobis(triphenyl phosphine)-rhenium. The two reaction mixtures were allowed to stir at room temperature (sulfoxide **9** for 2 h and sulfoxide **10** for 72 h). The products were purified by PLC to give the corresponding sulfides (+)-(1′*S*)-**11**; 8 mg, 44% yield; $[\alpha]_D$=+12 (*c* 0.75, Me_2CO) from 1*R*,2*S*,1′*S*-**10** and (−)-(1′*R*)-**11**; 9 mg, 49% yield; $[\alpha]_D$=−12 (*c* 0.9, Me_2CO) from 1*R*,2*R*,1′*R*-**9**); HRMS found: M^+260.0342, $C_{14}H_{12}OS_2$ requires 260.0330; δ_H (300 MHz, $CDCl_3$) 4.66 (1H, d, *J*=8.2 Hz, 2-H), 4.89 (1H, d, *J*=8.2 Hz, 1′-H), 7.03–7.06 (2H, m, Ar-H), 7.18–7.25 (3H, m, Ar-H), 7.32–7.40 (5H, m, Ar-*H*); *m*/*z* 260 (M^+, 9%) and 153 (100).

4.4.5. Sodium periodate oxidation of (+)-(*R*)-1,3-benzodithiole-1-oxide 1B. (+)-(*R*)-1,3-Benzodithiole-1-oxide **1B** (260 mg, 1.52 mmol) in methanol (15 ml) was oxidized using a solution of sodium periodate (360 mg, 1.68 mmol) in water (10 ml) at 50 °C for 24 h. PLC (5% MeOH in $CHCl_3$), of the reaction mixture, afforded pure samples of (1*R*,3*R*)-1,3-benzodithiole-1,3-dioxide **1C**$_{trans}$ and the more polar *cis*-1,3-benzodithiole-1-oxide **1C**$_{cis}$.

(1R,3R)-1,3-Benzodithiole-1,3-dioxide **1C**$_{trans}$. White crystalline solid, 156 mg, 75% yield; R_f 0.33 (5% MeOH in $CHCl_3$); mp 123–126 °C (from $CHCl_3$/hexane); $[\alpha]_D$=+646 (*c* 1.5, $CHCl_3$); HRMS found: M^+185.9810, $C_7H_6O_2S_2$ requires 185.9809; δ_H (300 MHz; $CDCl_3$) 4.46 (2H, s, CH_2), 7.82–7.85 (2H, m, Ar-H), 7.99–8.02 (2H, m, Ar-H); *m*/*z* 186 (M^+, 32%) and 156 (100). Benzo-1,3-dithiole-1,3-dioxide **1C**$_{cis}$. 40 mg, 19% yield; R_f 0.22 (5% MeOH in $CHCl_3$); mp 179–180 °C; HRMS found: M^+185.9814, $C_7H_6O_2S_2$ requires 185.9809; δ_H (300 MHz, $CDCl_3$) 4.24 (1H, d, $J_{A,B}$=13.2 Hz, CH_AH$_B$), 5.03 (1H, d, $J_{B,A}$=13.2 Hz, CH$_A$$H_B$), 7.85–7.88 (2H, m, Ar-*H*), 8.08–8.11 (2H, m, Ar-*H*); *m*/*z* 186 (M^+, 33%) and 156 (100).

4.5. Ring opening reactions of bicyclic disulfoxide 1C$_{trans}$ and monosulfoxides 7B and 8B with alkyl and aryllithium reagents

4.5.1. Reaction of (1*R*,3*R*)-1,3-benzodithiole-1,3-dioxide 1C$_{trans}$ with *n*-butyllithium and benzaldehyde. *n*-Butyllithium solution in hexane (1.6 M, 0.53 ml) was added to stirred solution (1*R*,3*R*)-1,3-benzodithiole-1,3-dioxide

1C$_{trans}$ (100 mg, 0.54 mmol, $[\alpha]_D$=+646) in dry THF at −78 °C. After 0.5 h, benzaldehyde (0.06 ml, 0.6 mmol) was added to the reaction mixture; it was allowed to warm to room temperature, stirred overnight, and worked up as described earlier. Purification by PLC (5% MeOH in $CHCl_3$) afforded a pure sample of (−)-(butane-1-sulfinyl-methanesulfinyl)-benzene **13**; white solid, 64 mg, 49% yield; R_f 0.43 (5% MeOH in $CHCl_3$); mp 82–84 °C (from CH_2Cl_2/hexane); $[\alpha]_D$=−180 (*c* 1.69, $CHCl_3$); HRMS found: M^+244.0582, $C_{11}H_{16}O_2S_2$ requires 244.0592; δ_H (500 MHz, $CDCl_3$) 0.93 (3H, t, *J*=7.5 Hz, $(CH_2)_3$*Me*), 1.46–1.53 (2H, m, $(CH_2)_2CH_2CH_3$), 1.75–1.82 (2H, m, $CH_2CH_2CH_2CH_3$), 3.03–3.09 (1H, m, CH_AH_B-$(CH_2)_2CH_3$), 3.18–3.24 (1H, m, $CH_AH_B(CH_2)_2CH_3$), 3.91 (1H, d, *J*=13.3 Hz, SO−CH_AH_B−SO), 4.15 (1H, d, *J*=13.3 Hz, SO−CH_AH_B−SO), 7.55–7.59 (3H, m, Ar-*H*), 7.68–7.71 (2H, m, Ar-*H*); *m/z* 244 (M^+, 2%) and 126 (100); CD (λ, nm) 251 (Δε −11.18), 220 (Δε 16.67).

4.5.2. Reaction of (+)-(1*R*,3*R*)-benzo-1,3-dithiole-1,3-dioxide with phenyllithium. *General procedure*. The lithium reagent (1.1 molar equiv.) was added to a stirring solution (−78 °C, under nitrogen) of the disulfoxide (**1C**$_{trans}$) or mono-sulfoxide (**7B**, **8B**) (1.0 mmol in ~20 ml of dry THF). The reaction mixture was allowed to warm up to room temperature, stirred overnight, and then quenched with water; it was extracted with ethyl acetate, the extract dried ($MgSO_4$), concentrated under reduced pressure, and the crude product purified by PLC.

(1R,3R)-Benzo-1,3-dithiole-1,3-dioxide **1C**$_{trans}$. (50 mg, 0.27 mmol, $[\alpha]_D$=+646) and phenyllithium solution in cyclohexane (1.8 M, 0.25 ml) yielded meso *bis*-(phenylsulfinyl) methane **14**; white solid, 23 mg, 32% yield; mp 124–125 °C (from $CHCl_3$); lit.[14] 123 °C; R_f 0.25 ($CHCl_3$); δ_H (500 MHz; $CDCl_3$) 4.10 (1H, d, $J_{A,B}$=12.7 Hz,−SO−CH_AH_B−SO−), 4.20 (1H, d, $J_{B,A}$=12.7 Hz,−SO−CH_AH_B−SO−), 7.51–7.59 (6H, m, Ar-H), 7.68–7.74 (4H, m, Ar-H).

4.5.3. Reaction of (+)-(1*R*,3*R*)-benzo-1,3-dithiole-1,3-dioxide 1C$_{trans}$ with methyllithium. (1*R*,3*R*)-Benzo-1,3-dithiole-1,3-dioxide **1C**$_{trans}$ (45 mg, 0.24 mmol, $[\alpha]_D$=+646) and methyllithium solution in hexane (1.6 M, 0.25 ml) yielded (−)-(1*R*)-[(3*S*)-methylsulfinyl] methylphenyl sulfoxide **15**; white solid, 38 mg, 59% yield; mp 135 °C (from $CHCl_3$); R_f 0.22 ($CHCl_3$); $[\alpha]_D$=−234 (*c* 0.8, $CHCl_3$); HRMS found: M^+202.0118, $C_8H_{10}O_2S_2$ requires 202.0122; δ_H (500 MHz, $CDCl_3$) 2.98 (3H, s, *Me*), 3.91 (1H, d, $J_{A,B}$=13.1 Hz, CH_AH_B), 4.13 (1H, d, $J_{B,A}$=13.1 Hz, CH_AH_B), 7.55–7.60 (3H, m, Ar-H), 7.67–7.70 (2H, m, Ar-H); *m/z*: 202 (M^+1%),139 (26), 125 (53), 109 (72), 91 (100); CD (λ, nm) 251 (Δε −18.15), 220 (Δε 20.42).

4.5.4. Deoxygenation of disulfoxide 15. To a solution of (−)-(1*R*)-[(3*S*)-methylsulfinyl] methylphenyl sulfoxide **15** (22 mg, 0.11 mmol, $[\alpha]_D$=−234) in CH_2Cl_2 (3 ml) was added triphenylphosphine (25 mg, 0.1 mmol) along with a catalytic amount of trichlorooxobis(triphenylphosphine)-rhenium; the reaction mixture was stirred (2 h) at room temperature. Triphenylphosphine oxide was removed from the reaction mixture by reverse phase PLC (70% MeOH in H_2O). The product was further separated into two sulfoxides by PLC on silca gel (5% MeOH in $CHCl_3$); (−)-(*S*)-(phenylsulfanylmethyl)methyl sulfoxide **20**; 8 mg, 40% yield; $[\alpha]_D$=−27 (*c* 0.81, $CHCl_3$); 24% ee (Chiralcel OJ) and (−)-(*R*)-(methylsulfanylmethyl)phenyl sulfoxide **21**; 5 mg, 22% yield; $[\alpha]_D$=−110 (*c* 0.45, $CHCl_3$); 87% ee (Chiralcel OD). Spectral data for sulfoxides **20** and **21** were in agreement with the literature values.[6]

4.5.5. Reaction of (+)-(1*R*,3*R*)-benzo-1,3-dithiole-1,3-dioxide 1C$_{trans}$ with *n*-hexyllithium. (1*R*,3*R*)-Benzo-1,3-dithiole-1,3-dioxide **1C**$_{trans}$ (40 mg, 0.22 mmol, $[\alpha]_D$=+646) and *n*-hexyllithium solution in hexane (2.0 M, 0.15 ml) yielded (−)-(1*R*)-[(3*S*)-*n*-hexylsulfinyl]-methylphenyl sulfoxide **16**; white solid, 38 mg, 65% yield; mp 128–130 °C (from benzene); R_f 0.2 ($CHCl_3$); $[\alpha]_D$=−247 (*c* 1.7, $CHCl_3$); HRMS found: M^+272.0902, $C_{13}H_{20}O_2S_2$ requires 272.0905; δ_H (500 MHz, $CDCl_3$) 0.89 (3H, t, J_{Me,CH_2}=7.1 Hz, Me), 1.30–1.34 (4H, m, $(CH_2)_3$-$(CH_2)_2$Me), 1.45–1.47 (2H, m, $(CH_2)_2CH_2(CH_2)_2$Me), 1.77–1.82 (2H, m, $CH_2CH_2(CH_2)_3$Me), 3.04–3.08 (1H, m, CH_AH_B−$(CH_2)_4$Me), 3.16–3.21 (1H, m, CH_AH_B−$(CH_2)_4$Me), 3.90 (1H, d, $J_{A'B'}$=13.2 Hz, SO−$CH_{A'}H_{B'}$−SO), 4.13 (1H, d, $J_{B'A'}$=13.2 Hz, SO−$CH_{A'}H_{B'}$−SO), 7.55–7.60 (3H, m, Ar-H), 7.68–7.71 (2H, m, Ar-H); *m/z* 272 (M^+, 1%), 210 (5), 126 (81) and 43 (100); CD (λ, nm) 256 (Δε −21.39), 221 (Δε 23.55).

4.5.6. Reaction of (+)-(1*R*,3*R*)-benzo-1,3-dithiole-1,3-dioxide 1C$_{trans}$ with *t*-butyllithium. (1*R*,3*R*)-Benzo-1,3-dithiole-1,3-dioxide **1C**$_{trans}$ (60 mg, 0.32 mmol) and *t*-butyllithium solution in pentane (1.7 M, 0.3 ml) yielded (+)-(1*R*)-[(3*S*)-*t*-butylsulfinyl]methylphenyl sulfoxide **17** as a light yellow coloured oil, 20 mg, 30% yield; R_f 0.2 ($CHCl_3$); $[\alpha]_D$=+75 (*c* 1.0, $CHCl_3$); HRMS found: M^++H 245.0666, $C_{11}H_{16}S_2O_2$+H requires 245.0670; δ_H (500 MHz; $CDCl_3$) 1.21 (9H, s, Bu^t), 3.91 (1H, d, J_{AB}=12.4 Hz, CH_AH_B), 3.95 (1H, d, J_{BA}=12.4 Hz, CH_AH_B), 7.58 (3H, m, Ar-H), 7.79 (2H, m, Ar-H); *m/z* (CI) 245 (M^++H, 7%), 189 (15), 126 (96) and 57 (100); CD (λ, nm) 250 (Δε −14.66), 218 (Δε 26.49).

4.5.7. Reaction of (−)-(*R*)-2,3-dihydrobenzothiophene-1-oxide 7B with *n*-butyllithium. *n*-Butyllithium solution in hexane (1.6 M, 0.53 ml) and (−)-(*R*)-2,3-dihydrobenzo[*b*]thiophene-1-oxide **7B** (90% ee) (118 mg, 0.78 mmol) yielded (−)-(*R*)-*n*-butylphenethyl sulfoxide **18** as a colourless oil, 45 mg, 28% yield; R_f 0.51 (5% MeOH in $CHCl_3$); $[\alpha]_D$=−13.3 (*c* 1.98, $CHCl_3$); HRMS found: M^+210.1072, $C_{12}H_{18}OS$ requires 210.1078; δ_H (500 MHz; $CDCl_3$) 0.95 (3H, t, $J_{1,2}$=7.4 Hz, Me), 1.39–1.55 (2H, m, CH_2Me), 1.71–1.78 (2H, m, $CH_EH_FCH_2$Me), 2.60–2.66 (1H, ddd, $J_{C,D}$=13.0 Hz, $J_{C,A}$=9.0 Hz, $J_{C,B}$=8.5 Hz, $PhCH_2$−H_CH_D), 2.70–2.76 (1H, ddd, $J_{D,C}$=13.0 Hz, $J_{D,A}$=7.8 Hz, $J_{D,B}$=5.9 Hz, $PhCH_2$−H_CH_D), 2.86–2.93 (1H, ddd, $J_{E,F}$=12.8 Hz, $J_{E,G}$=10.0 Hz, $J_{E,H}$=7.2 Hz, $SOCH_EH_F$), 2.93–2.98 (1H, ddd, $J_{F,E}$=12.8 Hz, $J_{F,G}$=9.3 Hz, $J_{F,H}$=5.6 Hz, $SOCH_EH_F$), 3.04–3.10 (1H, ddd, $J_{A,B}$=14.0 Hz, $J_{A,C}$=9.0 Hz, $J_{A,D}$=7.8 Hz, Ph−CH_AH_B), 3.11–3.17 (1H, ddd, $J_{B,A}$=14.0 Hz, $J_{B,C}$=8.5 Hz, $J_{B,D}$=5.9 Hz, Ph−CH_AH_B), 7.23–7.26 (3H, m, Ar-H), 7.31–7.34 (2H, m, Ar-H); *m/z* (EI) 210 (M^+, 13%), 194 (24), 193 (32), 154 (7), 135 (8), 105 (100), 91 (69), 77 (57), 57 (92), 41 (47), 29 (55).

4.5.8. Reaction of (−)-(*S*)-1,3-benzoxathiole 8B with *n*-butyllithium. *n*-Butyllithium solution in hexane (1.6 M, 0.16 ml) and (−)-(*S*)-1,3-benzoxathiole-1-oxide **8B** (73% ee) (35 mg, 0.23 mmol) yielded (+)-(*R*)-*n*-butylphenoxymethyl sulfoxide **19** as an oil; 16 mg, 34% yield; R_f 0.52 (5% MeOH in $CHCl_3$); $[\alpha]_D$=+74 (*c* 0.6, $CHCl_3$); HRMS found: M^+, 212.0866, $C_{11}H_{16}O_2S$ requires 212.0871; *m/z* 212 (M^+, 70%), 108 (97), 107 (88), 94 (96), 79 (95), 77 (87), 65 (92), 57 (66), 55 (99), 51 (94), 41 (97), 39 (95), 29 (100), 27 (95); δ_H (500 MHz; $CDCl_3$) 0.98 (3H, t, $J_{CH_2,Me}$=7.3 Hz, Me), 1.44–1.59 (2H, m, CH_2Me), 1.78–1.85 (2H, m, CH_2CH$_2$Me), 2.80–2.91 (2H, m, CH_2CH$_2$CH$_2$Me), 4.92 (1H, d, $J_{A,B}$=10.2 Hz, OCH_AH$_B$), 5.02 (1H, d, $J_{B,A}$= 10.2 Hz, OCH$_A$$H_B$), 7.02–7.08 (3H, m, Ar-H), 7.31–7.33 (2H, m, Ar-H); 49% ee (CSP-HPLC, Chiralcel OD Column α=1.1).

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Combined biological and chemical catalysis in the preparation of oxycodone

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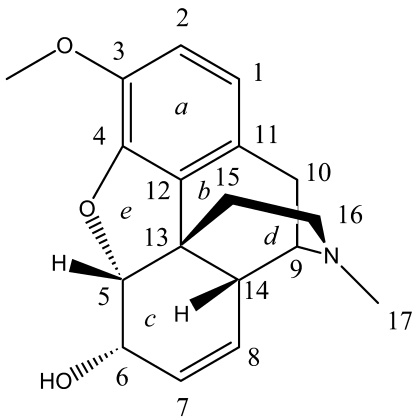
Abstract—The opioid oxycodone was produced from codeine, using a combination of chemical and biological catalysis. The use of novel functionalized ionic liquids permitted this reaction to be performed in a single solvent.

1. Introduction

The opioid oxycodone (14β-hydroxydihydrocodeinone, **1**) is finding increasing application in clinical medicine as both an analgesic and antitussive agent,[1,2] as well as being an intermediate synthon in the preparation of narcotic antagonists such as naloxone (**2**) and naltrexone (**3**).[3] Current methods for the production of oxycodone proceed from thebaine (**4**), an opium alkaloid of comparatively low abundance. These preparations additionally suffer from the use of harsh reaction conditions, hazardous reagents and the need for extensive chromatography to purify the final product.[4] Preparations from more readily available feedstocks, such as morphine (**5**) and codeine (**6**), are available[5,6] but suffer from poor yields and the need to isolate sensitive intermediates. The use of biocatalysis as a clean and efficient alternative for the production of specific opioids from morphine and codeine has been extensively investigated; enzymes isolated from a strain of *Pseudomonas putida* have been previously shown to be capable of performing a number of chemical transformations upon the morphinan nucleus.[7]

In particular, the $NADP^+$-dependent morphine dehydrogenase (MDH) from this organism is known to oxidize the hydroxyl group in the 6-position of both morphine and codeine, leading to the α,β-unsaturated ketones morphinone (**7**) and codeinone (**8**), respectively.[8] Additional enzymes have been observed to elicit catalytic hydrogenation of the 7,8-double bond (see Scheme 1), N-demethylation and other processes.[9,10]



Scheme 1. The structure and labelling of the morphinan nucleus in (−)-codeine.

Despite these advances, the fundamental step in the conversion of **6** to **1**-namely the hydroxylation of the 14-position—has proven difficult to accomplish either chemically or biocatalytically. The direct allylic hydroxylation of codeine is nowadays generally considered impossible;[11] the few published methods proceed through preliminary oxidation to **8** followed by hydroxylation of the dienol tautomer (**9**), in an analogous manner to the commercial hydroxylation of thebaine.[12] These processes are in turn hampered by the reactivity and sensitivity of this intermediate, leading to poor yields and the formation of copious by-products. Enzyme catalysed 14-hydroxylation of codeinone has likewise been reported[13] and appears to proceed through a similar mechanism;[14] again, however, yields are poor and the aqueous environment leads to the decomposition of much of the substrate. Additionally, in both of the above cases, hydroxylation at the 14-position of **8** leads to 14β-hydroxycodeinone (**10**). A further hydrogenation step is then required to give **1**. Consequently, a method was sought

Keywords: Biocatalysis; Ionic liquids; Oxycodone; Hydration.
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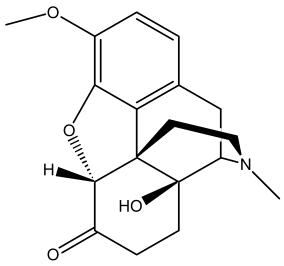

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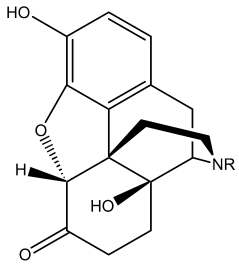
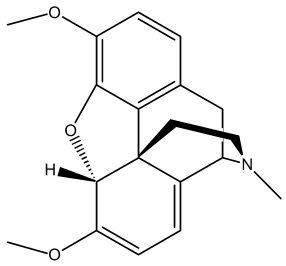


2 (R = allyl)
3 (R = cyclopropylmethyl)



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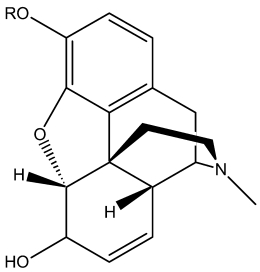
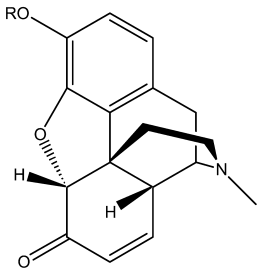
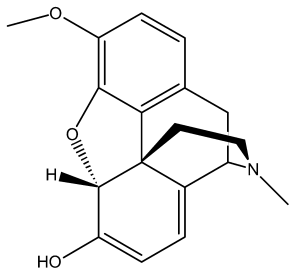


5 (R = H)
6 (R = methyl)



7 (R = H)
8 (R = methyl)



9

whereby the codeinone sourced from the MDH-catalysed oxidation of codeine may be directly converted to oxycodone.

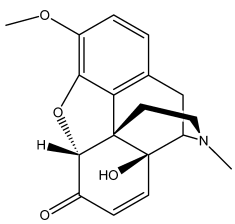
2. Results and discussion

2.1. Conversion of codeine to oxycodone

In order to achieve this conversion, the 14-hydroxyl group must be introduced with concomitant saturation of the 7,8-double bond. Hydration of this bond in codeinone proceeds in the classical manner of conjugate addition to yield 8-hydroxydihydrocodeinone (**11**).[15] Of interest in this respect was the observation that, in aqueous solution, codeinone exists in a state of dynamic equilibrium with its β,γ-unsaturated isomer, neopinone (**12**), proceeding via the dienol tautomer **9** (Scheme 2).[16] Direct Markovnikov addition of water across the double bond of this isomer, in contrast to that of codeinone, leads directly to **1**.

In the absence of any known hydratase capable of performing this hydration biocatalytically, a series of chemical reagents known to elicit Markovnikov hydration of substituted alkenes were therefore applied to equilibrated mixtures of codeinone and neopinone, with generally poor results. The majority of these reagents either failed to hydrate at all or resulted in the predominant formation of unwanted by-products such as **11**. In several cases, the entire morphinan skeleton suffered rearrangement or lysis. Nevertheless, a small number of reagents were observed to elicit the formation of oxycodone. In particular, the use of bis(acetylacetonato)cobalt (II) to catalyse the reaction of the alkene moiety with molecular oxygen and phenylsilane (as reported by Isayama and Mukaiyama[17]) not only resulted in the high yielding conversion of **12** to **1**, but did so without any effect upon **8**. This was interpreted to be a result of the deactivation of the codeinone double bond towards electrophilic addition (and thus silylperoxide formation), due to its α,β-conjugation with the carbonyl group on carbon 6; furthermore, the absence of any 7-hydroxydihydrocodeinone argued for the inability of this system to elicit conjugate hydride reduction (with subsequent α oxidation) even in the presence of dioxygen, which might otherwise be expected.[18] Steric constraints inhibiting α-attack ensured the correct orientation of the introduced hydroxyl group, whilst the tertiary nature of the substituted carbon atom rendered impossible the lysis of the silylperoxide to ketonic products. When the reaction was performed in tetrahydrofuran, an equilibrium mixture of 74:26% codeinone/neopinone was thus converted to an effectively isoproportional mixture of codeinone and oxycodone (Scheme 3). The efficacy of a cobalt-based catalyst was particularly interesting in view of Coop and Rice's observation of 14-hydroxylation of the codeinone/neopinone system using cobalt (III) acetate.[12]

In view of its observed inactivity towards codeinone, this catalytic process was examined in protic solvents. Since THF did not permit the enolization of either **8** or **12**, it was



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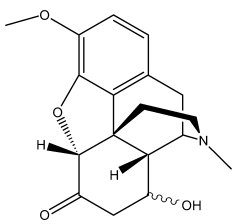
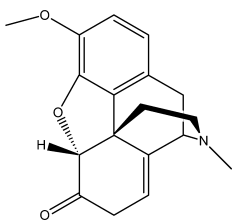
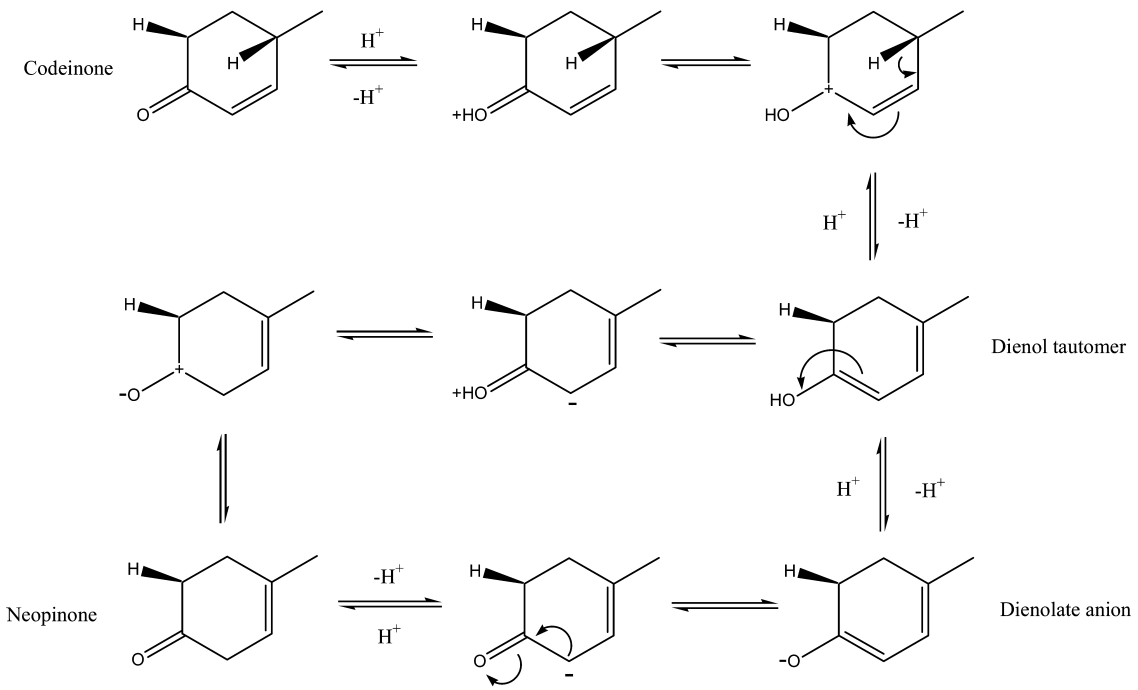


11



12



Scheme 2. Protic dienolization of the c-ring in the codeinone–neopinone isomerization.

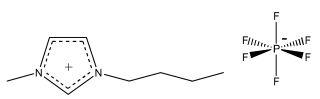
impossible to convert any of the considerable codeinone in the isolated equilibrium mixtures to neopinone and thence to oxycodone. If this enolization was permitted to occur through a protic medium, then the total morphinan content of the reaction should become available to the catalyst; as **12** was removed by conversion to **1**, so the perturbation of the equilibrium would result in further conversion of **8** to **12**. In practice, the limitations imposed by the pK_a of standard solvents severely hampered this; the enolization rate in primary alcohols was too slow to significantly enhance the yield on a reasonable timescale, whereas stronger acids gave rise to protonation of the amine group and a significant degree of decomposition. Addition of a small quantity of water to the THF solution was more successful in promoting equilibration and did not significantly affect the hydration reaction. Since the hydration was irreversible, this procedure ultimately resulted in the conversion of codeinone to oxycodone in 68% yield.

The preparation of oxycodone was thus achieved from codeine in a two-stage process, using a combination of enzymatic and chemical catalysis. The need to extract the codeinone/neopinone mixture from the aqueous biocatalytic reaction prior to the hydration step remained nonetheless a considerable disadvantage; being necessitated by the occurrence of side-reactions and the poor activity of the cobalt catalyst in aqueous solution, together with its destructive effect upon the biological components. The insolubility of phenylsilane in water and the ready availability of hydroxide led to the gradual accumulation of undesirable alternative products, notably **11**. Consequently, a means was sought whereby the enzymatic oxidation, codeinone/neopinone isomerization and hydration may be performed in a concomitant fashion.

2.2. Ionic liquids as solvents for combined catalysis

The absence of a suitable solvent in which to perform this type of combined bio/chemical catalytic process in 'one pot' has long been a major impediment to the industrial application of biocatalysis. Conventional organic solvents are incapable of functioning as satisfactory solvents for most enzymes[19] and, unless anhydrous, generally elicit denaturation. Water is an equally poor solvent for many organic substrates. Ionic liquids have recently elicited much interest as solvents for both chemical and biological catalysis,[20,21] but have not previously been shown to permit the combination of these two methods in a single reaction vessel. However, by utilizing ionic liquids incorporating specific functional groups, it has proven possible to perform both enzyme-catalysed and transition metal-catalysed reactions upon a common substrate in a single solvent.

Dialkylimidazolium-based ionic liquids such as 1-*n*-butyl-3-methylimidazolium hexafluorophosphate (BMIm PF_6, **13**) have been used in biocatalysis in both single- and two-phase systems.[22] The enzymes used in the pure ionic liquids have been primarily rugged lyases and lipases,[23] which have also shown activity in molecular organic solvents. In the two-phase systems, water-immiscible ionic liquids have been effectively used as substrate reservoirs, with the actual catalytic processes taking place in the aqueous phase.[24] Cofactor-dependent enzyme biocatalysis has not previously been observed in pure ionic liquids.



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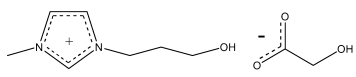
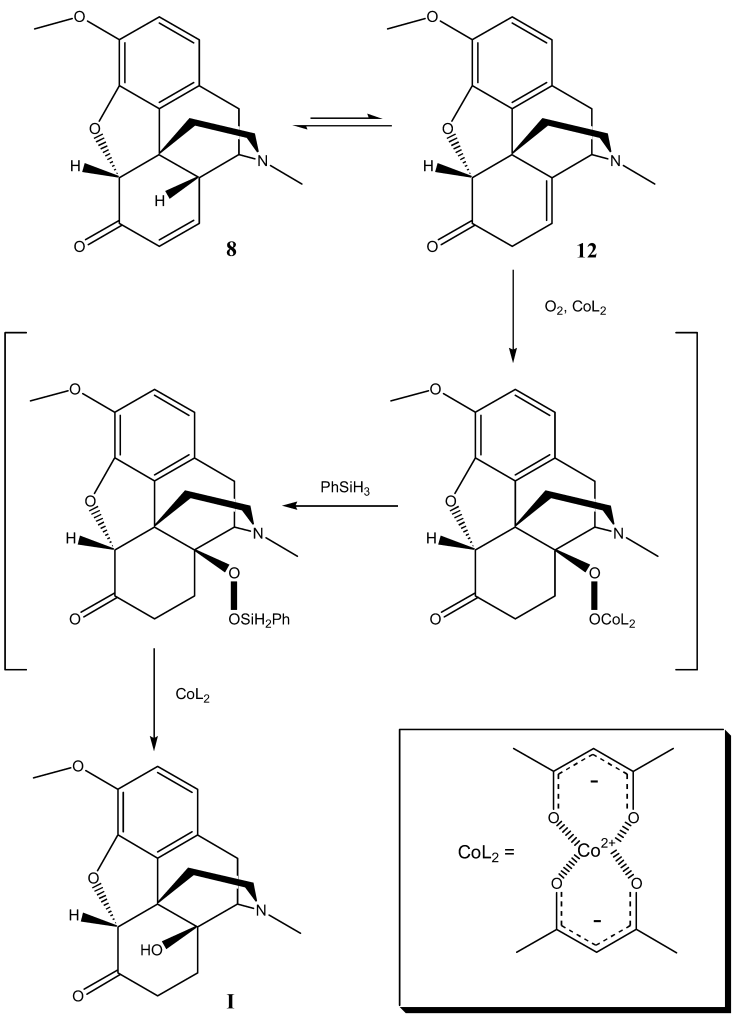


14

This is unsurprising in view of the hydrophobic nature of



Scheme 3. Conversion of codeinone (**8**) to oxycodone (**1**), by Markovnikov hydration of neopinone (**12**).[17] *Conditions* used: 200 mM alkaloid (64 mM **12**) in 5 mL THF, 0.64 alkaloid equiv. (2 equiv. **12**) $PhSiH_3$, 6.3 total alkaloid wt% $Co(acac)_2$, O_2 atmosphere. Yield: 92% from **12**, 29% overall.

solvents such as **13**, which exhibit poor hydrogen bond donor/acceptor properties and whose interaction with highly polar, hydrophilic enzyme surfaces is consequently very limited. By incorporating hydrogen bonding functional moieties into this type of ionic liquid structure, it has proven possible to elicit dissolution of enzymes and their cofactors, thus permitting homogeneous biocatalysis to occur.[25] The most dramatic improvements in enzyme dissolution and activity for the MDH system were observed when hydroxylated ionic liquids were used as reaction solvents. Initial experiments were performed using the glycolate salt of the simple hydroxylated **13** analogue 1-(3-hydroxypropyl)-3-methylimidazolium (3-HOPMIm glycolate, **14**). This solvent proved to be capable of simultaneously dissolving the substrate, enzyme and cofactor. Dissolution of the biological molecules in the ionic liquids was hampered by slow diffusion resulting from their relatively high viscosity. It was consequently elicited by vortexing followed by centrifugation, with the supernatant solutions being micro-filtered to remove any suspended matter. Dissolution was confirmed by means of infra-red spectroscopy, based on the characteristic shifts of the protein amide absorbances in ionic solution.[25] The activities demonstrated by the enzyme under these conditions was manifestly greater than that shown by suspensions (or extremely small concentration solutions) of the enzyme in anhydrous molecular organic solvents or in **13**, suggesting homogeneous catalysis with little denaturation.

When provided with a stoichiometric equivalent of NADP, MDH was found to be active against codeine in **14** solution. The observed activity was, as expected, considerably diminished relative to that in water, but was far in excess of that observed in either **13** or conventional organic solvents, with an equilibrium concentration of 84:16 in favour of codeine being reached in 24 h. The codeinone thus produced was found to be considerably more stable in ionic solution than in aquo, such that decomposition of the accumulated product did not occur to any appreciable extent, even after several days at room temperature. Enolization was found to occur in **14**, but the equilibrium was found to lie more in favour of codeinone than was the case in water. The equilibration rate was much slower than in water, but reproducibly faster than that observed in ethanol. In view of this, the action of bis(acetylacetonato)-cobalt (II) upon the neopinone/codeinone mixture was investigated in **14**. Although the codeinone/neopinone could be extracted from ionic solution by conventional methods,

this was found to be destructive to both MDH and NADP; hence the enzyme was removed from solution by dialysis. The cobalt catalyst was then added directly to the equilibrated solution of the ketones in the ionic liquid, albeit at the risk of destroying the cofactor. The poor solubility of oxygen in ionic liquids necessitated the use of a continuous oxygen stream being bubbled through the solution. In this way, the catalytic process was found to take place smoothly, with practically all of the neopinone being converted to oxycodone within 12 h. It was found that the addition of the full equivalence of phenylsilane at the start of this reaction led to the reduction of a significant proportion of the codeinone to codeine, a phenomenon which was not observed in THF solution. This was controlled by the gradual addition of the hydride donor as the reaction progressed. The enolization rate of the ketones in **14** was unfortunately still too slow to result in the conversion of significant residual codeinone. However, the stability of this compound in ionic solution allowed for the re-establishment of equilibrium over time, after which the catalysis could be repeated. Hence it was found to be ultimately possible to convert up to 42% of the initial codeinone/neopinone mixture to oxycodone, albeit slowly. The addition of a small quantity of water was found to permit this equilibrium to establish more rapidly and with a more favourable bias towards neopinone, without exerting a major deleterious effect upon yield.

3. Conclusions

It has been shown that hydrophilic, functionalized ionic liquids can effectively act as solvents for both chemical and biological homogeneous catalysis. The specimen ionic liquid employed was found to be capable of dissolving both morphine dehydrogenase and its associated nicotinamide cofactor whilst permitting the retention of considerable catalytic activity against codeine. The same solvent was also found to permit both the codeinone/neopinone equilibration and the hydration of the neopinone double bond under bis(acetylacetonato)cobalt (II) catalysis. Consequently it has proven possible to convert codeine to oxycodone using a combination of biological and chemical catalysis in a common solvent. It is reasonable to expect that the application of a cofactor-recycling system within the biocatalytic system would enable this yield to be increased, rather than it being determined by the equilibrium position of the $NADP^+$/NADPH couple. This would also enable the exploitation of the much higher solubility of the alkaloid substrate in ionic liquids as opposed to water.

Although the sensitivity of the biological components to the chemical catalyst prevents this from being a true 'one pot' reaction, the solubility of substrate, enzyme, cofactor and chemical catalyst within the same solvent provides the potential for the development of continuous flow combined catalytic processes, whereby a solvent stream containing the substrate may be passed through a bioreactor, either using tethered enzymes or with subsequent dialysis and then on to further (biological or chemical) catalysts. In this way, the need to isolate and purify intermediate products such as codeinone is eliminated and the entire process may be operated as a continuous phase. We conclude that solvents of this type are ideal candidates for the development of combined biological/chemical catalytic processes and that they offer the potential to obviate several major current obstacles to the industrial practicality of biocatalysis.

4. Experimental

4.1. General procedures

Reagents were purchased from Sigma-Aldrich Co. Ltd, Gillingham, UK or Fisher Scientific UK Ltd, Loughborough, UK and were of the highest purity available. All reactions were performed in oven-dried glassware under an argon atmosphere unless otherwise stated. Tetrahydrofuran was twice distilled under argon. Ionic liquids were dried by freezing in liquid nitrogen and placing under high vacuum on an Edwards Modulyo freeze drier for 48 h, followed by heating to 80 °C under high vacuum for 18 h. Water content was determined using a KEM MKS-500 volumetric Karl Fischer titrator. Reactions were followed by TLC on Polygram SIL G/UV_{254} 0.20 mm silica coated plates, using an eluent of 84:14:2 chloroform/methanol: 0.880 ammonia solution. Column chromatography was performed using YMC-Gel 60A I-400/230 mesh silica.

^1H NMR spectra were recorded using a Jeol Lambda LA400 400 MHz spectrometer, at 23 °C in deuterated chloroform unless otherwise stated. Chemical shift values are given in ppm relative to a tetramethylsilane internal standard. Infrared spectra were taken on a Perkin–Elmer Spectrum One spectrophotometer, using KBr discs unless otherwise stated.

Codeine, codeinone, thebaine and oxycodone standards were supplied by Macfarlan Smith Limited. Neopinone was prepared from thebaine according to the method of Conroy.[26]

4.1.1. Synthesis of ionic liquids. BMIm PF_6 was prepared according to the method of Koel.[27] HOPMIm glycolate was prepared from 1-methylimidazole as follows.

20.53 g 1-Methylimidazole (redistilled, 250 mmol) and 3-chloro-1-propanol (1.1 equiv., 26.00 g) were placed in a 500 mL round-bottomed flask equipped with reflux condenser, magnetic stirrer and calcium chloride drying tube. The reaction mixture was heated to 80 °C for 48 h with continuous stirring, at the conclusion of which it was cooled to room temperature and washed three times with diethyl ether. The resultant 1-(3-hydroxypropyl)-3-methylimidazolium chloride (40.5 g) was dissolved in dry acetone and anhydrous potassium glycolate (1.05 equiv., 27.36 g) was added with stirring. Stirring was continued for a further 12 h, after which the reaction mixture was filtered to remove the precipitated potassium chloride. The acetone was removed in vacuo and the residue was diluted to 80% with dry acetonitrile. Following treatment with activated charcoal, the product was chromatographed on a 7 cm 60A silica column and the solvent was removed to yield 1-(3-hydroxypropyl)-3-methylimidazolium glycolate as a pale yellow liquid, yield 30.7 g (62%).[28]

^1H NMR [400 MHz, d_6-DMSO, δ (ppm)]: 1.92 (2H, q, 16,

β-CH_2), 2.0 (0.9H, br, $HOCH_2CO_2^-$), 3.40 (2H, t, 6, α-CH_2), 3.52 (2H, s, $CH_2CO_2^-$), 3.75 (1H, br, CH_2CH_2OH), 3.83 (3H, s, NCH_3), 4.21 (2H, t, 8, γ-CH_2), 7.65 (1H, d, 2, 4-*H*), 7.72 (1H, d, 2, 5-*H*), 9.13 (1H, s, 2-*H*).

FT-IR (Nujol mull, cm^{-1}): 1731, 1634, 1576, 3390, 1167, 871, 1061, 1088, 1228.

4.1.2. Codeine to codeinone in water using MDH. Codeine hydrochloride dihydrate (200 mg, 0.54 mmol) was dissolved in 10 mL argon-flushed p*H* 9.0, 50 mM dibasic potassium phosphate buffer containing 250 μL acetone (200 mg, 6.38 equiv.). 100 μg each of morphine dehydrogenase from *Pseudomonas putida* M10 and alcohol dehydrogenase from *Thermoanaerobium brockii* were added, together with 25 mg (0.06 equiv.) of β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt). The solution was stirred in the dark at 30 °C under an argon atmosphere. The reaction was followed by time-course sampling and analysis of the samples by thin layer chromatography and ^{1}H nuclear magnetic resonance spectroscopy. After 24 h the reaction mixture was adjusted to pH 11 with aqueous sodium hydrogen carbonate and extracted three times with 25 mL volumes of chloroform. The organic fractions were pooled and the solvent was removed in vacuo to yield 134 mg of crude crystalline residue, which was chromatographed on silica gel to yield codeine (72 mg) and a pure equilibrium (68:32) mixture of codeinone (**8**) and neopinone (**12**) (40 mg, 25%) as a pale brown crystalline material, which could not be further separated chromatographically and was analysed by ^{1}H NMR. If the reaction was left for 48 h, chromatography was hampered by the presence of decomposition products and the codeinone/neopinone yield was not increased.

^{1}H NMR [400 MHz, $CDCl_3$, δ (ppm)]: 1.81 (1H, m, codeinone 15α-*H*), 1.88 (0.47 H, m, neopinone 15α-*H*), 1.92 (0.45H, br, neopinone 15β-*H*), 2.03 (1H, t, 10, codeinone 15β-*H*), 2.27–2.34 (2.9H, dd+m, codeinone and neopinone 16-H_2), 2.42 (3H, s, codeinone N–CH_3), 2.48 (1.42H, s, neopinone N–CH_3), 2.57 (1H, d, 16, codeinone 10α-*H*), 2.77 (0.45H, d, 6, neopinone 10α-*H*), 3.07 (1H, d, 24, codeinone 10β-*H*), 3.16 (1H, s, codeinone 9-*H*), 3.27 (0.47H, d, 10, neopinone 10β-*H*), 3.33 (0.45H, br, neopinone 7α-*H*), 3.38 (1H, s, codeinone 14-*H*), 3.64 (0.47H, d, 6, neopinone 9-*H*), 3.81 (3H, s, codeinone 3-O–CH_3), 3.91 (1.4H, s, neopinone 3-O–CH_3), 3.95 (0.48H, d, 14, neopinone 7β-*H*), 4.67 (1H, s, codeinone 5-*H*), 5.00 (0.47H, s, neopinone 5-*H*), 5.50 (0.47H, d, 6, neopinone 8-*H*), 6.05 (1H, d, 14, codeinone 7-*H*), 6.58 (1H, d, 16, codeinone 2-*H*), 6.63–6.70 (2.94H, m, codeinone 1-*H* and 8-*H* and neopinone1-*H* and 2-*H*).[29]

FT-IR showed the characteristic carbonyl absorption bands at 1670 cm^{-1} (codeinone) and 1735 cm^{-1} (neopinone).

4.1.3. Codeine to codeinone in ionic liquid using MDH. Lyophilized MDH (100 μg) was dissolved in 10 mL dry (<100 ppm H_2O) 3-HOPMIm glycolate (**14**) with vortexing over 2 h. The resultant solution was centrifuged at 13,000 rpm for 5 min to remove suspended material and the supernatant was filtered through 0.2 μm Sartorius syringe filters. Dissolution of the protein was confirmed by Fourier transform infra-red spectroscopy. Anhydrous codeine free base (150 mg, 0.5 mmol) was added along with 380 mg (1 equiv.) NADP (sodium salt) and the solution was placed under argon. The reaction was shaken at 110 rpm, 30 °C with hourly sampling. Samples were taken by removing 500 μL of solution, diluting 20 times with water, adjusting to pH 10 with aqueous sodium carbonate and extracting three times with 5 mL volumes of diethyl ether. The organic fractions were pooled and the solvent was removed in vacuo. The residual solid was redissolved in $CDCl_3$ and analysed by ^{1}H NMR. This showed the progressive production of codeinone up to a yield of 16% after 24 h. A solution of MDH in the same solvent left to stand, in a desiccated environment, at room temperature for four weeks showed reduced activity, but nonetheless showed 10% conversion of codeine to codeinone when it was used under otherwise identical conditions to the above. When codeinone was allowed to stand in **14** solution for 24 h, NMR analysis gave a codeinone/neopinone ratio of 91:9 (see Table 1).

Attempts to repeat the above reaction in various molecular organic solvents and in **13** failed. FT-IR showed no significant protein dissolution after centrifugation and filtration. In both instances, unchanged codeine was recovered in practically quantitative yield, identical to standards. Codeinone was recovered in 98% yield after standing in dry **13** solution for 24 h. No neopinone was detected.

The addition of 5% water to the ionic liquid biotransformation did not significantly denature the enzyme. Rather, the repetition of the above procedure in 95:5 **14**/H_2O resulted in 26% conversion to an 85:15 mixture of codeinone/neopinone after 24 h. This was a significant improvement upon the corresponding two-phase reaction involving water and **13**, in which a maximum yield of 6% after 24 h was obtained.

4.1.4. Hydration of codeinone and neopinone. *(a) In THF*. An equilibrium mixture of codeinone (195 mg) and neopinone (92 mg) was dissolved in 5 mL dry tetrahydrofuran,

Table 1. Variation in catalytic yields with solvent

Solvent	Yield **8** from **6**, MDH (10 μg mL^{-1})	Equilibrium **8**:**12** ratio	Yield **1** from **12**, $Co(acac)_2$ (3.6 mg mL^{-1})
50 mM phosphate buffer, pH 9	37–44%	68:32%	Not performed due enolization and insolubility of **12**
Tetrahydrofuran, <100 ppm H_2O	<1%	100:0%	80–97%
BMIm PF_6 (**13**), <100 ppm H_2O	<1%	100:0%	75–88%
3-HOPMIm glycolate (**14**), <100 ppm H_2O	12–18%	91:9%	90–98%
3-HOPMIm glycolate (**14**), 5% H_2O	23–27%	85:15%	Not quantifiable due enolization.

containing 18 mg bis(acetylacetonato)cobalt (II). Phenylsilane (70 mg, 2 neopinone equiv.) was added and the reaction was stirred under an oxygen atmosphere for 12 h, being monitored by TLC. At the conclusion of this period, the volatiles were removed in vacuo, the solid residue was resuspended in 15 mL chloroform and washed three times with 10 mL portions of dilute aqueous potassium carbonate. The organic fraction was dried over magnesium sulfate, filtered and chromatographed on silica gel to yield 90 mg (29% based on total ketone content, 92% based on neopinone alone) 14β-hydroxydihydrocodeinone (oxycodone, **1**).

^{1}H NMR [400 MHz, $CDCl_3$, δ (ppm)]: 1.55 (1H, m, 12, 15α-*H*), 1.63 (1H, d, 12, 8α-*H*), 1.83 (1H, d, 12, 8β-*H*), 2.15 (1H, t, 12, 15β-*H*), 2.26 (2H, m, 7α -*H*+16α-*H*), 2.37–2.41 (4H, s+m, 16α-*H*+N–CH_3), 2.42 (1H, m, 16β-*H*), 2.57 (1H, dd, 16, 10α-*H*), 2.83 (1H, d, 4, 9-*H*), 2.98 (1H, t, 12, 7β-*H*), 3.12 (1H, d, 16, 10β-*H*), 3.88 (3H, s, 3-O–CH_3), 4.64 (1H, s, 5-*H*), 5.05 (1H, br, O*H*), 6.61 (1H, d, 8, 2-*H*), 6.67 (1H, d, 8, 1-*H*).

FT-IR (KBr disc, cm^{-1}): 1722, 1501, 1446, 1260, 1036, 2936, 1346, 1165, 1145, 1018.

The balance of the material was almost entirely composed of codeinone; no neopinone could be detected in the codeinone fraction by NMR and no quantifiable amounts of 7- or 8-hydroxylated products were found.

When the above reaction was repeated with 200 mg pure neopinone, oxycodone (205 mg, 97%) was recovered as the sole product. When 200 mg pure codeinone was used, no oxycodone was detected and the balance of the starting material was recovered.

(b) In ionic liquid. An equilibrium mixture of codeinone and neopinone (as above) was dissolved in 5 mL dry 1-(3-hydroxypropyl)-3-methylimidazolium glycolate, containing 18 mg bis(acetylacetonato)cobalt (II). Phenylsilane (105 mg, 1 equiv., 120 μL) was added in 20 μL portions over 12 h, with vigorous stirring and under constant oxygen sparging. The reaction was monitored by TLC. At the conclusion of the reaction, the solution was diluted 10 times with water, basified with aqueous sodium carbonate and extracted five times with 10 mL portions of diethyl ether. The organic extracts were pooled, washed with brine, dried over magnesium sulfate and the solvent was removed in vacuo. The semi-solid residue was taken up in a small volume of terahydropyran and chromatographed on silica gel to yield **1** (96 mg, 98% from neopinone, 31% from codeinone/neopinone, 5% from codeine). The balance was composed primarily of unchanged codeinone, with smaller amounts of neopinone, codeine, neopine and an additional unknown compound which was not characterised. The isolated codeinone was redissolved in **14** and allowed to equilibrate for 72 h. The reaction was then repeated as above and the process was performed a third time with the recovered codeinone. After three runs, the total yield of oxycodone was 42% from codeinone/neopinone.

When the reaction was performed in an identical manner to (a) above, with simple substitution of 3-HOPMIm glycolate for THF, significant (up to 30%) reduction of codeinone and neopinone to (+/−)-codeine and (+/−)-neopine was observed. This was attributed to the poor solubility of oxygen in ionic liquids preventing sufficient silylperoxide formation and consequent reduction by the phenylsilane.

The repetition of reactions (a) and (b) above with the addition of 5% v/v water to the solution had a profound effect upon yields. As the enolisation of codeinone was facilitated, so a greater proportion of the total ketone content was found to undergo hydration, giving total yields of 68% (THF) and 55% (**14**) from the total ketone content.

Hence, if the entire reaction sequence were performed in 95:5 **14**/H_2O, **6** could be converted to **1** in 14% yield.

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Oxidation of secondary amines by molecular oxygen and cyclohexanone monooxygenase

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Abstract—Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* catalyzed the oxidation of tertiary and secondary amines to *N*-oxides and nitrones, respectively. The formation of a hydroxylamine intermediate was involved with secondary amines as starting substrates.

1. Introduction

The increasing demand for enantiomerically pure compounds for the pharmaceutical and agrochemical industries implies a great incentive for the study of the biotransformations. Oxidative biotransformations are amongst the most useful of all identified biologically mediated conversions.[1] They usually involve monooxygenases or dioxygenases, that catalyze the insertion of one oxygen or two oxygen atoms at a specific point of a molecule, often with high stereo and/or regioselectivity. The cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CHMO) (EC 1.14.13.22) is an interesting enzyme for its application in the manufacture of fine chemicals and in organic synthesis.[2]

CHMO is a yellow FAD-containing enzyme of about 60,000 Da, it is active as a monomer and contains one tightly but non-covalently bound FAD per monomer.[2a] This enzyme catalyzes the Baeyer–Villiger oxidation of cyclohexanone with the formation of the corresponding ε-caprolactone; the only reagents consumed are O_2 and NADPH. The enzyme shows an absolute specificity for the electron donor (NADPH) and a wide specificity for the ketonic substrates.

The bacterium NCIMB 9871 can grow on cyclohexanol or cyclohexanone as sole carbon source; the key step is the ring expansion with formation of ε-caprolactone. The subsequent hydrolysis leads to 6-hydroxyhexanoate, that is oxidized to adipate, and then to the acetyl-CoA and succinyl-CoA by β-oxidation.[2] Mechanistic studies have suggested that the reactive intermediate is the FAD-4a-hydroperoxyflavin, that has a twofold activity since it can react either as a nucleophilic species, as in the case of the Baeyer–Villiger reaction and the boronic acid oxidation, or as an electrophilic species, as in the sulfoxidation reaction.[2a] More recent investigations would, however, indicate that the oxygenating intermediate is the anionic 4a-peroxide and not its protonated form, the 4a-hydroperoxyflavin.[3]

Apart from cyclohexanone, CHMO is able to transform a great variety of other substituted cyclic ketones, bicyclic ketones and aldehydes.[4] These biotransformations have wide applications for the stereoselective oxidations of prochiral ketones and constitute the only efficient methodology to resolve racemic ketones.[5] Furthermore, CHMO can oxidize a wide series of organic compounds containing electron rich heteroatoms; the sulfides are converted to sulfoxides,[2b] the sulfites to sulfates,[6] the selenides to selenoxides,[7] tertiary amines to *N*-oxides[8] and phosphines to phosphinoxides.[9] In many cases these reactions are highly enantioselective. Recently, our group has shown that the enzyme can epoxidize electron poor olefins but with severe limitations; in fact, only two compounds are accepted as substrates, namely, the dimethyl and the diethylvinylphosphonate, compounds structurally related to Fosfomycin. They are converted into the corresponding epoxides with ee >98%.[10]

The main goal of this last work was to extend the synthetic repertoire of CHMO; for this reason we have started investigating the oxidation of secondary amines to nitrones,

Keywords: Cyclohexanone monooxygenase; Nitrones; Amines; Hydroxylamines; *N*-Oxides.
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a reaction previously studied with 4a-hydroperoxyflavin as oxidizing species.[11] It is known that mammalian liver contains two enzymatic systems responsible for the oxidation of *N*-substituted amine drugs, one containing a flavoprotein (NADPH cytochrome P-450 reductase) and the other a cytochrome P-450. The former catalyzes the *N*-oxidation of secondary and tertiary amines in a NADPH and O_2-dependent process similar to CHMO and the latter the *C*-oxidation of amines, that leads to *N*-dealkylation.[12]

Another point of interest is that the reaction products, the nitrones, are highly versatile synthetic intermediates, in particular as 1,3-dipoles and for the preparation of various nitrogen-containing biologically active compounds such as antibiotics, alkaloids, amino-sugars and β-lactams.[13] They have also pharmacological activity, e.g., the *tert*-butylbenzylnitrone (PBN) acts as a spin trap in physiological buffer, and exhibits antioxidant and neuroprotective activity against oxidative damage.

Nitrones are currently synthesized either by condensation of carbonyl compounds with *N*-monosubstituted hydroxylamines[14] or by oxidation of secondary amines or of the corresponding hydroxylamines.[15] The oxidative approach provides the most direct and general method for their preparation. This methodology uses hydrogen peroxide as primary oxidant in the presence of catalysts such as Na_2MoO_4 or Na_2WO_4,[15] $MeReO_3$[16] or SeO_2.[17] Very recently, alkyl hydroperoxides have been used as primary oxidant and titanium alkoxide complexes as catalyst,[18] and protected from the water formed as a co-product by the combined use of trialkanolamine ligands and molecular sieves.[18]

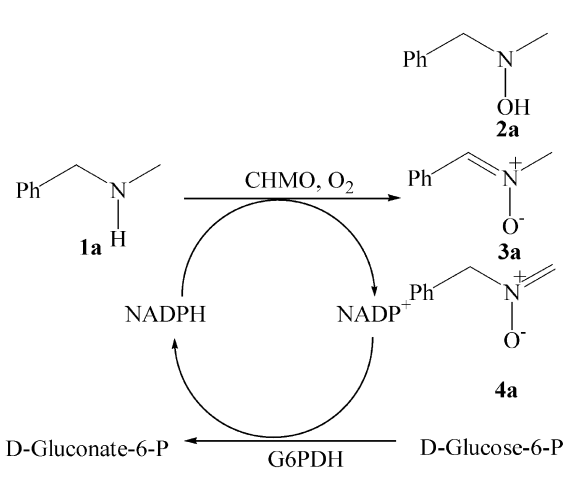
2. Results and discussion

We have examined more extensively the enzymatic oxidation of secondary amines, hydroxylamines and tertiary amines mediated by CHMO, and preliminary results have been reported.[19]

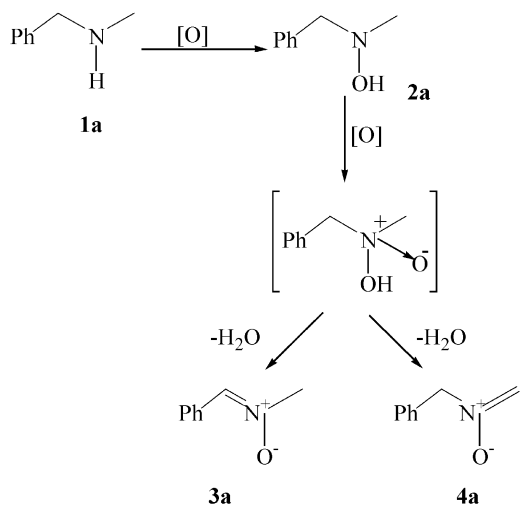
For economic reasons it is essential to regenerate NADPH in situ at the expenses of a cheap co-substrate, by means of a second enzymatic reaction This has been achieved using glucose-6-phosphate/glucose-6-phosphate dehydrogenase (G6PDH) (Scheme 1). The conversions and the identities of the products have been determined by HPLC analysis by comparison of their elution order with nitrones and hydroxylamines synthesized ad hoc. The first screening of potential amine substrates for CHMO showed that the reaction is quite sensitive to steric and electronic effects. Indeed, aliphatic amines such as diisopropylamine and pipecoline or sterically bulky amines such as dibenzylamine were not substrates for CHMO.

We have chosen *N*-methylbenzylamine (**1a**) as the model substrate in order to investigate the mechanism involved in this biooxidation. We have found that the formation of the nitrones occurs through a double oxidation, i.e., of the starting secondary amine and, then, of the hydroxylamine intermediate (**2a**) which leads to the two regioisomeric nitrones (Scheme 2). The occurrence of hydroxylamine was proved by HPLC comparison with a standard of *N*-methylbenzylhydroxylamine (**2a**). Moreover, hydroxylamine **2a** was converted by CHMO faster than the starting amine (**1a**), in agreement with its higher specificity constant value (V_{max}/K_m) (Table 1). The ratio of the two regioisomeric nitrones formed was independent of the enzymatic reaction and in favour of the most stable nitrone, in agreement with the results found by Bruice et al.[11a] in the oxidation with 4a-hydroperoxyflavin.

We have tested other substrates structurally related to the model **1a**, considering, first of all, the influence of the alkyl chain bound to the nitrogen. *N*-Ethylbenzylamine (**1b**), *N-iso*-propylbenzylamine (**1c**) and *N-n*-butylbenzylamine (**1d**) were all substrates, the specificity constant (V_{max}/K_m) order being: *N*-ethylbenzylamine>*N*-methylbenzylamine>*N-iso*-propylbenzylamine>*N-n*-butylbenzylamine (Table 1). These results were in fairly good agreement with the conversion data (Table 2). The conversions decreased when the branching of the alkyl chain was increased; *N-tert*-butylbenzylamine (**1e**) was not transformed by CHMO (Table 2). This result demonstrates the strict steric requirements of substrates. Also methyl-(1-phenyl-ethyl)-amine (**1f**) was not transformed; the substitution of a



Scheme 1. CHMO catalyzed oxidation of *N*-methylbenzylamine with in situ coenzyme regeneration.



Scheme 2. Mechanism of oxidation of *N*-methylbenzylamine by CHMO.

Table 1. Kinetic constants of CHMO with amine substrates[a]

Amines	V_{max} (μM×min^{-1})	K_m (μM)	$V_{max}/K_m \times 10^6$ (min^{-1})
N-Methylbenzylamine (**1a**)	0.21	2400	87.5
N-Methylbenzylhydroxylamine (**2a**)	0.24	2300	104.3
N-Ethylbenzylamine (**1b**)	0.20	2120	95.2
N-*iso*-Propylbenzylamine (**1c**)	0.25	4000	62.5
N-*n*-Butylbenzylamine (**1d**)	0.30	5100	58.8
N-Methyl-*p*-methoxybenzylamine (**1h**)	0.27	3170	86.4
N-Methyl-*p*-methylbenzylamine (**1i**)	0.22	2520	89.4
N-Methyl-*p*-nitrobenzylamine (**1j**)	0.21	1830	112.7
N-Methylaniline (**1l**)	0.22	2650	84.6
N,*N*-Dimethylbenzylamine (**1m**)	0.24	3060	78.4
Methyl-(4-methylthiobenzyl)-amine (**1p**)	0.40	1570	255.7

[a] For conditions see Section 3.

Table 2. CHMO catalyzed oxidation of amines

Amines	Substrate conversion (%)	Product	Product formed (%)
N-Methylbenzylamine (**1a**)	98	$C_6H_5CH_2N(OH)CH_3$ (**2a**)	80
		$C_6H_5CH{=}N(O)CH_3$ (**3a**)	10
		$C_6H_5CH_2N(O){=}CH_2$ (**4a**)	8
N-Methylbenzylhydroxylamine (**2a**)	52	$C_6H_5CH{=}N(O)CH_3$ (**3a**)	36
		$C_6H_5CH_2N(O){=}CH_2$ (**4a**)	16
N-Ethylbenzylamine (**1b**)	68	$C_6H_5CH{=}N(O)CH_2CH_3$ (**3b**)	30
		$C_6H_5CH_2N(O){=}CHCH_3$ (**4b**)	18
N-*iso*-Propylbenzylamine (**1c**)	49	$C_6H_5CH{=}N(O)CH(CH_3)_2$ (**3c**)	13
N-*n*-Butylbenzylamine (**1d**)	57	$C_6H_5CH{=}N(O)(CH_2)_3CH_3$ (**3d**)	14
		$C_6H_5CH_2N(O){=}CH(CH_2)_2CH_3$ (**4d**)	2
N-*tert*-Butylbenzylamine (**1e**)	N.R.		
Methyl-(1-phenylethyl)-amine (**1f**)	N.R		
N-Methyl-*o*-methoxybenzylamine (**1g**)	N.R		
N-Methyl-*p*-methoxybenzylamine (**1h**)	98	*p*-MeO–$C_6H_4CH{=}N(O)CH_3$ (**3h**)	10
N-Methyl-*p*-methylbenzylamine (**1i**)	68	*p*-Me–$C_6H_4CH{=}N(O)CH_3$ (**3i**)	9
N-Methyl-*p*-nitrobenzylamine (**1j**)	34	*p*-NO_2–$C_6H_4CH{=}N(O)CH_3$ (**3j**)	26
N-Methyl-*p*-chlorobenzylamine (**1k**)	N.R		
N-Methylaniline (**1l**)	60	$C_6H_5N(OH)CH_3$ (**3l**)	58
N,*N*-Dimethylbenzylamine (**1m**)	63	$C_6H_5N(O)(CH_3)_2$ (**3m**)	62
4-(Ethylaminomethyl)-pyridine (**1n**)	N.R		
Methyl-thiophen-2-ylmethylamine (**1o**)	N.R		
Methyl-(4-methylthiobenzyl)-amine (**1p**)	58	*p*-CH_3SO–$C_6H_4CH_2NCH_3$ (**5p**)	55 ee=66%

N.R.: no reaction.

benzylic hydrogen with a methyl group led to total loss of reactivity (Table 2). This fact prevented the possibility of carrying out the kinetic resolution of the starting amine.

In order to examine the stereoelectronic effects of substituents on the aromatic ring, we have investigated *N*-methyl-*o*-methoxybenzylamine (**1g**) and *N*-methyl-*p*-methoxybenzylamine (**1h**). While the *ortho*-substituted amine **1g** was recovered unchanged, the *p*-substituted one **1h** was totally transformed, giving the expected nitrone **3h** (10%) and a product deriving from the decomposition of hydroxylamine **2h** (Table 2). The instability of **2h** was demonstrated with a control experiment carried out under the same reaction conditions in the absence of CHMO. For shorter reaction times (15 min), the formation of hydroxylamine was observed together with that of its decomposition product.

The research has been extended to other substrates substituted in the para position of the aromatic ring; *N*-methyl-*p*-methylbenzylamine (**1i**) and *N*-methyl-*p*-nitrobenzylamine (**1j**) were substrates for CHMO. Again the hydroxylamine **2i** decomposed in the usual reaction conditions. In the case of *N*-methyl-*p*-chlorobenzylamine (**1k**), the starting amine was recovered unchanged. These results indicate that also the electronic requirement plays an important role. *N*-Methylaniline (**1l**) and *N*,*N*-dimethylbenzylamine (**1m**), structurally similar to the amine model **1a** were substrates for CHMO; the former reacted to give the corresponding hydroxylamine while the latter was transformed into the *N*-oxide. A further confirmation of the fact that the electronic factors are important was the total loss of reactivity in the case of 4-(ethyl-amino-methyl)-pyridine (**1n**) and of methyl-thiophen-2-yl methylamine (**1o**) (Table 2).

Finally, we took in consideration the behaviour of a substrate having two functions potentially able to undergo oxidation by CHMO, namely, two different heteroatoms, i.e. nitrogen and sulfur. Therefore, we prepared and tested methyl-(4-methylthiobenzyl)-amine (**1p**). Since the k_{cat} values of CHMO for sulfur versus nitrogen are in the 8:1 ratio,[2] a preferential attack at sulfur was expected. That was indeed the case and the corresponding sulfoxide was formed in 55% chemical yield and 66% ee (Table 2).

It should be stressed that the catalytic efficiencies of FAD-4a-hydroperoxyflavin towards secondary and tertiary amines are of the same order of magnitude (Table 1), whereas in the case of 4a-hydroperoxyflavin as oxidant, *N*,*N*-dimethyl aniline was reported to be twice as reactive with respect to *N*-methyl aniline.[12]

In conclusion, we have shown that cyclohexanone monooxygenase reacts with secondary amines, hydroxylamines and tertiary amines. The reaction has a limited versatility from the synthetic point of view since, basically, only substituted benzylic amines are accepted as substrates by the enzyme. On the other hand, we have shown that the mechanism of CHMO oxidation at nitrogen in secondary amines is similar to that of organosulfur derivatives, since both heteroatoms undergo an electrophilic attack by the terminal oxygen of the 4a-hydroperoxyflavin. In spite of the synthetic limitations, these biotransformations have a remarkable mechanistic interest if they are compared to the 4a-hydroperoxyflavin system studied by Bruice and co-workers and to hepatic flavoproteins involved in the microsomial oxidation.[12]

3. Experimental

The ^{1}H NMR spectra were recorded on a Bruker AC 300 apparatus with $CDCl_3$ as solvent. The kinetic measurements were carried out with a Jasco V-530 UV spectrophometer. Low-resolution mass spectra were run with a Fisons MD 800 spectrometer using the EI method. HPLC analyses were performed on Chiralcel OD column on a Jasco HPLC instrument (model 980-PU pump, model 975-UV detector) using *n*-hexane/2-propanol (9:1) for compounds **1a–f** and **1l–n** and *n*-hexane/2-propanol (8:2) for compounds **1g–k** and **1o–p** as the mobile phase; the flow rate was 1 ml/min and readings were made at 254 nm. Melting points were recorded on a Stuart Scientific apparatus. Chemical reactions were monitored by analytical TLC, performed on Merck silica gel 60 F_{254} plates and visualized by UV irradiation or by iodine. Columns were packed using Merck silica gel 60 (230–400 mesh) as the stationary phase and eluted using the flash chromatographic technique. CHMO was as a partially purified preparation obtained from an *E. coli* strain in which the gene of the enzyme was cloned and overexpressed.[20] Glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. *N*-Methylbenzylamine, *N*-ethylbenzylamine, *N*-*iso*-propylbenzylamine, *N*-*tert*-butylbenzylamine, *N*-*n*-butylbenzylamine, *N*-methylaniline, 4-(ethylamino-methyl)-pyridine, *N*,*N*-dimethyl-benzylamine were purchased from Sigma-Aldrich and used without further purification.

3.1. Preparation of non-commercially available secondary amines 1g–k and 1o–p

Amines **1g–k** and **1o–p** were prepared by condensation of the corresponding aldehydes with methylamine (30% in aqueous solution), followed by reduction with $NaBH_4$ according to known methods.[21] The amines were characterized as follows.

3.1.1. *N*-Methyl-*o*-methoxybenzylamine (1g). Yellow liquid, bp 104 °C (10 Torr), (lit.[22] 103 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.41 (3H, s, NCH_3), 3.74 (2H, s, CH_2N), 3.83 (3H, s, CH_3O), 6.90 (1H, m, Ar-H), 7.23 (3H, m, Ar-H). Yield=85%.

3.1.2. *N*-Methyl-*p*-methoxybenzylamine (1h). Colourless liquid, bp 106 °C (10 Torr), (lit.[23] 105 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.68 (1H, bs, N–H), 2.44 (3H, s, NCH_3), 3.68 (2H, s, CH_2N), 6.86 (2H, d, J_{H-H}=12.0 Hz, Ar-H), 7.23 (2H, d, J_{H-H}=12.0 Hz, Ar-H). Yield=95%.

3.1.3. *N*-Methyl-*p*-methylbenzylamine (1i). Orange liquid, bp 84 °C (10 Torr), (lit.[24] 83 °C, 11 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.63 (1H, bs, N–H), 2.34 (3H, s, Ar-CH_3), 2.44 (3H, s, NCH_3), 3.71 (2H, s, CH_2), 7.18 (4H, m, Ar). Yield=95%.

3.1.4. *N*-Methyl-*p*-nitrobenzylamine (1j). Orange liquid, 147 °C (10 Torr), (lit.[25] 156–158, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (1H, bs, N–H), 2.46 (3H, s, NCH_3), 3.85 (2H, s, CH_2), 7.90 (2H, d, Ar-H, J_{H-H}= 9.2 Hz), 8.18 (2H, d, Ar-H, J_{H-H}=9.2 Hz). Yield=96%.

3.1.5. *N*-Methyl-*p*-chlorobenzylamine(1k). Orange liquid, bp 119 °C (10 Torr), (lit.[25] 120–123, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.74 (1H, bs, N–H), 2.38 (3H, s, NCH_3), 3.66 (2H, s, CH_2), 7.22 (4H, m, Ar-H). Yield=76%.

3.1.6. Methyl-thiophen-2-ylmethylamine (1o). Yellow liquid, 79 °C (10 Torr), (lit.[26] 75–80 °C, 14 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (1H, bs, N–H), 2.48 (3H, s, NCH_3), 3.95 (2H, s, CH_2), 6.96–7.23 (4H, m, Ar). Yield=99%.

3.1.7. Methyl-(4-methylthiobenzyl)-amine (1p). Yellow liquid, 108 °C (10 Torr), ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.52 (1H, bs, N–H), 2.44 (3H, s, SCH_3), 2.47 (3H, s, NCH_3), 3.70 (2H, s, CH_2), 7.24 (4H, m, Ar-H). Yield=94%.

3.2. Preparation of *N*-methyl-*N*-(1-phenylethyl)amine 1f

Amine **1l** was prepared from the corresponding primary amine by condensation with ethyl-chloroformate, followed by reduction with $LiAlH_4$.[27]

The amine was characterized as follows.

3.2.1. *N*-Methyl-*N*-(1-phenylethyl)amine 1f. Pale yellow liquid, bp 82 °C (10 Torr), (lit.[28] 83–85 °C, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35 (1H, d, CH_3, J_{H-H}= 9.9 Hz), 1.57 (1H, bs, N–H), 2.30 (3H, s, NCH_3), 3.62 (1H, q, CHN, J_{H-H}=9.9 Hz), 7.30 (5H, m, Ar). Yield=57%.

3.3. General procedure for the preparation of hydroxylamines 2a and 2h–i

$NaBH_4$ (2.0 mmol) was added to a solution of the corresponding nitrone (1 mmol) in EtOH (5 ml) and the mixture was stirred at room temperature for 5 h. The EtOH was removed under reduced pressure. H_2O was added (5 ml) and the reaction mixture extracted with CH_2Cl_2 (3×15 ml). The organic layer was dried with Na_2SO_4, filtered and the

solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave hydroxylamines **2a**, **2h**, **2i**.

3.3.1. *N*-Benzyl-*N*-methylhydroxylamine 2a. Colourless solid, mp 39 °C (lit.[29] 40–41 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.58 (1H, s, NCH_3), 3.72 (1H, s, NCH_2), 7.27–7.44 (5H, m, Ar-H), OH proton signal was not observed. Yield=84%.

3.3.2. *N*-*p*-Methoxybenzyl-*N*-methylhydroxylamine 2h. White solid, mp 53–54 °C (lit.[29] 56–58 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.57 (1H, s, NCH_3), 3.67 (2H, s, NCH_2), 3.78 (3H, s, OCH_3), 7.27–7.44 (5H, m, Ar-H), OH proton signal was not observed. Yield=84%.

3.3.3. *N*-*p*-Methylbenzyl-*N*-methylhydroxylamine 2i. White solid, mp 52 °C (lit.[29] 52–54 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.32 (1H, s, Ar-CH_3), 2.57 (3H, s, NCH_3), 3.70 (2H, s, NCH_2), 7.10–7.21 (5H, m, Ar-H), OH proton signal was not observed. Yield=85%.

3.4. General procedure for the preparation of nitrones 3a–i, 3k, 3n–o, 4b, 4d and 4n

Aqueous H_2O_2 (3.06 g, 27 mmol) was added dropwise to a stirred solution of the amines **1a–i**, **1k** and **1n–o** (9.0 mmol) and $Na_2WO_4{\cdot}2H_2O$ (0.148 mg, 0.45 mmol) in MeOH (20 ml) with ice cooling The reaction mixture was stirred at room temperature for 3–12 h until TLC (CH_2Cl_2/MeOH, 95:5) revealed the complete disappearance of the amine. MeOH was removed under reduced pressure. The reaction mixture was washed with a saturated solution of Na_2SO_3 (15 ml) and extracted with CH_2Cl_2 (3×30 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave the nitrones **3a–i**, **3k**, **3n–o**, **4b**, **4d** and **4n**.

3.4.1. *N*-Benzylidenemethylamine *N*-oxide 3a. White solid, mp 82–83 °C (lit.[30] 84 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (1H, s, CH_3), 7.37 (4H, m, Ar-H and CHN), 8.22 (2H, m, Ar-H). Yield=53%.

3.4.2. *N*-Benzylideneethylamine *N*-oxide 3b. Pale yellow liquid, bp 118 °C (0.8 Torr), (lit.[31] 116 °C, 0.8 Torr). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.53 (3H, t, CH_3, J_{H-H}=7.0 Hz), 3.93 (2H, q, NCH_2, J_{H-H}=7.0 Hz) 7.33 (4H, m, Ar-H and CHN), 8.22 (2H, m, Ar-H). Yield=56%.

3.4.3. *N*-Ethylidenebenzylamine *N*-oxide 4b. Pale yellow solid, mp 76 °C (lit.[32] 77 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.95 (3H, d, CH_3, J_{H-H}=7.0 Hz), 4.89 (2H, s, NCH_2), 6.72 (2H, q, CHN, J_{H-H}=7.0 Hz), 7.37 (5H, m, Ar-H). Yield=15%.

3.4.4. *N*-Benzylideneisopropylamine *N*-oxide 3c. Yellow liquid, bp 119 °C (0.8 Torr), (lit.[31] 118 °C, 0.8 Torr). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50 (6H, d, CH_3, J_{H-H}=6.7 Hz), 4.17 (1H, q, NCH, J_{H-H}=6.7 Hz) 7.37 (4H, m, Ar-H and CHN), 8.24 (2H, m, Ar-H). Yield=48%.

3.4.5. *N*-Benzylidene-*n*-butylamine *N*-oxide 3d. Pale yellow solid, mp 72–73 °C (lit.[33] 73 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.94 (3H, t, CH_3, J_{H-H}=7.0 Hz), 1.44 (2H, sest., CH_2, J_{H-H}=7.0 Hz), 1.96 (2H, quint., CH_2, J_{H-H}=7.0 Hz), 3.97 (2H, t, NCH_2, J_{H-H}=7.0 Hz), 7.33 (4H, m, Ar-H and CHN), 8.24 (2H, m, Ar-H). Yield=28%.

3.4.6. *N*-Butylidenbenzylamine *N*-oxide 4d. Pale yellow solid, mp 74–75 °C (lit.[34] 76 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.86 (3H, t, CH_3, J_{H-H}=7.3 Hz), 1.44 (2H, sest., CH_2, J_{H-H}=7.3 Hz), 2.37 (2H, q, CH_2, J_{H-H}=7.0 Hz), 4.80 (2H, s, Ar-CH_2), 6.63 (1H, t, CHN, J_{H-H}=7.3 Hz), 8.24 (2H, m, Ar-H). Yield=42%.

3.4.7. *N*-Benzylidene-*tert*-buthylamine *N*-oxide 3e. White solid, mp 72–74 °C (lit.[35] 75–76 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (9H, s, CH_3,) 7.41 (3H, m, Ar-H) 7.54 (1H, s, CHN), 8.29 (2H, m, Ar-H). Yield=91%.

3.4.8. *N*-Methyl-(1-phenylethylidene) amine *N*-oxide 3f. Pale yellow solid, mp 114–115 °C (lit.[36] 115 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.43 (1H, s, CH_3), 3.63 (1H, s, NCH_3), 7.23–7.44 (5H, m, Ar-H). Yield=54%.

3.4.9. 2-Methoxybenzylidenemethylamine *N*-oxide 3g. White solid, mp 84 °C (lit.[30] 85 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.77 (3H, s, NCH_3), 3.82 (3H, s, OCH_3), 6.81–6.97 (2H, m, Ar-H), 7.20 (1H, *dt*, Ar-H, J_{H-H}=9.9, 2.7 Hz) 7.78 (1H, s, CHN), 9.20 (1H, dd, Ar-H, J_{H-H}=11.9, 2.7 Hz). Yield=78%.

3.4.10. 4-Methoxybenzylidenemethylamine *N*-oxide 3h. White solid, mp 75 °C (lit.[30] 76 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.81 (6H, bs, CH_3), 6.90 (2H, d, Ar-H, J_{H-H}=9.0 Hz) 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=91%.

3.4.11. 4-Methylbenzylidenemethylamine *N*-oxide 3i. White solid, mp 117 °C (lit.[37] 119 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (3H, s, Ar-CH_3), 3.83 (3H, s, NCH_3), 6.92 (2H, d, Ar-H, J_{H-H}=9.0 Hz), 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=68%.

3.4.12. 4-Chlorobenzylidenemethylamine *N*-oxide 3k. White solid, mp 126 °C (lit.[30] 128 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (3H, s, NCH_3), 7.37 (3H, m, Ar-H and CHN), 8.18 (2H, d, Ar-H, J_{H-H}=11.0 Hz). Yield=64%.

3.4.13. *N*-Ethyl-4-pyridylmethylidene *N*-oxide 3n. Orange liquid, bp 125 °C, 10 Torr. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.97 (3H, t, CH_3, J_{H-H}=7.3 Hz), 3.97 (2H, q, NCH_2, J_{H-H}=7.3 Hz), 7.41 (1H, s, NCH), 7.96 (2H, d, Ar-H, J_{H-H}=8.0 Hz), 8.63 (2H, d, Ar-H, J_{H-H}=8.0 Hz). EI MS: *m*/*z* (%)=150.1 (100) [M^+]. Yield=44%.

3.4.14. *N*-Ethylidenepyridyl-4-methylamine *N*-oxide 4n. Orange solid, mp 82 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.96 (3H, d, CH_3, J_{H-H}=6.0 Hz), 4.83 (2H, s, NCH_2), 6.87 (1H, q, NCH, J_{H-H}=6.0 Hz), 7.25 (2H, d, Ar-H, J_{H-H}=7.5 Hz), 8.52 (2H, d, Ar-H, J_{H-H}=7.5 Hz). EI MS: *m*/*z* (%)=150.1 (100) [M^+]. Yield=22%.

3.4.15. *N*-Methyl-thienyl-2-methyleneamine *N*-oxide 3o.

Pale yellow solid, mp 118–119 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.82 (3H, s, CH_3), 7.12 (1H, dd, Ar-H, J_{H-H}=4.8, 4.0 Hz), 7.38 (1H, d, Ar-H, J_{H-H}=4.0 Hz), 7.42 (1H, d, Ar-H, J_{H-H}=4.8 Hz), 7.83 (1H, s, CHN). EI MS: *m/z* (%)=141.1 (100) [M^+]. Yield=94%.

3.5. General procedure for the preparation of nitrones 3j and 3p

The appropriate aldehyde (1.0 mmol) was added to a solution of *N*-methylhydroxylamine hydrochloride (1.0 mmol) and sodium acetate (1.1 mmol) in EtOH (10 ml). The reaction mixture was stirred at 50–60 °C for 3 h. EtOH was removed under reduced pressure, water was added (5.0 ml) and the reaction mixture extracted with CH_2Cl_2 (3×10 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave nitrones **3j** and **3p**.

3.5.1. 4-Nitrobenzylidenemethylamine *N*-oxide 3j. Yellow solid, mp 205 °C (lit.[30] 208 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (3H, s, Ar-CH_3), 3.83 (3H, s, NCH_3), 6.92 (2H, d, Ar-H, J_{H-H}=9.0 Hz), 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=68%.

3.5.2. 4-Methylthiobenzylidenemethylamine *N*-oxide 3p. White solid, mp 103–104 °C (lit.[38] 105 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.45 (3H, s, SCH_3), 3.80 (3H, s, NCH_3), 7.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz) 7.27 (1H, s, CHN), 8.09 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=78%.

3.6. Preparation of (4-methanesulfinyl-benzyl)-methyl-amine 5p

Methyl-(4-methylthiobenzyl)-amine (0.420 g, 2.50 mmol) was added to a solution of $NaIO_4$ (0.560 mg, 2.60 mmol) in H_2O (25 ml) cooled with an ice bath. The reaction mixture was stirred at 0 °C for 15 h and then filtered through a Büchner funnel. The filter cake was washed with 50 ml of CH_2Cl_2. The filtrate was extracted with CH_2Cl_2 (2×25 ml), the organic layer was dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography with CH_2Cl_2/MeOH (7:3) gave **5l** (yield=45%).

3.6.1. (4-Methanesulfinyl-benzyl)-methyl-amine 5p. Yellow liquid, bp 135 °C (10 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.45 (1H, s, NCH_3), 2.70 (3H, s, SCH_3), 3.81 (2H, s, NCH_2), 7.48 (2H, d, Ar-H, J_{H-H}=9.2 Hz), 7.59 (2H, d, Ar-H, J_{H-H}=9.2 Hz). EI MS: *m/z* (%)=183.1 (100) [M^+]. Yield=45%.

3.7. General procedure for enzymatic oxidation

The amines (15 mM) were reacted, at 25 °C, under stirring, in 0.85 ml of 0.05 M Tris–HCl buffer, pH 8.6, containing $NADP^+$ (0.5 mM), 5 units of CHMO, glucose-6-phosphate (50 mM) and 18 units of glucose-6-phosphate dehydrogenase (G6PDH) that served to regenerate the cofactor. After 24 h, the reaction mixtures were extracted with AcOEt (3×1 ml). The solvent was removed with gentle stream of N_2 and the product analyzed.

3.8. General procedure to determine the kinetic constants

The experiments were carried out in 50 mM Tris–HCl buffer at pH 8.6, 25 °C, in 1 ml cuvettes, 1 cm path length. The reaction mixture contained CHMO (30 milliunits), 0.1 mM NADPH and 0.4–5 mM substrate. The consumption of NADPH was spectrophotometrically monitored at 340 nm.

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Regioselective biocatalytic hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile for production of (*E*)-2-methyl-2-butenoic acid

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Abstract—*Acidovorax facilis* 72W nitrilase catalyzed the regioselective hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile, producing only (*E*)-2-methyl-2-butenoic acid with no detectable conversion of (*Z*)-2-methyl-2-butenenitrile. (*E*)-2-Methyl-2-butenoic acid, produced in aqueous solution as the ammonium salt, was readily separated from (*Z*)-2-methyl-2-butenenitrile, and isolated in high yield and purity. The combination of nitrile hydratase and amidase activities of several *Comamonas testosteroni* strains were also highly regioselective for the production of (*E*)-2-methyl-2-butenoic acid from (*E*,*Z*)-2-methyl-2-butenenitrile.

1. Introduction

(*E*)-2-Methyl-2-butenoic acid (**1**), commonly known as tiglic acid, and (*Z*)-2-methyl-2-butenoic acid (**2**), commonly known as angelic acid, are useful starting materials for the preparation of flavor and fragrances, and in the preparation of pharmaceutical intermediates. **1**[1–4] and **2**[1,4,5] have each been prepared by a variety of chemical methods, including oxidation of the corresponding (*Z*)-alcohol, dehydration/hydrolysis of 2-hydroxy-2-methylbutyronitrile, carboxylation of 2-butenyl-2-lithium, and carbonylation of 2-chloro-2-butene or 2-bromo-2-butene. 2-Methyl-3-butenenitrile (**3**), a commercially-available by-product of adiponitrile manufacture,[6] can be readily isomerized to a mixture of (*E*)-2-methyl-2-butenenitrile (**4**) and (*Z*)-2-methyl-2-butenenitrile (**5**) using basic alumina,[7] but separation of this mixture by distillation prior to hydrolysis of the individual nitriles is difficult due to the similar chemical properties of the geometric isomers. Chemical processes for nitrile hydrolysis are not known to result in the regioselective hydrolysis of mixtures of geometric isomers; for example, reaction of (*E*,*Z*)-2-methyl-2-butenenitrile (**6**) with sulfuric acid at elevated temperatures followed by distillation produced a mixture of 19.5% **1** and 80.5% **2**.[8]

Enzyme-catalyzed hydrolysis of nitriles to the corresponding carboxylic acids is often preferred to chemical methods because the reactions are often run at ambient temperature, do not require the use of strongly acidic or basic reaction conditions, and produce the desired product with high selectivity at high conversion.[9] Regioselectivity for hydrolysis of the (*E*)-isomer of several (*E*,*Z*)-3-substituted-acrylonitriles has been demonstrated using the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana*.[10] Nitrilase substrate specificity for α,β-unsaturated nitriles can be fairly limited; *Bacillus pallidus* Dac521 nitrilase showed no activity for hydrolysis of (*Z*)-2-pentenenitrile (**7**), but was capable of hydrolyzing acrylonitrile, methacrylonitrile, or crotononitrile.[11] The combined nitrile hydratase and amidase activities of *Rhodococcus* sp. AJ270 has been used for the enantioselective biotransformation of racemic β-substituted-α-methylenepropionitriles, where the amidase was shown to discriminate between the two amide hydration products produced by the nitrile hydratase.[12] The present report describes the use of *Acidovorax facilis* 72W nitrilase (Scheme 1), or the combination of nitrile hydratase and amidase activities of several strains of *Comamonas testosteroni*, for the facile preparation of **1** from **6**.

2. Results and discussion

2.1. Regioselective hydrolysis of 6 by *Acidovorax facilis* 72W nitrilase

The reaction of **4**, **5** and **6** with *A. facilis* 72W nitrilase was first performed using intact cells of *E. coli* SS1001,[13] a recombinant catalyst which expresses *A. facilis* 72W nitrilase. **4** was completely hydrolyzed to **1**, whereas under identical reaction conditions there was no detectable

Keywords: Nitrile hydratase; Amidase; Nitrilase; Enzyme catalysis; Tiglic acid; Angelic acid.
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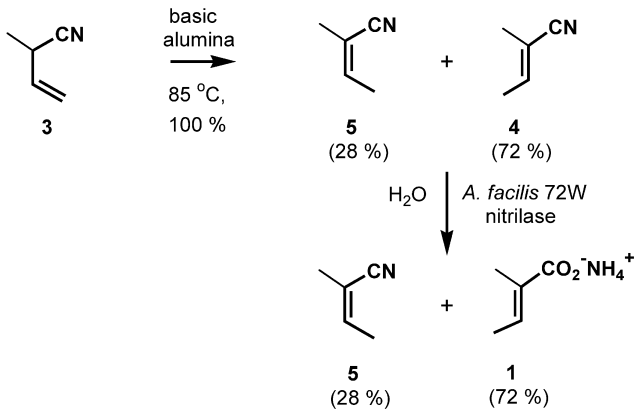

Scheme 1. Conversion of **3** to **1** and **5** by racemization of **3** to **6**, and subsequent regioselective hydrolysis of **4** using *A. facilis* 72 W nitrilase.

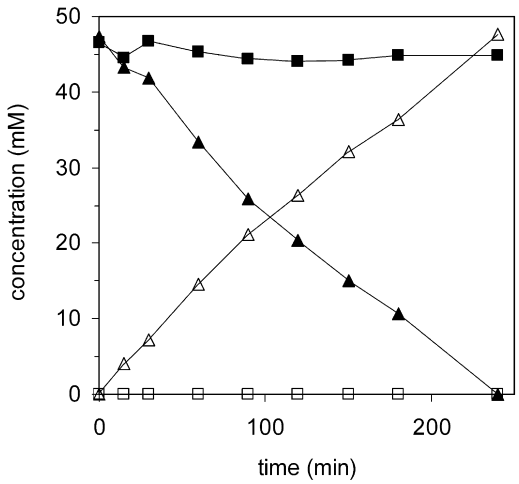

Figure 1. Time course for reaction of **6** ([**4**]=47 mM, [**5**]=47 mM) with *E. coli* SS1001 (9.5 mg dcw/mL) at 25 °C; **4** (▲), **5** (■), **1** (△), **2** (□).

hydrolysis of **5** (Table 1), even at extended reaction times. *E. coli* SS1001 also hydrolyzed only the (*E*)-isomer of a 1:1 mixture of **6** prepared from the two individual geometric isomers (Fig. 1), and a similar result was obtained using a 72:28 mixture of **6** prepared by isomerization of **3**. Several additional nitriles were tested as substrates for the *A. facilis* 72W nitrilase in order to determine if the presence or absence of an α-alkyl substituent, or the geometric isomerism of α,β-unsaturated nitriles, were a contributing factor for *A. facilis* 72W nitrilase substrate specificity. There was no regioselectivity observed for hydrolysis of a mixture of (*E*, *Z*)-2-pentenenitrile (**8**), and both isomers were converted to the corresponding carboxylic acids (*E*)-2-pentenoic acid (**9**) and (*Z*)-2-pentenoic acid (**10**) at similar rates. **3** was completely converted to 2-methyl-3-butenoic acid (**11**) at a significantly faster rate than for **4**.

A. facilis 72 W nitrilase was previously shown to regioselectively hydrolyze 2-methylglutaronitrile (**12**) or 2-methyleneglutaronitrile (**13**), with little or no hydrolysis of the cyano group adjacent to the 2-methyl- or 2-methylene substituent of these two dinitrile substrates.[14] Hydrolysis of **12** produced 4-cyanopentanoic acid (**14**, >98% yield) and 2-methylglutaric acid (**15**, <2% yield) with no detectable production of 2-methyl-4-cyanobutanoic acid at complete conversion of **12**, whereas **13** produced 4-cyano-4-pentenoic acid (**16**) as the only hydrolysis product, with no detectable hydrolysis of the cyano group vicinal to the α-methylene substituent at extended reaction times. This nitrilase has now been found to completely hydrolyze **3**, and to show no regioselectivity for hydrolysis of **8** (*E*/*Z*=22:78); in contrast, the AtNit1 nitrilase from *Arabidopsis thaliana* was completely (*E*)-regioselective for the hydrolysis of (*E*,*Z*)-2-butenenitrile.[10] The presence or absence of a methyl or methylene substituent at the α-position relative to a cyano group, and the geometric isomerism of α,β-unsaturation are each not independently predictive of the substrate specificity of *A. facilis* 72W nitrilase. The lack of significant hydrolysis of the more sterically-hindered cyano group of **12** or **13** was apparently related to the presence of a second, less-hindered cyano group, and not the presence of the α-methyl or α-methylene substituent.

2.2. Immobilized-cell nitrilase reactions

E. coli SS1001 cells were immobilized in both alginate[15] and carrageenan beads,[16] and *A. facilis* 72 W cells were immobilized in alginate beads,[15] and the resulting immobilized cell catalysts examined for the hydrolysis of **4** in **6** (*E*/*Z*=72:28) (Table 2). At concentrations of **6** greater than ca. 1.1 wt% (0.14 M) at 25 °C, the aqueous phase of the reaction mixture was saturated with **6**, and a second organic

Table 1. Regioselectivity of *E. coli* SS1001 and *A. facilis* 72W nitrilase

Substrate	Conc. (mM)	Catalyst	mg dcw/mL[a]	Temp. (°C)	Time (h)	Product or recovered substrate (% yield)[b]
4	99	*E. coli* SS1001	9.5	35	2.5	**1** (98)
5	98	*E. coli* SS1001	9.5	35	3	**5** (100)
6 (*E*/*Z*=1:1)	95	*E. coli* SS1001	9.5	25	4	**1** (99), **5** (100)
6 (*E*/*Z*=1:1)	199	*E. coli* SS1001	9.5	35	6	**1** (99), **5** (100)
6 (*E*/*Z*=72:28)	101	*E. coli* SS1001	9.5	25	6	**1** (96), **5** (100)
6 (*E*/*Z*=72:28)	104	*A. facilis* 72W[c]	12	25	16	**1** (100), **5** (100)
7	99	*E. coli* SS1001	9.5	35	2	**10** (100)
8 (*E*/*Z*=22:78)	101	*E. coli* SS1001	9.5	35	4	**9** (100), **10** (100)
3	101	*E. coli* SS1001	9.5	35	1	**11** (100)
12	100	*E. coli* SS1001	10	25	1	**14** (98.7), **15** (1.3)
13	100	*A. facilis* 72W[c]	13	27	2	**16** (100)

[a] Dry cell weight (dcw) *E. coli* SS1001 or *A. facilis* 72W unimmobilized cells.
[b] For reactions with **6** and **8**, yields were based on initial concentration of corresponding isomer.
[c] A suspension of *A. facilis* 72W cells in 0.35 M phosphate buffer (pH 7.0) was heated at 50 °C for 0.5 h prior to use to inactivate undesirable nitrile hydratase/amidase activity.[14]

Table 2. Regioselective hydrolysis of **6** using immobilized nitrilase catalysts

Substrate	Conc. (M)	Catalyst[a,b]	Temp. (°C)	Time (h)	Product or recovered substrate (% yield)[c]
6 (*E*/*Z*=72:28)	2.00	*E. coli* SS1001/alginate beads	35	22	**1** (100), **5** (100)
6 (*E*/*Z*=72:28)	0.40	*E. coli* SS1001/carrageenan beads	35	18	**1** (96), **5** (100)
6 (*E*/*Z*=72:28)	0.40	*A. facilis* 72W/alginate beads	35	18	**1** (100), **5** (100)

[a] 7.5% dry cell weight (dcw) *E. coli* SS1001 or *A. facilis* 72W immobilized in 2.75 wt% alginate or 3.0 wt% carrageenan.
[b] 20 wt% immobilized cell catalyst beads in reaction mixture.
[c] Yields based on initial concentration of corresponding isomer.

Table 3. Hydrolysis of **4**, **5**, and **6** at 25 °C using *C. testosteroni* microbial cell catalysts

Substrate	Conc. (M)	*C. testosteroni* catalyst	mg dcw/mL[a]	Time (h)	Product or recovered substrate (% yield)[b]
4	0.10	5-MGAM-4D	8	0.25	**1** (100)
5	0.10	5-MGAM-4D	8	0.25	**5** (73), **18** (27), **2** (0)
4	0.10	S5C	10	2.5	**1** (99)
5	0.10	S5C	10	2.5	**5** (75), **18** (23), **2** (0)
4	0.10	S2B-1	12	1.5	**1** (99)
5	0.10	S2B-1	2.4	1.5	**5** (92), **18** (8), **2** (0),
6 (*E*/*Z*=72:28)	0.10	S2B-1	1.2	47	**1** (100), **5** (18), **18** (75), **2** (0)

[a] Dry cell weight (dcw) *C. testosteroni* unimmobilized cells.
[b] For hydrolysis of **6**, yields were based on initial concentration of corresponding isomer.

phase consisting of undissolved **6** was present; rapid stirring of this mixture produced an emulsion of **6** in the aqueous phase containing the suspended catalyst beads, and complete conversion of the **4** was obtained. Reactions employing the immobilized-cell nitrilase catalysts were run in the absence of added buffer. As the reaction proceeded, the reaction mixture was self-buffering at a pH of ca 7.2 due to the formation of the ammonium salt of **1**, and the reaction was run to complete conversion of **4** with no pH control. At the end of a reaction, the catalyst was recycled by decanting the product mixture from the immobilized-cell catalyst and adding a fresh charge of water and **6** (*E*/*Z*=72:28); no significant loss of activity of the catalyst over 2–3 catalyst recycles was observed.

The nitrilase activity of the immobilized cell biocatalysts was stable at concentrations of **6** as high as 2.0 M (as a two-phase reaction) and to similar concentrations of **1** over the course of a single reaction, as well as over the course of a series of reactions which employed catalyst recycle. The nitrilase utilizes the sulfhydryl group of a cysteine in the active site of the enzyme for its catalytic activity,[13] and α,β-unsaturated nitriles, amides and carboxylic acids can be highly reactive towards sulfhydryl groups, irreversibly inactivating the enzyme.[17] The lack of significant inactivation of enzyme activity by the starting material or product is an indication of the robustness of *A. facilis* 72W nitrilase in the present application. The production of **1** as the ammonium salt allows for both the facile removal of unreacted **5** by extraction, and for recovery of **1** in the acid form in high yield and purity. Although not demonstrated in the present work, it should be possible to recover unreacted **5** in high purity, and convert it to **2** using an appropriate biological or chemical catalyst.

2.3. Regioselective hydrolysis of 6 using microbial nitrile hydratase/amidase catalysts

Microbial cell catalysts having a combination of nitrile hydratase and amidase activities can also be used to separate **6** into a mixture of products that are readily separated by extraction. The nitrile hydratase of *Comamonas testosteroni* 5-MGAM-4D,[14] *C. testosteroni* S5C, and *C. testosteroni* S2B-1 were each found to have significantly greater specific activity for hydration of **4** to (*E*)-2-methyl-2-butenamide (**17**) than for hydration of **5** to (*Z*)-2-methyl-2-butenamide (**18**). Similarly, the amidase of these three microbial catalysts were each found to have significantly greater specific activity for hydrolysis of **17** to **1** than for hydrolysis of **18** to **2** (Table 3). A time course for the reaction of **6** (*E*/*Z*=72:28) with *C. testosteroni* S2B-1 is presented in Figure 2. S2B-1 completely hydrolyzed both geometric isomers of **8** (*E*/*Z*=22:78) to the corresponding acids, so regioselectivity of these nitrile hydratase/amidase biocatalysts was limited to (*E*)-and (*Z*)-2-methyl-substituted α,β-unsaturated nitriles.

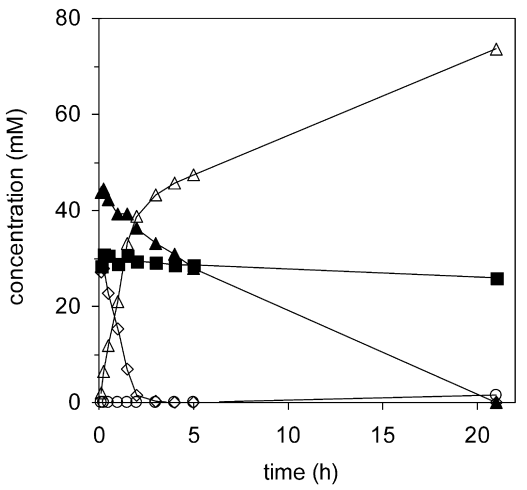

Figure 2. Time course for reaction of **6** ([**4**]=74 mM, [**5**]=29 mM) with *C. testosteroni* S2B-1 (1.2 mg dcw/mL) at 25 °C; **4** (▲), **5** (■), **1** (△), **18** (○), **17** (◇).

C. testosteroni S2B-1 and *C. testosteroni* S5C were each isolated and characterized for the first time in this application. The nitrile hydratase and amidase activities of *C. testosteroni* 5-MGAM-4D have previously been reported as catalysts for the regioselective hydrolysis of aliphatic α,ω-dinitriles to the corresponding ω-cyanocarboxylic acids.[14] In that application, *C. testosteroni* 5-MGAM-4D exhibited both non-regioselective and regioselective nitrile hydratase activities, and heating a suspension of *C. testosteroni* 5-MGAM-4D at 50 °C for 30–60 min was required to inactivate the non-regioselective nitrile hydratase activity of the microbial cell catalyst. For regioselective production of **1** by hydrolysis of **6**, no heat-treatment of *C. testosteroni* 5-MGAM-4D cells was required.

3. Conclusions

A method has been developed for the facile preparation of **1** from **6**, where **6** was first prepared by the facile isomerization of commercially-available **3**. Although *C. testosteroni* microbial cell catalysts had higher specific activities for hydrolysis of **4**, regioselective hydrolysis of **6** by *A. facilis* 72W nitrilase was preferred over the combination of nitrile hydratase and amidase activities of *C. testosteroni* 5-MGAM-4D, S2B-1 or S5C, since the latter catalysts each produced significant conversion of **5** to the corresponding amide at complete conversion of **4** to **1**, whereas there was no detectable conversion of **5** by the nitrilase at extended reaction times. The immobilized-cell nitrilase catalysts were robust under reaction conditions employing high concentration of **6** and catalyst recycle, and high yields of **1** were obtained with the added advantages of low temperature and energy requirements, and low waste production when compared to chemical methods of nitrile hydrolysis.

4. Experimental

4.1. General

Chemicals were obtained from commercial sources unless otherwise noted, and used as received. Isolated yields are unoptimized and melting points uncorrected. **3**[18] and **4**[18] were each isolated from a mixture of **6** by fractional distillation under vacuum using a 10-plate Oldershaw column and a reflux ratio of >10:1. The calculated recovery of nitriles and yields of the hydrolysis products were based on initial nitrile concentration, and determined by HPLC using a refractive index detector and either a Supelcosil LC-18 DB column (30 cm×4.6 mm dia.) and 10 mM acetic acid/10 mM sodium acetate in 2.5% methanol/water as mobile phase (for hydrolysis of **4** and **6**), or a Supelcosil LC-18 DB column (15 cm×4.6 mm dia.) and 10 mM acetic acid/10 mM sodium acetate in 7.5% methanol/water as mobile phase (for hydrolysis of **3**, **7**, and **8**). Gas chromatographic analysis of nitriles was performed on a J&W Scientific DB1701 column (30 m, 0.53 mm ID, 1 μm film thickness). Chemical shifts for ^{1}H and ^{13}C NMR spectra are expressed in parts per million positive values downfield from internal TMS. Identification of hydrolysis products of **6**, **3** and **8** were made by comparison of HPLC retention times to commercially available samples.

The isolation and growth of *A. facilis* 72W,[14,19] *E. coli* SS1001,[13] and *C. testosteroni* 5-MGAM-4D[14] has been reported. *C. testosteroni* S2B and S5C were isolated from soil samples using standard enrichment procedures and S12-N medium (S12 medium with ammonium sulfate replaced with sodium sulfate),[20] then grown aerobically in E2 medium containing 0.2% (w/v) 3-hydroxyvaleronitrile at 30 °C. Cell paste isolated from fermentation was frozen at −80 °C without pre-treatment with glycerol or DMSO. Wet cell weights (wcw) of microbial catalysts were obtained from cell pellets prepared by centrifugation of fermentation broth or cell suspensions in buffer. Dry cell weights (dcw) were determined by microwave drying of wet cells. Microbial cell enzyme activity was measured by stirring a suspension of 8.5–12.5 mg dry cell weight/mL in 25 mM phosphate buffer (pH 7.0) and 0.14 M substrate at 25 °C. *E. coli* SS1001 cells were immobilized in both alginate[15] and carrageenan beads,[16] and *A. facilis* 72 W cells were immobilized in alginate beads,[15] using previously reported procedures.

4.2. Isomerization of 2-Methyl-3-butenenitrile (3)

A mixture of **3** (50 g, 0.62 mol) and activity I basic alumina (5 g) was heated with stirring at 85 °C. After 18 h, the mixture was cooled to ambient temperature and filtered to yield 49 g (98% isolated yield) of a 72:28 (mole/mole) mixture of (*E*)- and (*Z*)-2-methyl-2-butenenitrile, determined by analysis of the reaction product by gas chromatography.

4.3. Isomerization of (*Z*)-2-pentenitrile (7)

A mixture of **7** (402.7 g, 4.96 mol), triphenylphosphine (16.0 g, 61.1 mmol), and zinc chloride (8.4 g, 61.6 mmol) (anhydrous) was heated at 70 °C for 22 h. The resulting mixture was cooled to ambient temperature, the insoluble zinc chloride filtered from the mixture, and the mixture vacuum distilled at 82 °C and 130 Torr to separate the mixture of (*Z*)- and (*E*)-2-pentenitriles from triphenylphosphine and soluble zinc chloride. The resulting distillate was analyzed by gas chromatography, using authentic samples of (*Z*)- and (*E*)-2-pentenitrile as standards; the ratio of (*Z*)- and (*E*)-isomers in the distillate was 78:22 (mole:mole).

4.4. General procedure for reactions using unimmobilized cells

In a typical procedure, an aqueous solution (10.0) mL containing 0.10 M nitrile, cell paste (0.50 g wcw) and 50 mM potassium phosphate buffer (pH 7.0) was stirred at 35 °C At pre-determined times, a 0.100 mL aliquot of the reaction was removed and mixed with 0.900 mL of 60 mM *N*-ethylacetamide (HPLC external standard) in 1:1 acetonitrile:methanol, the sample centrifuged, and the supernatant analyzed by HPLC. Results are tabulated in Tables 1 and 3.

4.5. Immobilized-cell catalyst recycle reactions

In a typical procedure, distilled, deionized water (15.0 mL),

0.20 M calcium acetate buffer (0.2 mL) (pH 7.0, 2.0 mM final calcium ion concentration in reaction mixture), glutaraldehyde/polyethylenimine-crosslinked *E. coli* SS1001 cell/alginate beads (4.0 g), and **6** (0.802 mL, 0.656 g) of (*E*/*Z*=72:28, 0.404 M total concentration) were placed in a 50 mL jacketed reaction vessel equipped with an overhead stirrer (temperature-controlled at 35 °C with a recirculating temperature bath), and the mixture stirred at 35 °C. After 20 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 100 and 0%, respectively. The product mixture was decanted from the catalyst beads, and distilled, deionized water (15.1 mL), 0.20 M calcium acetate buffer (pH 7.0) (0.2 mL) and **6** (0.809 mL, 0.662 g, 0.407 M) (*E*/*Z*=72:28) was again mixed with the immobilized-cell catalyst at 35 °C. After 20 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 97 and 0%, respectively. At the completion of the second reaction with catalyst recycle, the final concentrations of **1** and **5** were 357 and 78.9 mM, respectively.

4.5.1. Isolation of (*E*)-2-methyl-2-butenoic acid (1) from hydrolysis of (*E*,*Z*)-2-methyl-2-butenenitrile (6) with alginate-immobilized *E. coli* SS1001 cells. Into a 250 mL jacketed reaction vessel equipped with an overhead stirrer (temperature-controlled at 35 °C with a recirculating temperature bath) was placed GA/PEI-crosslinked *E. coli* SS1001 cell/alginate beads (30 g). To the reaction vessel was added distilled, deionized water (98.5 mL), 0.20 M calcium chloride (2.0 mM final calcium ion concentration) (1.5 mL) and **6** (20.0 mL, 16.32 g, 0.201 mole) (*E*/*Z*=72:28, 1.34 M total concentration), and the mixture stirred at 35 °C. After 22 h, the conversions of **4** and **5** were 100 and 0%, respectively, and the yields of **1** and **2** were 100 and 0%, respectively. The product mixture was decanted from the immobilized cell catalyst, and the catalyst washed twice with aqueous 2 mM calcium chloride (75 mL). The combined aqueous washes and decantate were extracted twice with ethyl ether (100 mL) to remove unreacted **5**, then the aqueous phase was adjusted to pH 1.5 with concentrated hydrochloric acid. After saturation with sodium chloride, the aqueous phase was extracted four times with ethyl ether (100 mL), the organic extracts combined, dried with magnesium sulfate, and the solvent removed by rotary evaporation. The resulting white solid was dried to constant weight under vacuum to yield (*E*)-2-methyl-2-butenoic acid (13.2 g, 90% isolated yield based on **4**): mp 63.6–64.8 °C (reported 64.5–65.5 °C);[4] ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.81–1.87 (6 H, m, H_3CCCO_2H, $HCCH_3$), 6.99–7.03 (1 H, d of quartets, J=7.0, 1.3 Hz, $HCCH_3$), 12.26 (1 H, bs, CO_2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 11.6, 14.6, 128.2, 140.0, 173.9; MS (CI) *m*/*z* 101 (MH^+, 100), 100 (30), 83 (72), 69 (58); HRMS calcd for $C_5H_9O_2$ (MH^+) 101.0603, found 101.0605.

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Chemo-enzymatic enantio-convergent asymmetric synthesis of (*R*)-(+)-Marmin

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Abstract—Asymmetric biohydrolysis of trisubstituted terpenoid oxiranes (*rac*-**1a**–*rac*-**3a**) was accomplished by employing the epoxide hydrolase activity *Rhodococcus* and *Streptomyces* spp. Depending on the biocatalyst, the biohydrolysis proceeded in an enantio-convergent fashion and gave the corresponding *vic*-diols in up to 97% ee at conversions beyond the 50%-threshold. In order to avoid a depletion of the ee of product by further oxidative metabolism, bioconversions had to be conducted in an inert atmosphere with exclusion of molecular oxygen. The synthetic applicability of this method was demonstrated by the asymmetric total synthesis of the monoterpenoid coumarin (*R*)-(+)-Marmin in 95% ee.

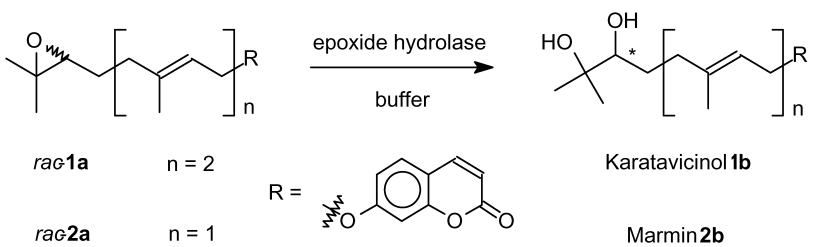
1. Introduction

A great variety of natural products possessing an isoprenylated coumarin structure of the umbelliferone family, such as Karatavicinol (**1b**) and Marmin (**2b**) have been isolated from various biological sources, such as roots, bark and fruit peel of various plants indigenous to Central Asia. They were found to be constituents of gum resins derived by incision of roots, which are applied in traditional medicine.[1,2] The sesquiterpenoid coumarin Karatavicinol[3] (**1b**) was isolated and fully characterised from *Ferula sinaica*.[1] The monoterpenoid analogue—Marmin (**2b**)[4]—was isolated from the bark of *Aegle marmelos*[4] and grapefruit peel oil[5] and was shown to be (*R*)-configurated. The (*S*)-(−)-antipode was found in *Pituranthos tridadiatus*.[6] (*R*)-(+)-Marmin has been synthesised through multistep sequences from L-glutamic acid[7] and via Sharpless epoxidation.[8] A recent more elegant approach involved a microbial 'dihydroxylation' of 7-geranyloxy-coumarin (aurapten) by *Aspergillus niger*.[9]

In light of the general nature of microbial oxidative pathways,[10,11] the latter pathway occurs presumably via epoxidation (catalysed by cytochrome P450 monooxygenases[12]) followed by hydrolytic ring-opening involving an epoxide hydrolase.[13]

2. Results and discussion

Based on the highly selective asymmetric biohydrolysis of trisubstituted oxiranes catalysed by bacterial epoxide hydrolases,[14] we envisaged that this strategy might be applicable to the synthesis of natural products bearing an isoprenoid (geranyl or farnesyl) side chain (Scheme 1). Due to the fact that many of these bioconversions proceeded in an enantio-convergent fashion,[15] the occurrence of an unwanted stereoisomer is avoided, which renders an excellent synthetic efficiency.



Scheme 1. Biosynthetic strategy for the synthesis of Karatavicinol and Marmin employing epoxide hydrolases.

Keywords: Epoxide hydrolase; Rhodococcus; Streptomyces; (*R*)-(+)-Marmin; Biotransformation.
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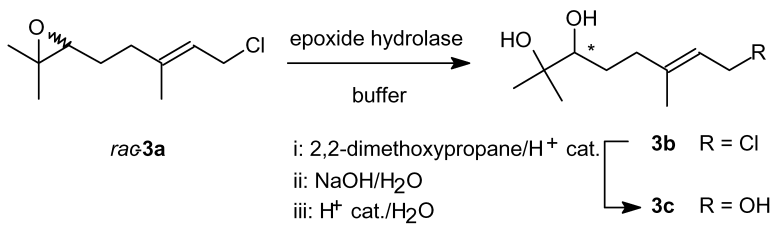


Scheme 2. Biohydrolysis of building block *rac*-**3a** and synthesis of reference material **3c**.

Table 1. Biohydrolysis of *rac*-**3a**

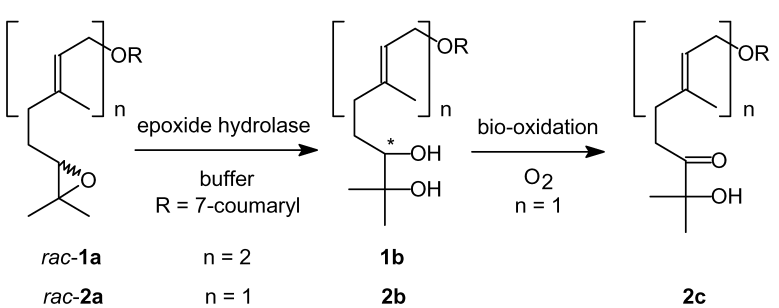
Strain	Conv. (%)	Ee (%)	
		3a	(*R*)-**3b**
Rhodococcus ruber DSM 43338	41	18	75
Rhodococcus ruber DSM 44541	49	86	79
Rhodococcus ruber DSM 44540	59	50	81
Streptomyces sp. FCC 003	64	74	93
Rhodococcus erythropolis DSM 312	60	31	83
Streptomyces venezuelae ATCC 10712	63	59	91

Our first approach was guided by the idea to create a common chiral building block bearing a terminal epoxy- or dihydroxy-functional group, which would allow to apply this strategy to a broad variety of terpenoid natural products. Thus we chose substrate *rac*-**3a** derived from geranyl chloride (**6**) by epoxidation bearing in mind that the allylic halogen moiety should allow facile coupling onto the main framework.

Oxirane *rac*-**3a** was subjected to biohydrolysis in aqueous buffer (pH 8.0) employing a range of lyophilised bacterial cells which are known to possess a broad secondary metabolism and epoxide hydrolase activity, in particular[16] (Scheme 2). A large number of strains showed good activity and produced the corresponding *vic*-diol **3b** in various amounts (data not shown). Inspection of the stereoselectivity of the reaction, however, revealed that the enantiomeric composition of diol **3b** and remaining non-converted oxirane **3a** was low to moderate (up to 70% ee) in most cases. Several strains gave sufficient stereoselectivity and produced (*R*)-**3b** in up to 93% ee (Table 1). Detailed analysis of the data (conversion, ee_P and ee_S) revealed that-depending on the strain used-mixed stereochemical pathways are found: Whereas *Rhodococcus ruber* DSM 43338, 44541 and 44540 (entries 1–3) largely followed a classic kinetic resolution pattern, enantio-convergent pathways[15] predominated with *Rh. erythropolis* DSM 312 (entry 5) and both *Streptomyces* spp. ATCC 10712 and FCC 003 (entries 4 and 6). The latter is indicated by the high ee_P (83 to 93%) at a conversion beyond 60%.

In order to investigate whether the presence of a large structural coumaryl-moiety in the side-chain would lead to enhanced stereoselectivity, substrates *rac*-**1a** and *rac*-**2a** were tested (Scheme 3). Conversion of substrate *rac*-**1a** bearing the sterically demanding sesquiterpene farnesyl-unit proved to be cumbersome and even prolonged exposure to a series of bacterial strains gave exceedingly low conversion (<5% to max 15%, data not shown). In addition, in those cases, where diol **1b** (Karatavicinol) could be detected in measurable amounts, the ee proved to be disappointingly low (<5%).

Much better results were obtained with the monoterpene analog *rac*-**2a** (Table 2). From a range of bacteria, *Rhodococcus ruber* DSM 44539 and DSM 43338 exhibited sufficient activity and formed (*R*)-**2b** (Marmin) in up to 72% ee (entries 1 and 3). Careful monitoring of the bioconversion revealed that the ee_P significantly dropped upon extended reaction times (entries 2 and 4), which is in contrast to an enantio-convergent transformation, where the ee_P should remain at a constant level.[15] When the reaction time for *Rh. ruber* DSM 43338 was extended to 216 h, the starting material *rac*-**2a** was entirely consumed and the ee of **2b** showed a mere 6%, but of the *opposite (S)-enantiomer* (entry 5). Analysis of the reaction mixture revealed that in addition to the expected biohydrolysis product **2b** a side-product was formed in ca. 20% yield. Spectroscopic analysis and comparison with independently synthesised reference material revealed that the latter was hydroxy-ketone **2c**, which was presumably formed from diol **2b** due to further oxidative metabolism. Since the initially formed diol **2b** was (*R*)-configured and prolonged exposure gave the (*S*)-enantiomer, it was deduced that the concurring bio-oxidation was (*R*)-selective.

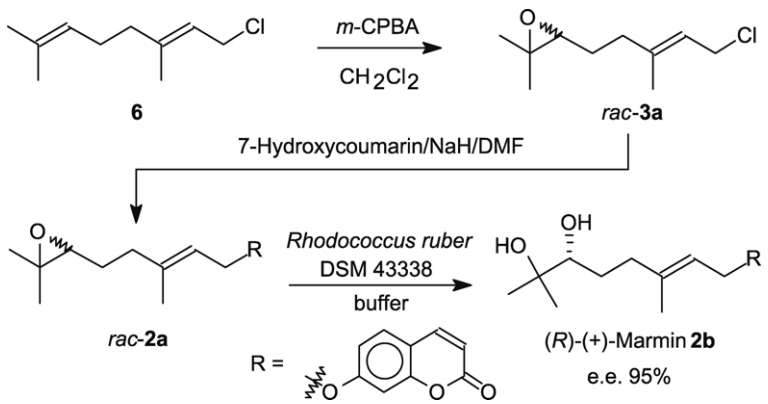


Scheme 3. Biotransformation of *rac*-**1a** and *rac*-**2a**.

Table 2. Bioconversion of *rac*-**1a**

Strain	Conditions	Time (h)	Conv.[a] (%)	ee (%)	
				1a	**1b**
Rhodococcus ruber DSM 44539	Aerobic	24	68	24	72/*R*
Rhodococcus ruber DSM 44539	Aerobic	48	87	28	62/*R*
Rhodococcus ruber DSM 43338	Aerobic	24	83	39	70/*R*
Rhodococcus ruber DSM 43338	Aerobic	48	89	44	59/*R*
Rhodococcus ruber DSM 43338	Aerobic	216	~100[b]	n.d.	6/*S*
Rhodococcus ruber DSM 43338	Ar	24	8[c]	6	97/*R*
Rhodococcus ruber DSM 43338	Ar	216	67[d]	59	96/*R*

[a] With respect to remaining epoxide *rac*-**1a**; nd=not determined.
[b] Hydroxy-ketone **1c** was formed in 20% yield.
[c] **1c** was formed in <1% yield.
[d] **1c** was formed in 1.8% yield.



Scheme 4. Chemo-enzymatic asymmetric total synthesis of (*R*)-(+)-Marmin.

In order to suppress the competing bio-oxidation, bio-hydrolysis experiments were conducted in the absence of O_2 in an Ar-atmosphere. We were pleased to see that under these conditions, the formation of hydroxy-ketone **2c** could be suppressed and that the expected biohydrolysis-product (*R*)-**2b** (Marmin) was formed in excellent ee's (up to 97%). In order to prove the synthetic value of the method, a preparative-scale experiment was conducted which gave (*R*)-(+)-Marmin in 95% ee (Scheme 4).

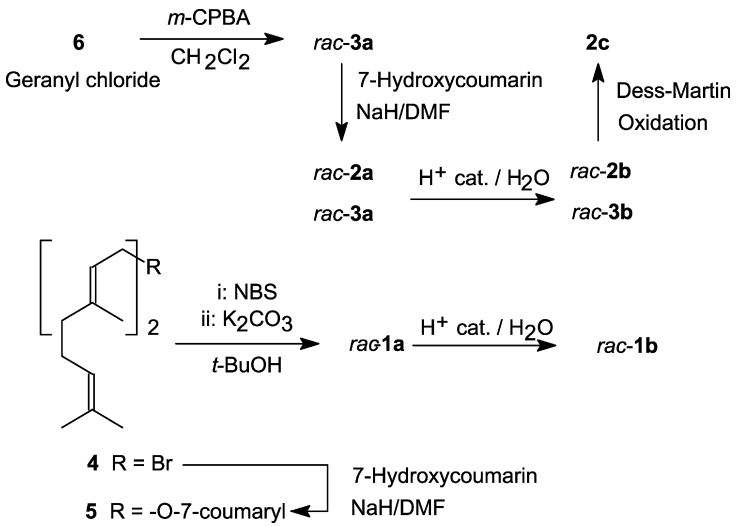
The absolute configuration of **2b** (Marmin) was revealed to be (*R*) by comparison of optical rotation values with literature data.[6] The absolute configuration of **3b** was determined by conversion of chloro-diol **3b** into triol **3c** by alkaline hydrolysis of the allylic chloride (NaOH aq.) with intermediate protection/deprotection of the *vic*-diol moiety as the corresponding acetonide. Comparison of the optical rotation of the latter material with literature data revealed its (*R*)-configuration.[17]

In summary, we have shown that the asymmetric biohydrolysis of trisubstituted terpenoid epoxides by bacterial epoxide hydrolases proceeds in an enantio-convergent fashion to furnish the corresponding *vic*-diols in high ee. This strategy offers a convenient and short access to terpenoid natural products, which was demonstrated by the asymmetric total synthesis of the monoterpenoid coumarin (*R*)-(+)-Marmin in 95% ee.

3. Experimental

3.1. General

NMR spectra were recorded in $CDCl_3$ using a Bruker AMX 360 at 360 (^{1}H) and 90 (^{13}C) MHz. Chemical shifts are reported relative to TMS (δ 0.00) with $CHCl_3$ as internal standard, coupling constants (*J*) are given in Hz. TLC plates were run on silica gel Merck 60 (F_{254}) and compounds were visualised by spraying with Mo-reagent [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (100 g L^{-1}) and $Ce(SO_4)_2\cdot 4H_2O$ (4 g L^{-1}) in H_2SO_4 (10%)] or by dipping into a $KMnO_4$ reagent (2.5 g L^{-1} $KMnO_4$ in H_2O). Compounds were purified by flash chromatography on silica gel Merck 60 (230–400 mesh). Petroleum ether had a boiling range of 60–90 °C. Enantiomeric purities were analysed on a HPLC JASCO-System equipped with a multiwavelength-detector (MD-910) and a temperature-chamber (AS-950) either on a DAICEL Chiralcel OD-H (0.46 cm d×25 cm) or a DAICEL Chiralcel AD (0.46 cm d×25 cm) column. High resolution mass spectra were recorded on a double focussing Kratos Profile Mass Spectrometer with electron impact ionization (EI,+70 eV). Optical rotation values were measured on a Perkin Elmer polarimeter 341 at 589 nm (Na-line) in a 1-dm cuvette. Solvents were dried or freshly distilled by common practice. For anhydrous reactions, flasks were dried at 150 °C and flushed with dry argon just before use. Organic extracts were dried over Na_2SO_4, and then the solvent was



Scheme 5. Synthesis of substrates and reference compounds.

evaporated under reduced pressure. Bacteria were obtained from culture collections, FCC stands for our in-house 'Fab-Crew-Collection'.

3.2. Syntheses of substrates

Substrates *rac*-**1a**–*rac*-**3a** were synthesised as follows (Scheme 5): Epoxidation of geranyl chloride (**6**) by *m*-chloroperbenzoic acid selectively gave *rac*-**3a**. Coupling of the coumaryl moiety using the anion of 7-hydroxy-coumarin furnished *rac*-**2a**. 7-O-Alkylation of 7-hydroxy-coumarin with farnesyl bromide (**4**) gave **5**. The side-chain of the latter was epoxidised at the terminal alkene moiety via the corresponding halohydrin (NBS/*t*-BuOH) followed by base-induced ring-closure (K_2CO_3) to give *rac*-**1a**. In order to verify the structural identity of the expected biohydrolysis products, diols **1b**–**3b** were independently synthesised in racemic form by acid-catalysed hydrolysis of epoxides *rac*-**1a**–*rac*-**3a**. Dess-Martin oxidation of *rac*-**2b** gave a sample of **2c** for proof of structure of the bio-oxidation product.

3.2.1. 7-[9-(3,3-Dimethyloxiranyl)-3,7-dimethylnona-2*E*,6*E*-dienyloxy]-chromen-2-one (*rac*-1a). To a stirred solution of 7-farnesyloxy-coumarin (**5**) (0.5 g, 1.4 mmol) in *tert*-BuOH (20 mL) and petroleum ether (20 mL), NBS (0.3 g, 1.7 mmol) was added at 0 °C. After stirring was continued for 24 h at room temperature, the bromohydrin intermediate was extracted with ether, the organic phase was dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography (petroleum ether/ethyl acetate 1:1) to give the bromohydrin in 50% yield. The latter was dissolved in MeOH (20 mL) and K_2CO_3 (0.2 g, 2.0 mmol) was added. After stirring was continued for 12 h at room temperature, solids were filtered and the solvent was evaporated. The crude product was purified by flash chromatography (petroleum ether/ethyl acetate 1:1) to give *rac*-**1a** as a white solid (0.1 g, 86%). Mp 141–143.5 °C.

^{1}H NMR ($CDCl_3$) δ=7.64 (d, J=6.0 Hz, 1H), 7.37 (d, J=6.5 Hz, 1H), 7.27 (d, J=5.7 Hz, 2H), 6.84 (d, J=4.3 Hz, 1H), 6.25 (d, J=4.0 Hz, 1H), 5.48 (m, 1H), 5.17 (m, 1H), 4.61 (d, J=3.7 Hz, 2H), 4.13 (d, J=4.3 Hz, 2H), 2.75 (t, J=3.4 Hz, 1H), 2.14 (m, 3H), 1.77 (s, 2H), 1.64 (s, 3H), 1.57 (s, 3H), 1.31 (s, 3H), 1.27 (s, 3H); ^{13}C NMR ($CDCl_3$) δ=162.2, 161.3, 155.9, 143.5, 142.2, 134.8, 128.7, 124.2, 118.6, 113.3, 113.0, 112.5, 101.6, 65.5, 64.2, 60.4, 39.5, 36.3, 27.5, 26.2, 24.9, 21.0, 16.8, 16.1.

3.2.2. 7-[5-(3,3-Dimethyloxiranyl)-3-methylpent-2*E*-enyloxy]-chromen-2-one (epoxyaurapten)[18] (*rac*-2a). NaH (0.3 g, 12.5 mmol, 60% dispersion in mineral oil) was suspended in abs. DMF (30 mL) under nitrogen and 7-hydroxycoumarin (1.0 g, 6.2 mmol) was added with stirring at room temperature. After ten minutes, *rac*-**3a** (1.1 g, 5.8 mmol) was added and stirring was continued for 12 h, until the starting material was consumed. The crude product was extracted with H_2O (300 mL) and a mixture of toluene/petroleum ether (1:1, 200 mL). The organic layers were dried (Na_2SO_4) and evaporated. The residue was flash chromatographed (petroleum ether/ethyl acetate 1:1) and recrystallised from MeOH to give *rac*-**2a** as white crystals (0.2 g, 10%). Mp 53–55 °C.

^{1}H NMR ($CDCl_3$) δ=7.62 (d, J=6.7 Hz, 1H), 7.34 (d, J=5.7 Hz, 1H), 6.81 (m, 2H), 6.22 (d, J=4.5 Hz, 1H), 5.50 (t, J=3.3 Hz, 1H), 4.59 (d, J=4.2 Hz, 2H), 2.69 (t, J=4.2 Hz, 1H), 2.22 (m, 2H), 1.77 (s, 3H), 1.69 (m, 2H), 1.28 (s, 3H), 1.25 (s, 3H); ^{13}C NMR ($CDCl_3$) δ=162.1, 161.3, 155.9, 143.5, 141.5, 128.8, 119.1, 113.2, 113.0, 112.5, 101.6, 65.4, 63.9, 58.4, 36.3, 27.1, 24.9, 18.8, 16.8; HRMS calculated for $C_{19}H_{22}O_4$: 314.1518 [M^+]; found: 314.1509 [M^+].

3.2.3. (*E*)-3-(5-Chloro-3-methylpent-3-enyl)-2,2-dimethyloxirane (*rac*-3a). To a solution of 1-chloro-3,7-dimethyl-octa-2,6-diene (geranyl chloride, **6**) (0.5 g, 2.9 mmol) in CH_2Cl_2 (30 mL) containing K_2CO_3 (0.6 g, 6.1 mmol), *m*-CPBA (0.8 g, 4.6 mmol, Fluka 25800, 70%) was added slowly. After stirring was continued for 24 h at 0 °C, excess peracid was destroyed by shaking with aq. Na-metabisulfite solution (10%), and removal of *m*-chlorobenzoic acid by extraction with sat. $NaHCO_3$. Product was extracted with ethyl acetate, the organic phase was dried (Na_2SO_4) and evaporated. The residue

was flash-chromatographed using petroleum ether/ethyl acetate (1:1) to give *rac*-**3a** as a yellow liquid (0.4 g, 73%).

^{1}H NMR (360.13 MHz, $CDCl_3$) δ=5.54 (t, J=8.0 Hz, 1H), 4.09 (d, J=8.0 Hz, 2H), 2.69 (t, J=6.5 Hz, 1H), 2.20 (m, 2H), 1.74 (s, 3H), 1.65 (t, J=7.0 Hz, 2H), 1.30 (s, 3H), 1.25 (s, 3H); ^{13}C NMR ($CDCl_3$) δ=141.6, 120.9, 63.7, 58.2, 40.7, 36.1, 27.0, 24.7, 18.7, 16.0.

3.2.4. 7-(3,7,11-Trimethyldodeca-2*E*,6*E*,10*E*-trienyloxy)-chromen-2-one (7-farnesyloxycoumarin, umbelliprenin, 5). To a stirred suspension of NaH (0.1 g, 4.2 mmol, 60% dispersion in mineral oil) in a mixture of freshly destilled anhydrous THF (30 mL) and DMSO (30 mL) under nitrogen 7-hydroxycoumarin (0.6 g, 3.7 mmol) was added at room temperature. After ten minutes, **4** (1.1 g, 3.9 mmol) was added and the mixture was refluxed for 2 h. The crude product was extracted with 0.5 M HCl (20 mL), H_2O (100 mL) and ether (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated. The residue was flash chromatographed (petroleum ether/ethyl acetate 1:1) and recrystallised from EtOAc/petroleum ether (2/98) to give **5** as white crystals (1.3 g, 98%). Mp 45–46 °C.

^{1}H NMR ($CDCl_3$) δ=7.63 (d, J=9.47 Hz, 1H), 7.36 (d, J=8.47 Hz, 1H), 6.84 (m, 2H), 6.24 (d, J=9.47 Hz, 1H), 5.47 (t, J=5.93 Hz, 2H), 5.08 (m, 1H), 4.61 (d, J=6.55 Hz, 2H), 2.1–1.9 (m, 8H), 1.77 (s, 3H), 1.68 (s, 3H), 1.60 (s, 6H); ^{13}C NMR ($CDCl_3$) δ=162.02, 161.20, 155.80, 143.29, 142.21, 135.64, 131.37, 128.51, 124.36, 123.56, 118.28, 113.08, 112.78, 112.28, 101.66, 65.54, 39.68, 39.57, 26.75, 26.19, 25.74, 17.73, 16.82, 16.08.

3.3. Synthesis of reference material

General procedure for the synthesis of reference material of diols rac-**1b**–*rac*-**3b**. Epoxide *rac*-**1a**–*rac*-**3a** was dissolved in a mixture of H_2O (5 mL) and THF (3 mL) containing 3 drops of H_2SO_4 (conc.) and stirred at room temperature for 2 h. The crude product was extracted with EtOAc, neutralised with solid $NaHCO_3$ and purified by flash chromatography (petroleum ether/ethyl acetate 1:1). Thus were obtained.

3.3.1. 7-(10,11-Dihydroxy-3,7,11-trimethyldodeca-2*E*,6*E*-dienyloxy)-chromen-2-one (*rac*-1b). Yield (30 mg, 98%) from *rac*-1a (30 mg, 0.08 mmol). ^{1}H NMR ($CDCl_3$) δ=7.64 (d, J=6.6 Hz, 1H), 7.36 (d, J=6.2 Hz, 1H), 6.83 (dd, J=6.0 Hz, 1H), 6.24 (d, J=4.7 Hz, 1H), 5.73 (t, J=4.2 Hz, 1H), 5.14 (t, J=5.5 Hz, 1H), 4.61 (d, J=4.3 Hz, 2H), 3.39 (dd, J=3.3 Hz, 1H), 1.76 (s, 3H), 1.63 (s, 3H), 1.20 (s, 3H), 1.16 (s, 3H).

3.3.2. 7-(6,7-Dihydroxy-3,7-dimethyloct-2*E*-enyloxy)-chromen-2-one (*rac*-2b). Yield (70 mg, 67%) from *rac*-2a (0.1 g, 0.3 mmol). ^{1}H NMR ($CDCl_3$) δ=7.63 (d, J=6.7 Hz, 1H), 7.36 (d, J=5.8 Hz, 1H), 6.83 (m, 2H), 6.24 (d, J=4.7 Hz, 1H), 5.74 (s, 1H), 4.62 (d, J=3.8 Hz, 2H), 3.39 (m, 2H), 2.12 (m, 3H), 1.76 (s, 3H), 1.63 (s, 3H), 1.28 (s, 3H); ^{13}C NMR ($CDCl_3$) δ=162.2, 155.9, 143.6, 135.5, 128.8, 124.3, 118.8, 113.4, 101.6, 78.2, 73.0, 39.4, 36.8, 29.7, 26.5, 26.0, 23.4, 16.7, 15.9.

3.3.3. 8-Chloro-2,6-dimethyl-oct-6*E*-en-2,3-diol (*rac*-3b). Yield (0.2 g, 26%) from *rac*-3a (0.7 g, 3.7 mmol). ^{1}H NMR ($CDCl_3$) δ=4.73 (d, J=7.3 Hz, 2H), 3.95 (m, 2H), 2.1 (m, 4H), 1.67 (s, 3H), 1.22 (s, 3H), 1.18 (s, 3H).

3.3.4. 7-(7-Hydroxy-3,7-dimethyl-6-oxooct-2*E*-enyloxy)-chromen-2-one (2c). Dess Martin reagent (20 mg, 0.05 mmol) was dissolved in anh. CH_2Cl_2 (5 mL) and stirred at room temperature. *Rac*-**2b** (7 mg, 0.02 mmol) was added and stirring was continued for 12 h. The crude product was extracted with ether and sat. aq. $NaHCO_3$, the organic layer was dried (Na_2SO_4) and evaporated. The crude product was purified by flash chromatography (petroleum ether/ethyl acetate 1:1) to give **2c** as a white solid (5 mg, 77%). Mp 59–60 °C.

^{1}H NMR ($CDCl_3$) δ=7.63 (d, J=6.9 Hz, 1H), 7.37 (d, J=5.7 Hz, 1H), 6.83 (m, 2H), 6.25 (d, J=5.4 Hz, 1H), 5.50 (t, J=4.6 Hz, 1H), 4.60 (d, J=4.4 Hz, 2H), 3.65 (s, 1H), 2.73 (t, J=5.1 Hz, 2H), 2.42 (t, J=4.5 Hz, 2H), 1.79 (s, 3H), 1.39 (s, 6H); ^{13}C NMR ($CDCl_3$) δ=213.5, 162.0, 161.3, 155.9, 143.3, 140.7, 128.8, 119.3, 113.3, 113.2, 112.6, 101.6, 76.6, 65.3, 33.8, 33.0,26.6, 17.0; HRMS calculated for $C_{19}H_{22}O_5$: 330.1467 [M^+]; found: 330.1432 [M^+].

3.3.5. (*R*)-3,7-Dimethyloct-2*E*-en-1,6,7-triol (*R*)-(3c). Biohydrolysis of *rac*-**3a** using *Rhodococcus ruber* DSM 44541 gave **3b** in 79% ee (see below). The latter material (154 mg, 748 mmol) was stirred in a mixture of 2,2-dimethoxypropane (20 mL) and cat. *p*-toluenesulfonic acid (20 mg). After 2 h, hydrolysis of the allylic chloride was affected by addition of conc. NaOH (4 M, 10 drops) and stirring was continued for 1 h. After the organic material was extracted with ethyl acetate and evaporated, deprotection of the acetonide was achieved by addition of H_2O (20 mL) containing cat. *p*-toluenesulfonic acid (20 mg). After 2 h, the solution was extracted with ethyl acetate and the pruduct was purified by column chromatography (petroleum ether/ethyl acetate 1:1) to give **3c** (74 mg, 53%) showing an optical rotation of $[\alpha]_D^{20}$+12.5 (c=0.95, $CHCl_3$, 73% ee), which nicely corresponds to literature data of $[\alpha]_D^{23}$+17.4 (c=1.4, $CHCl_3$) for the (*R*)-enantiomer.[17]

3.4. Biotransformations

3.4.1. Growth of strains. *Rhodococcus ruber* DSM 43338, DSM 44541, DSM 44540, *Rh. erythropolis* DSM 312 and *Streptomyces* sp. FCC 003 were maintained and grown on a medium as described before.[19] For *Streptomyces venezuelae* ATCC 10712 (=DSM 40230) medium #65 from DSMZ was used (http://www.dsmz.de).

3.4.2. General procedure for biotransformation. Lyophilised bacterial cells (50 mg) were rehydrated for 1 h in Tris–HCl buffer (1 mL, 0.05 M, pH=8.0) by shaking at 30 °C with 130 rpm. Racemic epoxides *rac*-**1a**–*rac*-**3a** (5 μL) were added, and the reaction was monitored by TLC. At intervals of 24 and 48 h, aliquots of 0.4 mL were withdrawn and extracted twice with EtOAc (0.4 mL each). To facilitate phase separation, the cells were removed by centrifugation. The combined organic layers were dried (Na_2SO_4) and analysed by HPLC (Table 3).

Table 3. HPLC-Data of enantioseparation

Compound	Column	Eluent[a]	Temp. (°C)	Retention time (min)
rac-**1a**	ODH	70/30	10	15.1/16.6
rac-**1b**	AD	70/30	10	12.5/13.5
rac-**2a**	ODH	90/10	10	50.4/55.6
rac-**2b**	AD	70/30	7	26.8 (*S*)/28.7 (*R*)
rac-**3a**	AD	70/30	18	7.1/7.4
rac-**3b**	AD	90/10	20	8.8 (*S*)/9.4 (*R*)

[a] Hexane/*i*-propanol.

3.4.3. Preparative-scale biotransformation. Epoxide *rac*-**2a** (100 mg, 0.3 mmol) was treated with 0.3 g of lyophilised cells of *Rhodococcus ruber* 43338 in Tris–HCl buffer (6 mL, 0.05 M, pH=8.0) as described above. After 24 h, products were extracted twice with CH_2Cl_2, the organic layer was dried (Na_2SO_4) and after evaporation the crude product was purified by flash chromatography (petroleum ether/ethyl acetate 1:1) to give 20 mg (15%) of (*R*)-(+)-Marmin *(R)*-**2b**: mp 104–109 °C, $[\alpha]_D^{20}=+9.1$ (c=2.5, MeOH, ee 95%); Lit. for (*S*)-**2b** $[\alpha]_D^{26}=-11$ (c=0.55, MeOH).[6] NMR-Spectroscopic data matched those previously reported.[6]

Acknowledgements

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Introduction of permanently charged groups into PEGA resins leads to improved biotransformations on solid support

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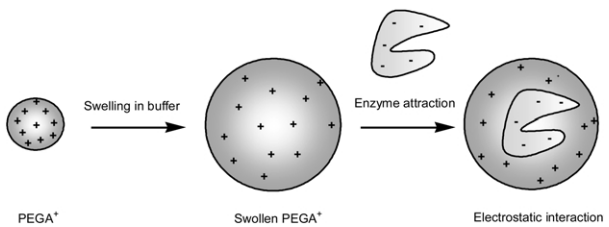
Abstract—The application of biotransformations in solid phase synthesis is an attractive alternative to chemical methodologies. An important issue that needs to be addressed in this context is accessibility of functional groups within a porous polymer to the biocatalyst. This paper shows that such accessibility can be improved for penicillin G amidase by introducing permanent charges into the polymer. The effect appears to be due to better swelling of the polymer in buffer and to electrostatic interactions between polymer and enzyme.

1. Introduction

In the screening approach to biomedical discovery, large libraries of compounds are commonly used to identify novel protein/ligand interactions. These compound libraries can be conveniently synthesized on solid phase polymer beads using combinatorial methods. Ideally, these compound libraries would then be used directly for on-resin biological screening, which requires polymer materials that are accessible to proteins and other biomolecules. Thus, Meldal et al. have successfully developed a range of PEGA polymers (cross-linked acrylamide and polyethylene glycol) for such screening experiments, in particular using peptide libraries for the screening of substrate specificity of small proteases.[1] $PEGA_{1900}$ was found to be completely permeable to proteins with molecular masses up to 35 kDa.[2] However, accessibility appears to be problematic for larger enzymes. Increasing the PEG chain to create $PEGA_{6000}$ or use of soluble PEG polymers has been shown to improve results but has compromised the ease of handling and loading of the polymers.[1d,3] Hence, there is a need for improved polymers that can be used both for combinatorial synthetic chemistry and biological screening.

We have recently introduced a conceptually different approach to improving enzyme access in polymer beads.[4] In this approach, we have exploited the observation that all proteins are charged. The net charge on a protein depends on its pI value. By introducing permanent charges into the PEGA resin, increased swelling is observed due to electrostatic repulsions between identical or similar charges. In addition, oppositely charged enzymes are attracted inside the polymer bead due to favourable electrostatic attraction between polymer and protein (see Scheme 1). For example, we have shown that penicillin G acylase (PGA), a 88 kDa enzyme could be used effectively for the hydrolysis of phenylacetamides within PEGA beads when the resin was derivatised with permanently charged quaternary amines ($PEGA^+$, see Fig. 1).

In this article, two important aspects related to the wider applicability of these novel polymers are investigated. Firstly, the suitability of such charged resins for solid phase chemical synthesis was explored. Secondly, the effects of polymer charges were systematically investigated on two hydrolases of different size.



Scheme 1. $PEGA^+$ resins allow for improved access of proteins due to (i) increased swelling resulting in increased pore size and (ii) improved access of oppositely charged proteins due to electrostatic attraction.

Keywords: Solid phase chemistry; PEGA; Charged PEGA; Penicillin G amidase; Thermolysin.
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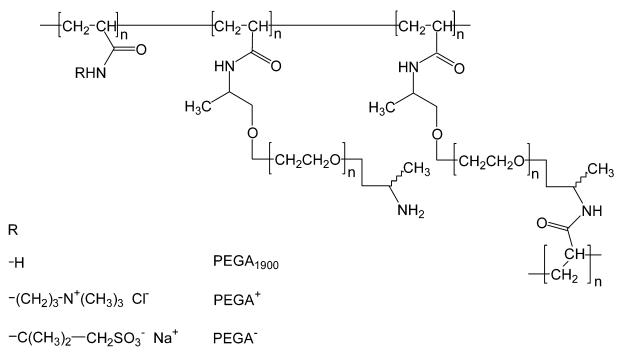


Figure 1. PEGA polymers used in the present study.

2. Results and discussion

2.1. Characterization of novel polymers

We recently reported on the successful incorporation of permanently charged monomers into PEGA supports.[4] $PEGA^+$ and $PEGA^-$ have a polyacrylamide backbone in which acrylamide units are partially substituted (10%) with charged units carrying 3-trimethylammonium chloride-propyl and 1,1-dimethyl-2-sulfonate-ethyl side-chains, respectively (Figure 1).

The first step in the further characterization of these novel polymers was to assess their chemical reactivity and loading. The presence of charged groups in $PEGA^+$ and $PEGA^-$ would probably influence the chemical reactivity of the polymer bound free amino groups. The reactivity was therefore monitored after each of four cycles of acylation with Fmoc-L-Phe. After each cycle the Fmoc group was removed with piperidine (20% in DMF) and coupling yield quantified by UV determination (Table 1).

The results obtained (Table 1) suggest that the chemical reactivity of the charged PEGA resins ($PEGA^+$ or $PEGA^-$) is lower than the reactivity of uncharged $PEGA_{1900}$. The presence of quaternary amino groups strongly decreased the reactivity of the polymer linked primary amino groups during the first acylation step. One reaction cycle was therefore not sufficient for the complete acylation of $PEGA^+$ since only 5% of the available amino groups were acylated. The presence of permanent negative charges also decreased the reactivity of the primary amino groups, since after one reaction cycle 50% of the available amino groups were acylated. However, after the second cycle complete acylation was obtained for both polymers. The loadings obtained were comparable to that of $PEGA_{1900}$.

Table 1. Loading determination of PEGA resins

Resin[a]	Loading after each acylation ($mmol/g_{dry}$)				
	1st	2nd	3rd	4th	Loading[b]
$PEGA_{1900}$	0.17	0.14	0.13	0.17	0.15
$PEGA^+$	0.01	0.23	0.24	0.22	0.23
$PEGA^-$	0.05	0.11	0.08	0.10	0.10

[a] Loading provided by manufacturer: 0.10–0.20 $mmol/g_{dry}$.
[b] Average of 2nd, 3rd and 4th acylations.

2.2. Swelling properties of the charged PEGA resins

The swelling properties of the polymers were investigated by measuring the weight of retained buffer after filtration of the polymers. Swelling properties are likely to be pH dependant due to interactions of the permanently charged monomers with functional amino groups.[5] These amino groups are expected to be protonated (and therefore charged) to an extent depending on the pH value of the buffer. In addition, protonation might depend on the presence of permanent charges in the polymer micro-environment: permanent positive charges suppress protonation while permanent negative charges can enhance protonation. The apparent pK value of the surface bound amino groups is around 7.0 and hence a significant proportion is protonated at pH 6.0, while most are deprotonated at pH 8.0. The effect of buffer pH on swelling was investigated by varying the pH value within this range (Fig. 2).

The electrostatic interactions that influence the swelling behaviour of charged polymers largely depend on the ionic strength of the buffer. Increasing concentrations of counter-ions that are present in higher concentration buffers are likely to shield the interactions. The swelling of the

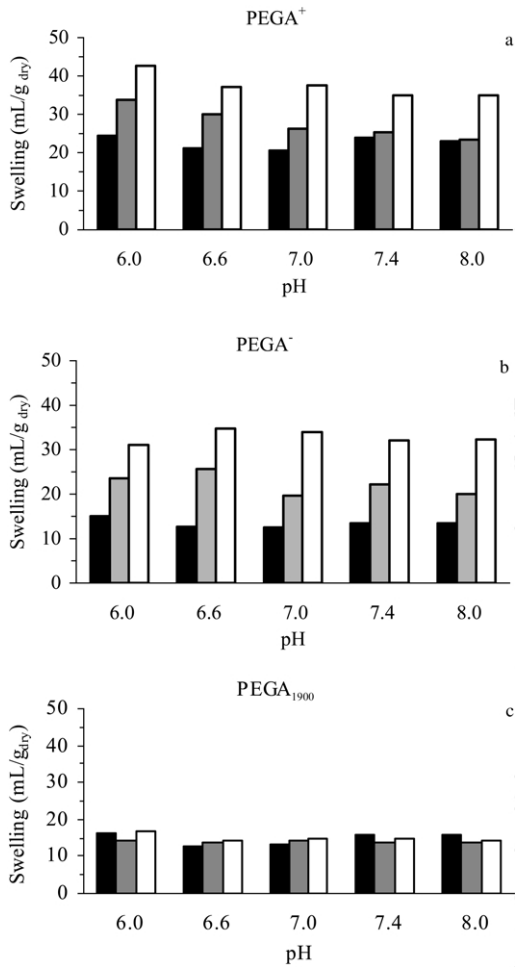


Figure 2. Swelling behaviour of PEGA polymers as a function of buffer concentration (0.1 M: black; 0.01 M: grey; 0.001 M: white) and the pH of the buffer (6.0–8.0) of the buffer solutions (a: $PEGA^+$; b: $PEGA^-$; c: $PEGA_{1900}$).

polymers was therefore studied in buffer concentrations varying from 0.1 to 0.001 M (Fig. 2).

The expected effect of buffer concentration on the swelling of charged PEGA polymers is evident for both charged polymers as more dramatic effects are observed at lower ionic strength (Fig. 2a and b). A pH change from 6.0 to 8.0 has a significant effect on $PEGA^+$ resulting in a better swelling at lower pH values. In this case, the presence of permanent quaternary amino groups and protonation of primary amino groups at lower pH values increases the electrostatic repulsion within the polymer, resulting in improved swelling. In $PEGA^-$ protonation of amino groups creates opposite charges counteracting repulsion resulting in decreased swelling at lower pH values.

In the absence of permanent charges ($PEGA_{1900}$) no measurable effect of either ionic strength or pH of the buffer on swelling is observed (Fig. 2c). The positively charged amines at low pH appear to be too distant from each other within the polymer beads to have a measurable effect on the swelling behaviour.

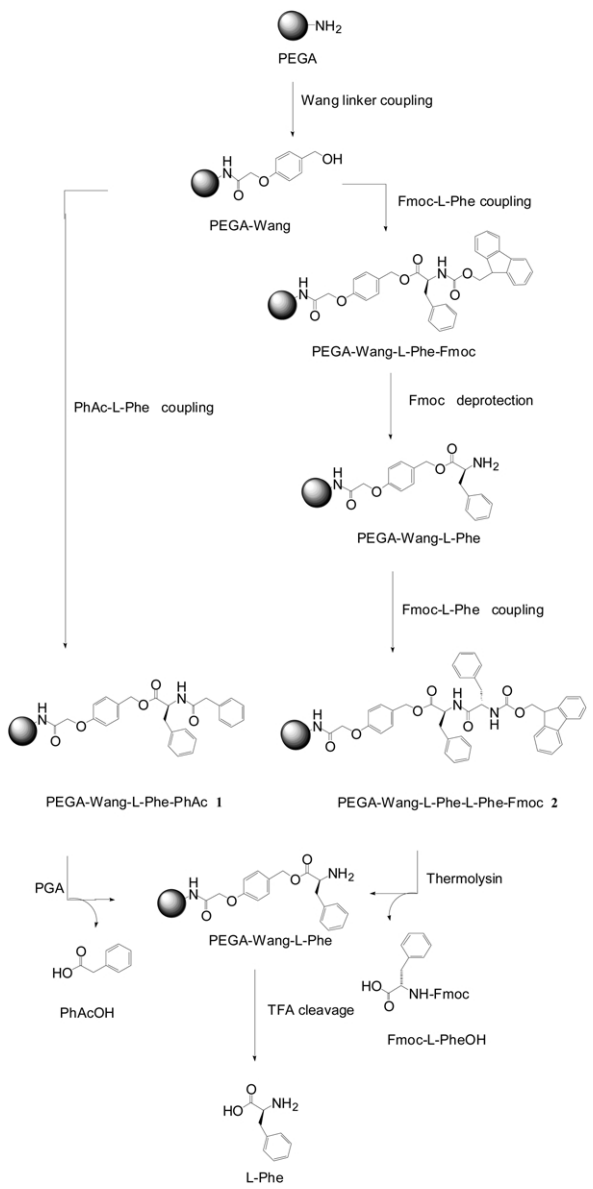
2.3. Biotransformations on novel polymers

Having established improved swelling, the charged polymers were subsequently studied for protein access to the core of the polymer beads. Two hydrolytic enzymes that had been previously investigated in our group and had yielded different accessibility were used as model systems. Thermolysin from *B. thermoproteolyticus* had been used in peptide synthesis/hydrolysis experiments and was found to be able to penetrate to the core of $PEGA_{1900}$ beads.[2b,c] In contrast, Penicillin G amidase (PGA) from *E. coli*,[6,7] had always given low yields. The two enzymes were particularly useful as model systems because of their difference in size (35 kDa for thermolysin and 88 kDa for PGA).

All three polymers were derivatised to give substrate **1** (specific substrate for PGA) and substrate **2** (specific substrate for thermolysin) using previously developed experimental procedures (Scheme 2). To enable full analysis the substrates were attached to the solid support by a cleavable Wang linker.

Using PEGA derivatives **1** and **2** as substrates for hydrolases the possible correlation of swelling behaviour of the charged polymers with the yield of enzymatic hydrolysis was further investigated. At first, hydrolysis of **1** by PGA was studied at pH 8.0, the value where the enzyme shows the highest hydrolytic activity. The reaction was studied at buffer concentrations from 0.5 to 0.001 M (Fig. 3).

While for $PEGA^-$ and $PEGA_{1900}$ no apparent effect was observed (conversions of $<15\%$ at all buffer concentrations studied), for $PEGA^+$ an increase in hydrolysis yields was obtained at decreasing ionic strength. When comparing Figures 2a and 3 correlation between the increased swelling of the resin in diluted buffers and the final conversion of the hydrolytic reaction is evident. Despite the increase of swelling of $PEGA^-$ (Fig. 2b) in diluted buffers, enzyme hydrolysis did not increase by lowering the buffer concentration from 0.5 to 0.001 M. These observations



Scheme 2. Chemical synthesis of PEGA-Wang-L-Phe-PhAc, **1**, of PEGA-Wang-L-Phe-L-Phe-Fmoc, **2** and enzymatic hydrolysis by PGA and thermolysin.

suggest that PGA accessibility is determined by pore size (swelling) but also by electrostatic interactions between polymer and protein. Indeed, PGA has an overall negative charge at pH 8.0 (pI=5.2–5.4), and would be attracted by $PEGA^+$ side chains, while it would repelled in $PEGA^-$.

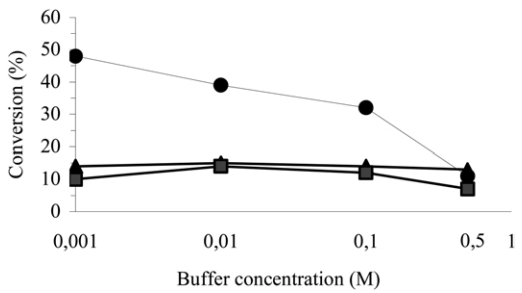


Figure 3. PGA catalysed release of PhAcOH from substrate **1** in Kpi buffer, pH 8.0, at different buffer concentration. $PEGA^+$: circles; $PEGA^-$: triangles; $PEGA_{1900}$: squares.

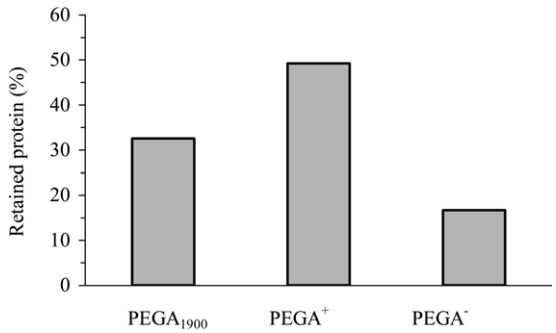


Figure 4. Retained PGA (in Kpi buffer, 0.001 M, pH 8.0) by PEGA polymers after filtration.

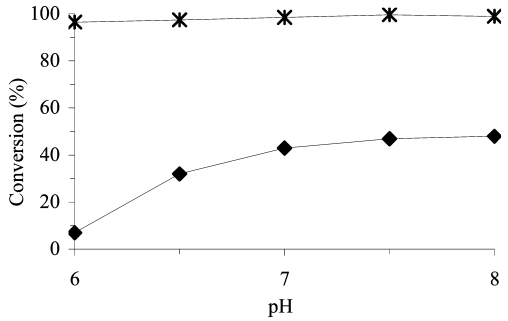


Figure 5. PGA (diamonds) and thermolysin (stars) catalysed hydrolysis of peptides **1** and **2** linked to PEGA$^+$in 0.001 M Kpi buffers of varying pH values.

Further evidence of increased access of PGA protein to the polymer beads was provided by the determination of protein retained in the polymer bead.[8] Figure 4 shows that 49% of the total amount of PGA present in a PEGA$^+$ suspension at pH 8.0 was absorbed by the polymer. In contrast, the unfavourable interactions between PGA and PEGA$^-$ is directly measured by only 20% of the enzyme being retained within the polymer. For PEGA$_{1900}$ an intermediate protein uptake of 32% was observed.

Clearly, the combined effects of polymer swelling and favoured electrostatic interactions between protein and permanent polymer charges result in protein retention by the charged polymer. It is not straightforward to establish what the relative importance of each of these two effects because both effects depend on the buffer concentration and cannot be studied independently. However, an indication of the relative importance of each of these effects could be obtained by lowering the pH of the buffer. For example, at pH 6.0 polymer swelling is significantly increased, while PGA is still overall negatively charged.

When the enzymatic hydrolysis was performed at optimal swelling conditions for PEGA$^+$ (corresponding to 0.001 M ionic strength and pH 6.0), the yield was less than 10%. Hence, despite increased swelling and electrostatic attractions between polymer and protein poor conversions are obtained. This unforeseen observation can be explained in terms of two possible effects. Firstly, the enzyme activity could be reduced at lower pH values. It is indeed known that amide hydrolysis is more favoured at pH 8.0 than at pH 6.0 (PGA has an optimal pH for the hydrolysis in the range 7.5–8.5). Secondly, the decrease of pH from 8.0 to 6.0 reduces the net negative charge on the enzyme. As a result the electrostatic attraction with the permanent charges within PEGA$^+$ decreases resulting in a decreased access of the enzyme.

In order to find out which of these two effects was more important, the results were compared with the thermolysin catalysed hydrolysis reaction of **2** on PEGA$^+$ (Fig. 5). Thermolysin has a pI of 5.1, similar to that of PGA, while its size is significantly smaller than that of PGA (only 35 kDa).

As stated before, thermolysin had been shown to access all chemical groups within PEGA$_{1900}$.[2a–c] One would anticipate this protein to access PEGA$^+$ equally well at pH values significantly over its pI, where the enzyme is negatively charged. This was indeed observed, and complete hydrolysis of peptide **2** was observed. Upon lowering the pH, and hence lowering the net negative charge of the protein, no significant decrease in conversion yields was observed. The dramatic decrease in enzymatic conversions as observed for PGA was clearly absent in thermolysin. These observations suggest that the decrease in hydrolysis observed for PGA is not related to limited enzyme accessibility. Instead, the decreased hydrolysis yields observed are probably related to reduced catalytic activity of the enzyme inside the polymer bead rather than net protein charge.

In summary, we have investigated novel PEGA polymers with superior swelling properties that vary with ionic strength and pH of the buffer used. We have also demonstrated that enzyme absorption by polymer beads can be improved by exploiting enzymes net charge.

3. Experimental

3.1. General

PEGA$_{1900}$, PEGA$^+$ and PEGA$^-$ were prepared by Polymer Laboratories (UK). 4-Hydroxymethyl phenoxyacetic acid (HMPA), *N*,*N*′-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBT), *N*-Fmoc-L-phenylalanine (Fmoc-L-Phe), 4-dimethylaminopyridine (DMAP), *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), *N*-ethyldiisopropylamine (DIPEA) phenylacetyl-L-phenylalanine (PhAc-L-Phe) and phenylacetic acid (PhA-cOH) were purchased from Sigma-Aldrich. Penicillin G amidase (PGA) (solution: 16–32 U/mg) was obtained from Fluka and Thermolysin was obtained from Sigma (100 units/mg protein). All the solvents were of HPLC grade and obtained from Labscan. *N*,*N*′-dimethylformamide (DMF) was Biotech grade, >99% (Aldrich).

3.2. Chemical synthesis

Before and after any chemical step resins were washed three times with MeOH/DMF (1:1), MeOH, DCM and DMF and filtered. All the reactions (loading determination, chemical syntheses and enzymatic hydrolyses) were performed at room temperature, under constant stirring in a blood rotator (40 rpm) and repeated at least three times.

3.2.1. Ninhydrin test. Three solutions were prepared:

solution A: 5 g ninhydrin in 100 mL of ethanol; solution B: 80 g phenol in 20 mL of ethanol; solution C: 2 mL of a KCN solution (0.001 mequiv. in pyridine) diluted to 100 mL. One drop of each solution was added on a small sample of resin. The mixture was then heated to 200 °C. A blue colour was evidence of the presence of unreacted amines. Yellow indicated all amines had reacted.

3.2.2. Determination of loading. The availability of amino groups of the resin was measured by reacting them with Fmoc-L-Phe (3 equiv.) in the presence of DIC (4 equiv.) and DMAP (0.1 equiv.) in dry DMF. The process was repeated until the ninhydrin test gave a negative result. The Fmoc group was removed and quantified by suspending the resins in a 20% solution of piperidine in DMF and filtering the mixture after 2 h. The loading of the resin was calculated on the filtrate using Eq. 1.

$$\text{Loading (mmol/g}_{\text{dry}}) = \frac{\text{Ads (290 nm)} \times V\ \text{(mL)}}{4950 \times g\ \text{(dry resin)}} \qquad (1)$$

where *Ads* (290 nm) represents the absorbance at 290 nm, *V* (mL) the volume of piperidine (20% in DMF) used, 4950 the ε and *g* (dry resin) the amount of dry resin.

3.2.3. Synthesis of peptides 1 and 2. Before chemical synthesis the resins were washed three times with MeOH/DMF, MeOH, DCM and DMF. The syntheses were performed on a gram scale (wet weight polymer) (Scheme 2). The solvent content after each chemical synthesis was calculated by drying samples at 110 °C (mmol/g_{dry}).

Wang linker coupling. The resins were weighed in reactor syringes and suspended in DMF. The Wang linker was attached using HMPA (3 equiv.) in the presence of DIC (4 equiv.) and HOBT (6 equiv.). The mixtures were allowed to mix on a blood rotator overnight. The resins were then filtered (VacuumSystem, Stepbio) and washed with MeOH/DMF, MeOH, DCM and DMF. The synthesis step was repeated until the ninhydrin test gave a negative result indicating all amines had reacted.

*Synthesis of **1***. Next, PhAc-L-Phe (3 equiv.) was added in the presence of DIC (4 equiv.) and DMAP (0.1 equiv.) in DMF. The reaction was performed in two cycles, the first of 2 h and the second overnight. After each cycle the resin was filtered and washed with MeOH/DMF, MeOH, DCM and DMF. The non-reacted OH groups were capped with acetic anhydride (10 equiv.) in DMF overnight.

*Synthesis of **2***. Fmoc-L-Phe was coupled in the presence of HOBT (12 equiv.), HBTU (12 equiv.) and DIPEA (7.5 equiv.) in dry DMF. Subsequently unreacted OH groups were capped with acetic anhydride as described above.

3.2.4. Enzymatic reactions. Prior to the enzymatic reactions, the peptide carrying polymers were washed three times with the buffer used for the enzymatic hydrolysis.

*PGA catalysed hydrolysis of **1***. The PGA solution was lyophilised and the specific activity of the lyophilised powder was 17 U/mg (benzylpenicillin units). The resin was suspended in 6 mL of the appropriate buffer in the presence of 5 mg of lyophilised PGA (85U). The reactions were incubated at room temperature for 24 h on a blood rotator. Afterwards, the reaction mixtures were filtered and washed using 36 mL (12×3 mL) of MeCN/H_2O (1:1). The filtrate was recovered in a flask, dried under vacuum, re-dissolved in 1 mL of MeCN/H_2O (1:1), centrifuged, and filtered through 0.45 μm membrane filters. The samples were then analysed with a RP-HPLC system as detailed below.

*Thermolysin catalysed hydrolysis of **2***. As above, but using 2 mg of thermolysin lyophilised powder directly as supplied.

To ensure that enzymatic reactions had been completed some reactions were left for longer reaction times after further addition of fresh enzyme.

3.2.5. Cleavage of Wang-linker. After the enzymatic reactions, the remaining peptide on the solid support was cleaved with a solution of TFA/H_2O (95:5) for 2 h. The filtrate was collected, the resins were then washed with a 1:1 mixture of MeCN/H_2O, filtered, collected, and dried under vacuum. The residues was re-dissolved in 1 mL of MeCN/H_2O (1:1) and analysed by RP-HPLC as detailed below.

3.2.6. HPLC analysis. Reverse phase HPLC analysis was carried out on a Waters 2690 LC system equipped with a Waters 468 UV detector using a C_{18} column. A gradient from 20 to 50% MeCN/H_2O (with 0.1% TFA in both phases) was used and enzymatic conversions were then calculated by comparison with standard solutions. Analysis was at 256 nm.

3.2.7. Determination of protein inside polymer beads. The amount of protein that had accessed the PEGA polymers (Fig. 4) was determined by the Pierce method, using bicinchoninic acid kit (Sigma). Standard PGA solutions with known enzyme concentrations were used as standards. Analyses were performed as reported in literature.[8]

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Tetrahedron

Biotransformation of resveratrol: synthesis of *trans*-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*

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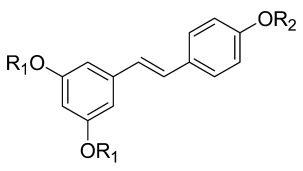
Abstract—Laccases from different sources have been used, for the first time, for the selective oxidation of the stilbenic phytoalexin *trans*-resveratrol (3,5,4′-trihydroxystilbene, **1a**) on a preparative scale. Specifically, the enzymes from *Myceliophtora thermophyla* and from *Trametes pubescens* gave the dehydrodimer **2a** in 31 and 18% isolated yields, respectively. These results compare favorably with the reported data for the chemically catalyzed dimerization of **1a** (18% yields). The antioxidant properties of **2a** have also been investigated.

1. Introduction

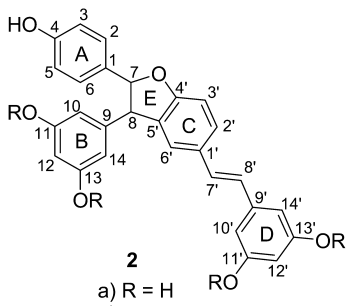
trans-Resveratrol (3,5,4′-trihydroxystilbene, **1a**) is a stilbenic phytoalexin produced by plants via a metabolic sequence induced in response to biotic or abiotic stress factors.[1] It has been found in a multitude of dietary plants, such as pines, legumes and grapevines: thus, relatively high concentrations of this compound are present in grape juice and in red wine.[2] Resveratrol is one of the phenolic compounds present in wine that could be responsible for the decrease in coronary heart disease observed among wine drinkers (French paradox).[3] Growing evidence suggest that resveratrol plays a role in the prevention of carcinogenesis.[4] In addition to resveratrol, its oligomers, the so-called 'viniferins', have also been found in plants[5] as a result of infection or stress. Oligomeric stilbenes are reported to exhibit a wide-array of biological activities, such as antimicrobial, anti-HIV, anti-inflammatory;[5] they are also reported to be potentially important cancer chemoprotective agents, being able to inhibit cellular events associated with carcinogenesis.[5,6] Due to their occurrence in various edible vegetables and fruits, these compounds may represent nutritionally important constituents.

Despite that, as many of these compounds are exclusively obtained by extraction from natural sources, the studies of their biological properties are limited by their very scarce availability.

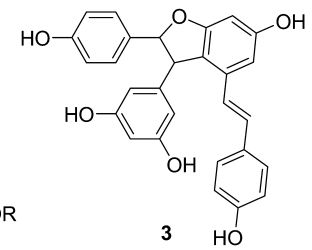
Few synthetic approaches to the oxidative coupling of resveratrol have been reported. By treating **1a** with 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) a major



1
a) $R_1 = R_2 = H$
b) $R_1 = Ac$; $R_2 = H$
c) $R_1 = H$; $R_2 = CH_3$



2
a) R = H
b) R = Ac



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Keywords: Resveratrol; Laccase; Polyphenols; Biotransformation.
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product was isolated in 18% yield and identified as trans-resveratrol dehydrodimer **2a**.[7] On the other hand, the treatment of **1a** with $FeCl_3$ failed to give **2a**, affording the isomeric dimer ε-viniferin (**3**) as the sole product.[8]

Different studies have shown that resveratrol is transformed in vivo (for instance by incubating **1a** with *Botrytis cinerea* cultures) into various oxidized products (dimers), such as **2a**.[9,10] On the other hand, to the best of our knowledge, there are only four reports on the in vitro biocatalyzed oxidation of **1a**: the first two describe a horseradish peroxidase–hydrogen peroxide promoted dimerization of **1a** to **2a**,[11] the third reports on the oxidative degradation of **1a** by action of a soybean lipoxygenase (products structures not identified),[12] while the fourth describes the performances of a purified laccase isolated from a *B. cinerea* strain.[13] The latter reaction was run on analytical scale, monitored by HPLC and the formed product was identified as ε-viniferin **3** simply by comparison with the HPLC elution time of an authentic sample.

As a part of our general interest in biocatalysis[14] and, more specifically, in enzyme-catalyzed carbon–carbon bond formation,[15] we have recently started an investigation on the synthetic potential of laccases. In this paper we report on the preparative-scale oxidation of *trans*-resveratrol and of some of its derivatives by action of two laccases, isolated from *Mycelyopthora thermophyla* and from *Trametes pubescens*, respectively.

2. Results and discussion

Laccases are oxidoreductases (the so-called 'blue oxidases'), widely distributed in fungi and in some bacteria and higher plants and able to catalyze the oxidation of various phenolic compounds at the expense of molecular oxygen.[16] The active site of these enzymes consists of a metallic cluster containing four copper atoms, all of them being involved in the redox process via a radical cyclic mechanism.[17] The oxidation of a specific phenolic derivative depends on the redox potential difference between the target compound and the so called 'T1'-copper. Laccases show an exceptional substrate versatility and therefore are potentially suitable biocatalysts for the mild oxidation of organic compounds. So far, the main limitation to their use has been their scarce availability. However, mainly to satisfy the demand for new 'green' processes by the textile and pulp and paper industries, some of these enzymes have recently been cloned and overexpressed and are becoming commercially available.[18]

Literature reports on the synthetic applications of laccases can be divided into two groups. In the first one, these enzymes are used to oxidize a suitable chemical compound (i.e., Tempo), which in turn acts as a 'mediator' for the oxidation of the synthetic target;[19] one example is the laccase-mediated production of aldehydes from the corresponding alcohols.[20]

The second group of reports describes the direct oxidation of phenolic derivatives, such as estradiol[21] and penicillin X,[22] to give dimers and higher oligomers. The main drawback of these biotransformations is the extensive polymerization that may arise from the radical mechanism of the oxidative process,[23] producing a complex mixture of poly-phenolic oligomers.

At variance, this has not been the case with *trans*-resveratrol. Following an optimized protocol for the use of laccases in organic solvents,[24] resveratrol (a compound that is poorly soluble in aqueous buffer solutions) has been dissolved in *n*-BuOH (pre-saturated with 25 mM phosphate buffer, pH 6.5) and submitted to the action of the laccase from *Myceliophtora thermophyla* supported on glass beads. The suspension has been shaken at 45 °C for 4 days and the formation of a more polar product has been observed by TLC. Similarly to resveratrol, this compound exhibits an intense purple color under UV irradiation (254 nm) on TLC plates: this information indicates that the new molecule maintains the extended conjugation of the stilbene system. The product has been isolated by flash chromatography in 31% yield and identified as the *trans*-dehydrodimer **2a** by EI-MS and extensive mono- and bi-dimensional NMR analysis.

The EI-MS shows a molecular ion peak at *m/z* 454, corresponding to the structure of a dehydrodimer of resveratrol (*m/z* calcd for $C_{28}H_{22}O_6$ is 454), while ^1H and ^{13}C NMR data, reported in Table 1, suggest the substituted dihydrobenzofurane structure **2a**, in accordance with the values reported in literature.[10b] To unambiguously assign the relative stereochemistry of the stereogenic centers of C-7 and C-8, a detailed NMR study has been performed.

Initial inspection of the ^1H NMR spectrum of **2a** in DMSO-d_6 has shown the presence of three different types of aromatic OH's at 9.14, 9.17 and 9.48 ppm (integral: 2H, 2H and 1H, respectively) suggesting a penta-phenolic structure. Additionally, the ^1H NMR spectrum exhibits signals due to one 4-hydroxybenzene moiety (multiplet at 6.85 ppm, AA′ system, 2H, and 7.24 ppm, BB′ system, 2H), to two 3,5-dihydroxybenzene moieties (doublet at 6.20 ppm, 2H; triplet at 6.26 ppm, 1H; triplet at 6.29 ppm, 1H; doublet at 6.54 ppm, 2H), to two *trans* olefinic protons (doublets at 6.90 and 7.06 ppm), and to two aliphatic protons (doublets at 4.47 and 5.45 ppm).

The assignments have been made on the basis of ^1H, ^{13}C-inverse detected single-quantum (HSQC)[25] and multiple-bond (HMBC)[26] correlation experiments. The long-range correlation between H-2,6 at 7.24 ppm and C-7 at 94.72 ppm, and between H-10,14 at 6.20 ppm and C-8 at 58.52 ppm allows to establish that the A ring is bonded to C-7 whereas the B ring is bonded to C-8. The low, but detectable, observed correlation between H-7 at 5.45 and C-4′ at 161.3 ppm confirms the presence of the fused oxygenated five-member ring and is in favor of a dihedral angle H-7–C-7–O–C-4′ close to 90°.

The value of ^3J(H-7,H-8) measured from ^1H NMR spectrum (7.97 Hz) suggests a predominant conformation with the two H-7 and H-8 protons in a pseudo *trans*-axial arrangement, whereas the ^3J(H-7′,H-8′) is 16.33 Hz, as expected for a double bond with a *trans* configuration. The distinction

Table 1. ^{1}H and ^{13}C NMR data for compounds **2a** and **2b**

	δ_H (mult., *J* Hz)			δ_C	
	2a[a,b]	2b[c]		2a[b]	2b[c]
			C-1	133.28	129.47
H-2,6	7.24 (BB′ multiplet)	7.20 (BB′ multiplet)	C-2,6	129.23	127.89
H-3,5	6.85 (AA′ multiplet)	6.76 (AA′ multiplet)	C-3,5	116.85	115.29
			C-4	159.10	157.65
H-7	5.45 (d, 7.96)	5.54 (d, 8.13)	C-7	94.72	92.08
H-8	4.47 (d, 7.96)	4.76 (d, 8.13)	C-8	58.52	54.80
			C-9	145.91	143.80
H-10,14	6.20 (d, 2.16)	6.92 (d, 2.10)	C-10,14	108.11	118.74
			C-11,13	160.44	151.08
H-12	6.29 (t, 2.16)	6.95 (t, 2.10)	C-12	103.08	114.79
			C-1′	132.43	130.08
H-2′	7.43 (dd, 8.26, 1.63)	7.47 (dd, 8.45, 1.36)	C-2′	129.29	128.24
H-3′	6.87 (d, 8.26)	6.94 (d, 8.45)	C-3′	110.81	109.67
			C-4′	161.30	159.14
			C-5′	132.81	130.76
H-6′	7.26 (broad s)	7.24 (broad s)	C-6′	124.62	123.07
H-7′	7.06 (d, 16.33)	7.26 (d, 16.35)	C-7′	129.80	130.24
H-8′	6.90 (d, 16.33)	7.02 (d, 16.35)	C-8′	127.92	124.07
			C-9′	141.46	139.79
H-10′,14′	6.54 (d, 2.10)	7.21 (d, 2.10)	C-10′,14′	106.42	116.73
			C-11′,13′	160.22	151.02
H-12′	6.26 (t, 2.10)	6.82 (t, 2.10)	C-12′	103.43	114.29
11,13-CH_3		2.22	11,13-CH_3		20.74
11′,13′-CH_3		2.26	11′,13′-CH_3		20.74
			11,13-C=O		168.80
			11′,13′-C=O		168.90
OH		9.53			

[a] Single broad signal due to hydroxy protons was found at 8.20.
[b] Aceton-d_6
[c] DMSO-d_6.

between the ethylenic protons has been made through the analysis of the H,C long-range correlation found in the HMBC spectrum: the signal of H-7′ at 7.06 ppm shows a correlation with the carbon signal at 124.62 ppm, directly bonded to H-6′ (i.e., C-6′), whereas the signal due to H-8′ at 6.90 ppm is correlated to the carbon signal at 106.42 ppm, directly bonded to H-10′, 14′ (i.e., C-10′,14′).

The relative stereochemistry of C-7 and C-8 carbons has been proposed on the basis of the analysis of the NOESY[27] NMR spectrum (400 ms mixing time) and of the computer-aided energy-minimized stereostructure obtained. The NOESY spectrum clearly shows the absence of NOE in-phase cross-peaks between H-7 and H-8, and the presence of positive in-phase cross peaks between H-7 and H-10,14 (of comparable intensity with respect to those between H-7 and H-2,6) and between H-8 and H-2,6 (of comparable intensity with respect to those between H-8 and H-10,14), thus confirming that H-7 and H-8 are in a prevalent *trans*-axial arrangement. The remaining NOE observed (i.e., between H-10,14 and H-6′) is in accordance with the conclusions drawn from the H,C correlation experiments. According to the modified Karplus-equation reported by Haasnoot,[28] the ^{3}J(H-7,H-8) value found for the compound **2a** (7.96 Hz) would result in a dihedral angle of about −140°, which is in good accordance with that found from the computer-aided calculation for the *trans*-dia-stereoisomer (−141°). In this way the isolated product has been unambiguously identified as the compound **2a**. The same product could be isolated, albeit in a lower yield (18%), when the bio-catalytic oxidation of **1a** was performed using a laccase from *Trametes pubescens* in a biphasic system acetate buffer—AcOEt.[24]

The structure of **2a** indicates that the dimerization reaction proceeds exclusively through the phenoxy radical derived from the 4′-OH of **1a**. As further evidence, it has been found that the 4′-*O*-methyl resveratrol derivative **1c** is unreactive under the same reaction conditions, whereas the 3,5-diacetylated derivative **1b** is oxidized to give the expected *trans*-dehydrodimer **2b**, whose structure has been assigned by ^{1}H and ^{13}C NMR analysis using HMBC, NOESY and ROESY experiments (Table 1).

The antioxidant activity of **2a** has been determined by the DPPH reduction method,[29] by plotting, as indicated in the Section 4, the remaining percentage of DPPH as a function of the molar ratios of **2a** over DPPH. The resveratrol dimer reacts with DPPH, reaching a steady state after about 3 h. An EC_{50} of 5.2±0.4 (mmol **2a**/mmol DPPH) has been determined, which is slightly greater than that observed for resveratrol [4.1±0.3 (mmol/mmol DPPH)] and comparable with that of its biologically active analogue pterostilbene [5.0±0.4 (mmol/mmol DPPH)].[4c]

3. Conclusions

In this paper we have shown the usefulness of isolated laccases for the synthesis of the resveratrol dimer **2a** in good yields and under very mild reaction conditions (atmospheric air, enzyme, solvent). To our knowledge, this is the first report on the preparative-scale oxidative dimerization of **1a** catalyzed by this group of oxidative enzymes. Due to the wide-array of biological activity exhibited by resveratrol oligomers, the compounds synthesized may serve as lead for the development of new drugs and as nutraceuticals. Furthermore, the bio-oxidation of resveratrol, affording dimeric product(s), is interesting not only from a chemical point of view but it has an utmost importance, for example, for the wine-making industry. The use of enzyme preparations in the wine-making industry to improve the quality of wine is well established:[30] for example, the color stabilization by removal of excess phenols using laccase has been proposed. Immobilization of enzymes for their use in wine making is another interesting possibility.

As regards the antioxidant activity of **2a**, this dimer is able to scavenge DPPH radical with an EC_{50} value comparable with that observed for resveratrol and its analogues, disclosing a preliminary positive information on the bioactivity of **2a**. Being easily scaled up, this simple and selective biotransformation can afford the dimeric product(s) in sufficient amounts to allow the evaluation of the biological activity of compound **2a** and of other resveratrol derivatives (i.e., **2b**): in particular, the protection against lipid peroxidation, the effects on cell proliferation and the ability to inhibit replicative DNA polymerase are under investigation in our laboratory and will be reported in due course.

4. Experimental

4.1. Materials and methods

Laccase from *Myceliophtora thermophyla* was from Novozymes A/S, Denmark, while the laccase from *Trametes pubescens* was provided by Professor Haltrich.[31] Compounds **1b**[32] and **1c**[4c] were synthesized following the procedures reported in the literature. TLC: precoated silica gel 60 F_{254} plates (Merck). Flash chromatography: silica gel 60 (70–230 mesh, Merck). Mass spectra were acquired with a combined HPLC particle beam MS-engine. NMR spectra were recorded in aceton-d_6 or DMSO-d_6 solutions at 400 MHz and the chemical shift reported are in ppm relative to tetramethylsilane as external standard. Energy minimized stereostructure of **2a** was obtained by MM+ calculation using HyperChem 5.0 molecular modeling program. Antiradical activity by DPPH: spectrophotometric data were acquired using a UV/VIS spectrometer.

4.2. Adsorption of the laccase from *M. thermophyla* on glass beads

M. thermophyla laccase was dialyzed against 25 mM phosphate buffer pH 6.5 and lyophilized. The enzyme was dissolved in the same buffer (60 mg/ml) and the solution was dropped onto glass beads (1 ml/3 g). The slurry was mixed and left to dry at room temperature with occasional mixing for nearly 2 days.

4.2.1. Oxidation of resveratrol catalyzed by *M. thermophyla* laccase. Resveratrol (100 mg) was dissolved in 6 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 200 mg (120 U) of *M. thermophyla* laccase supported onto glass beads was added and the suspension was shaken at 45 °C for 4 days. The enzyme and a little amount of a brown precipitate were separated by filtration, the solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=6:4:0.5) to give 31 mg of **2a** (31% yield). Pale yellow amorphous solid. ^{1}H NMR (acetone-d_6, TMS): 4.47 (d, J=8.0 Hz, 1H, H-8), 5.45 (d, J=8.0 Hz, 1H, H-7), 6.20 (d, J=2.2 Hz, 2H, H-10,14), 6.26 (t, J=2.1 Hz, 1H, H-12′), 6.29 (t, J=2.2 Hz, 1H, H-12), 6.54 (d, J=2.1 Hz, 2H, H-10′,14′), 6.85 (AA′ multiplet, 2H, H-3,5), 6.87 (d, J=8.3 Hz, 1H, H-3′), 6.90 (d, J=16.3 Hz, 1H, H-8′), 7.06 (d, J=16.3 Hz, 1H, H-7′), 7.24 (BB′ multiplet, 2H, H-2,6), 7.26 (broad s, 1H, H-6′), 7.43 (dd, J=8.3, 1.6 Hz, 1H, H-2′). ^{13}C NMR (acetone-d_6, TMS): 58.52, 94.72, 103.08, 103.43, 106.42, 108.11, 110.81, 116.85, 124.62, 127.92, 129.23, 129.29, 129.80, 132.43, 132.81, 133.28, 141.46, 145.91, 159.10, 160.22, 160.44, 161.30.

MS (EI, 70 eV): m/z (%)=454 (39, M^+); 228 (100); 179 (93); 123 (82).

Anal. calcd for $C_{28}H_{22}O_6$: C, 74.00%; H, 4.88%; O, 21.12%. Found C, 74.06; H, 4.93.

4.2.2. Oxidation of resveratrol catalyzed by *T. pubescens* laccase. Resveratrol (100 mg) was dissolved in 4 ml of ethyl acetate, while the laccase (110 U) was dissolved in 4 ml of 20 mM acetate buffer, pH 4.5. The biphasic system was shaken at room temperature for 24 h. The organic solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=6:4:0.5) to give 18 mg of **2a** (18% yield).

4.2.3. Attempted oxidation of 4′-*O*-methyl-resveratrol (1c) by *M. thermophyla* laccase. 4′-*O*-Methyl-resveratrol (**1c**, 3 mg) was dissolved in 0.5 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 50 mg (30 U) of *M. thermophyla* laccase supported onto glass beads were added and the system was shaken at 45 °C. No reaction was observed after 48 h.

4.2.4. Oxidation of 3,5-di-*O*-acetyl-resveratrol (1b) by *M. thermophyla* laccase. 3,5-Di-*O*-acetyl-resveratrol (**1b**, 35 mg) was dissolved in 8 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 150 mg (90 U) of the *M. thermophyla* laccase supported onto glass beads was added and the system was shaken at 45 °C for 4 days. The enzyme and a little amount of a brown precipitate were separated by filtration, the solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=75:25:5) to give 9 mg of **2b** (26% yield). Pale yellow oil. ^{1}H NMR (acetone-d_6): 2.22 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 4.76 (d, J=8.1 Hz, 1H, H-8), 5.54 (d, J=8.1 Hz, 1H, H-7), 6.76 (AA′ multiplet,

2H, H-3,5), 6.82 (t, J=2.1 Hz, 1H, H-12′), 6.92 (d, J=2.1 Hz, 2H, H-10,14), 6.94 (d, J=8.5 Hz, 1H, H-3′), 6.95 (t, J=2.1 Hz, 1H, H-12), 7.02 (d, J=16.4 Hz, 1H, H-8′), 7.20 (BB′ multiplet, 2H, H-2,6), 7.21 (d, J=2.1 Hz, 2H, H-10′,14′), 7.24 (broad s, 1H, H-6′), 7.26 (d, J=16.4 Hz, 1H, H-7′), 7.47 (dd, J=8.5, 1.4 Hz, 1H, H-2′), 9.53 (s, 1H, OH). ^{13}C NMR (acetone-d_6): 20.74 (2C), 54.80, 92.08, 109.67, 114.29, 114.79, 115.29, 116.73, 118.74, 123.07, 124.07, 127.89, 128.24, 129.47, 130.08, 130.24, 130.76, 139.79, 143.80, 151.02, 151.08, 157.65, 159.14, 168.80, 168.90.

MS (EI, 70 eV): m/z (%)=622 (33, M^+); 580 (36); 538 (27); 496 (15); 454 (7); 270 (33); 228 (69); 221 (86); 179 (100); 123 (77).

Anal. calcd for $C_{36}H_{30}O_{10}$: C, 69.44%; H, 4.86%; O, 25.70%. Found C, 69.47; H, 4.91.

4.3. Antiradical activity

The antioxidant activity of **2a** was determined using DPPH as a free radical, following the method described by Berset,[29] using different concentrations of **2a** (0.069, 0.137, 0.296, 0.468 mM). A methanolic solution of **2a** (100 μl) was added to 3.9 ml of a 0.06 mM DPPH methanolic solution. The decrease in absorbance at 515 nm was evaluated until the reaction reached a plateau (about 3 h). The percentage of residual DPPH at the steady state was reported onto a graph as a function of the molar ratio of antioxidant to DPPH. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (efficient concentration).

5. Supplementary material

NMR and mass spectra of **2a** and **2b**, and energy minimized stereostructure of **2a** are available on request.

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Enzymatic transformations. Part 55: Highly productive epoxide hydrolase catalysed resolution of an azole antifungal key synthon

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Abstract—A highly productive bioprocess for the preparation of enantiopure azole antifungal chirons is described. These are key building blocks for the synthesis of new triazole drug derivatives known to display valuable activity against such infections as for instance fluconazole-resistant oro-oesophageal candidiasis. Using commercially available recombinant *Aspergillus niger* epoxide hydrolase under optimised experimental conditions, the hydrolytic kinetic resolution of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane was performed in plain water, at room temperature, using a two-phase reactor. This methodology allowed the process to be run at a substrate concentration as high as 500 g/L (i.e., 2.5 M) and afforded the (unreacted) epoxide and the corresponding vicinal diol, both in nearly enantiopure form and quantitative yield.

1. Introduction

The preparation of enantio-enriched epoxides or of their corresponding vicinal diols, two types of chiral intermediates which offer high chemical versatility, has been considered for years as one of the most significant goals for asymmetric synthesis. Identification of new methodologies for access to these targets has been an area of active effort for several decades. Diverse conventional chemistry approaches have been described, the best ones based on the use of different types of transition-metal based catalysts.[1,2] We (and others) have also explored, over the last decades, the possibility offered by the use of appropriate enzymes. In particular, we have described that microbial epoxide hydrolases allowed efficient biocatalysed hydrolytic kinetic resolution (BHKR) of various epoxides.[3] Although this approach surprisingly seems to be sometimes ignored, for some unclear cultural reasons, by organic chemists, there is no doubt that this methodology could provide interesting complementarity[4] as well as valuable practical advantages to the conventional chemistry approach, particularly for industrial application. For example, the 'green chemistry' aspect, the very high turnover numbers generally observed, and the fact that some of these enzymes offer complementary regioselectivities (thus allowing to set up an enantioconvergent process, i.e., affording 100% ee, 100% yield starting from a racemic substrate) are important advantages which are surely worth considering.[5]

With the exception of lipases and baker's yeast, nowadays commonly accepted as 'classical reagents', the use of biocatalyts is very often considered to be hampered by several practical drawbacks. These are for instance: (a) the fact that the appropriate enzyme is rarely commercially available (b) the (possible) limited substrate specificity of a given enzyme (c) the low substrate concentration usable owing to the (generally) poor solubility of organic substrates in water (thus leading to low volumetric productivity of the bioreactor) or, else (d) the reaction limitation linked to substrate and/or product inhibition. Although, depending on the substrate, these limitations should indeed not be neglected, they may in many cases be circumvented by using appropriate methodologies. We describe herein our results, focused on the BHKR of epoxide **1**, where these drawbacks could be efficiently overcome. Thus, we show that using the commercially available recombinant *Aspergillus niger* epoxide hydrolase† it is possible to set up a highly productive bioprocess for the preparation of enantiopure epoxide **1** as well as of the corresponding chloro-diol **2**. Both these chirons are key building blocks for the synthesis of new triazole drug derivatives, like for instance D0870, known to display valuable activity against such infections—as for instance fluconazole-resistant oro-oesophageal candidiasis—often encountered in HIV-infected (and other immunocompromised) patients[6,7] (Scheme 1).

Keywords: Biocatalysis; Enzyme resolution; Epoxide; Kinetic resolution; Enantioselective; Hydrolysis.
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† This enzyme is presently commercially available from FLUKA (ref. 71832).

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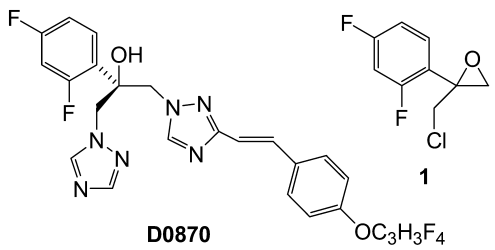


Scheme 1.

2. Results

We have recently described our preliminary results aimed at offering an alternative pathway to the synthesis of the enantiopure epoxide (*S*)-**1** and of the corresponding diol (*R*)-**2**.[8] This involved the BHKR of *rac*-**1**, performed using the commercially available (recombinant) *A. niger* epoxide hydrolase. We have shown that, when conducted at a 2 mM (0.4 g/L) concentration of *rac*-**1** in a 100 mM phosphate buffer solution (pH 7) containing 10% DMSO, this could efficiently lead to enantiopure (*S*)-**1** and to the corresponding chloro-diol (*R*)-**2**, also obtained in nearly enantiopure form (ees>98%) (Scheme 2). The observed *E* value of this resolution was higher than 200, and the α(*S*)/β(*S*) and α(*R*)/β(*R*) regioselectivity factors, determined for each one of the two enantiomers of **1**, were calculated to be 5/95 and 1/99, respectively, indicating that both enantiomers were essentially attacked at the less substituted carbon atom.

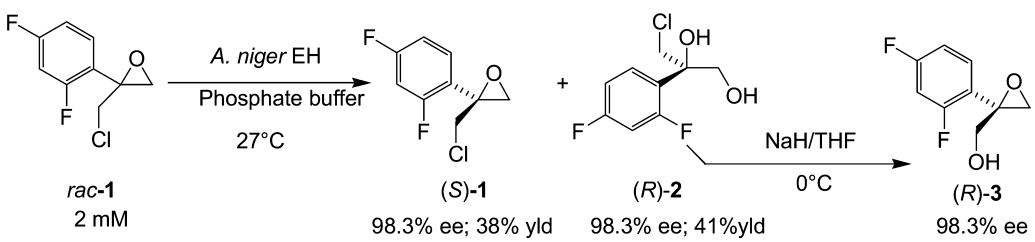
These interesting results encouraged us to pursue this study in order to set up an improved preparative scale process. In particular, we wanted to address some of the drawbacks cited above, in order to demonstrate the practical applicability of such a biocatalytic procedure. To reach this goal we explored the possibility (a) to simplify the experimental protocol (b) to increase the substrate concentration (c) to optimise the enzyme/substrate ratio (d) to perform this BHKR at a several-gram scale using the thus defined optimal experimental conditions.

2.1. Simplification of the experimental protocol

2.1.1. Use of plain water instead of phosphate buffer. As a general trend, one of the important issues for industrial application is, whatever the type of reactant or catalyst (chemical or enzymatic) involved, the set up of so-called 'salt free' processes. This obviously allows to simplify, and therefore to lower the cost, of the subsequent downstream processing. Our previously described procedure—which involved in the use of a 100 mM (pH 7) phosphate buffer—in fact implied an amount of 15 g/L of phosphate salts, which would translate into 15 kg/m^3 in case of an industrial application. Therefore, we have explored the possibility to perform this resolution using plain water instead of phosphate buffer. Indeed, we had shown previously—using rather water soluble pyridine oxirans—that to the contrary of many enzymes, the *A. niger* epoxide hydrolase retained nearly its total activity in plain water. It remained to be checked whether this was also applicable to the rather water insoluble epoxide **1**. The results of a comparative study between the BHKR of *rac*-**1**, using a phosphate buffer solution on one hand or plain water on the other hand, are reported on Figure 1. Interestingly no significant difference was observed between these two experiments, indicating that a salt free process could also be used in this case.

2.1.2. Optimisation of the substrate concentration. As far as the practical/industrial applicability of such a process is concerned, one major bottleneck obviously could be the usable substrate concentration. We have determined that the aqueous solubility of **1** is about 0.7 g/L, precluding any high concentration application using a monophasic aqueous medium. Addition of a water miscible solvent, i.e., DMSO as described in our preliminary results, obviously would allow to slightly increase the aqueous concentration, but only to a very limited extend keeping in mind that this solvent is only tolerated by the enzyme at less than about 10% concentration (at this concentration the residual activity is about 70%). Therefore, we decided to explore the possibility to set up a 'high concentration' two-phase reactor, the substrate playing itself the role of the organic phase. We have already described the possibility to efficiently operate such a two-phase BHKR reaction in the case of *para*-bromo-α-methylstyrene oxide[9] or *para*-chlorostyrene oxide,[5] for which a substrate concentration as high as 80 and 300 g/L, respectively, could be reached. It should be noted that these two reactions were carried out at low temperature (4 and 0 °C respectively) in order to limit the spontaneous chemical hydrolysis of these substrates. In the present case, epoxide **1** appeared to be very stable towards chemical hydrolysis (less than 0.1% per hour), thus allowing the preparative experiments to be carried out at nearly room temperature (27 °C).

The possibility to increase the substrate concentration was explored in the range of 2 mM–3.6 M concentration. The reaction was performed using plain water at a constant enzyme/substrate ratio of 93 U/mmol of *rac*-**1**, and was followed by monitoring the ee of the remaining epoxide at 20, 40 and 60 min reaction time periods (Fig. 2). Interestingly, it thus appeared that (a) the reaction did very nicely proceed in such a two-phase system (b) no mass transfer limit was apparent (c) the BHKR reaction was very efficient



Scheme 2.

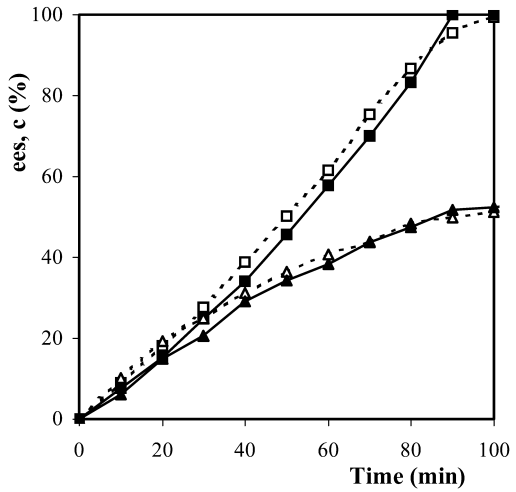


Figure 1. Comparative study between the BHKR of *rac*-**1** using a phosphate buffer solution or plain water. Full line: phosphate buffer; dashed line: plain water; squares: ee of **1**; triangles: conversion ratio c.

up to a substrate concentration as high as 2.5 M (500 g/L) (d) above this value, the reaction rate slowed down noticeably (e) surprisingly—at an identical reaction time—the ee of the remaining epoxide appeared to reach a higher value for experiments conducted at higher substrate concentration. Thus, a comparative study, carried out in a homogeneous system (at 2 mM, i.e., 0.4 g/L) on one hand, and using a two-phase system (2.5 M, i.e., 500 g/L) at the other hand, clearly showed that at high substrate concentration the rate of reaction was faster by a factor of about 1.5 than at low concentration. In these cases, the ee of the remaining epoxide reached nearly 100% after only 40 min in the biphasic system, whereas 60 min were necessary to reach the same value for the monophasic system (Fig. 3). A more detailed study of this interesting effector effect, which will be published elsewhere, showed that this activation was due to the presence of diol **2** formed during biohydrolysis.

2.1.3. Optimisation of the enzyme over substrate ratio. One other important aspect of the cost effectiveness of such a bioprocess is obviously the amount of enzyme necessary to run the reaction, and therefore, the enzyme/substrate ratio is one of the important factors to be optimised. This is particularly true in the case of epoxide resolution, racemic epoxides being generally relatively cheap compounds. We

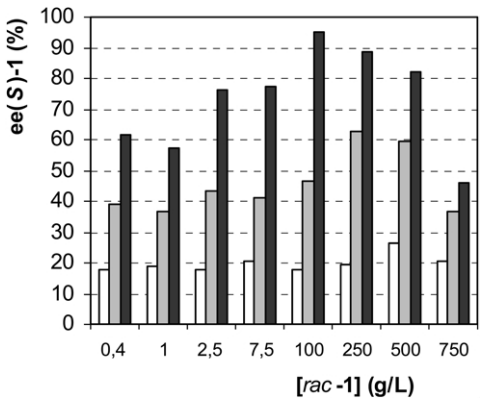


Figure 2. Substrate concentration increase. The enzyme/substrate ratio was kept constant at 93 U/mmol of *rac*-**1** throughout the scale up. Reaction time: white box: 20 min; grey box: 40 min; black box: 60 min.

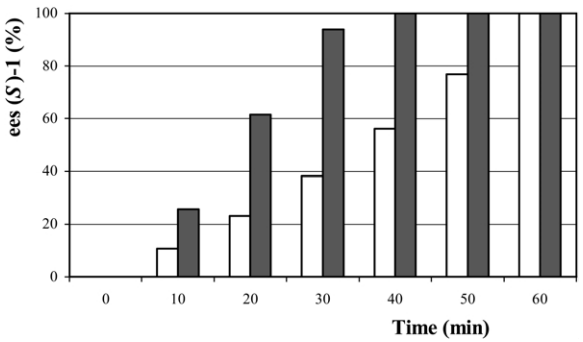


Figure 3. Influence of the substrate concentration on the ee of the residual epoxide-**1** as a function of time. □ %ee of (*S*)-**1** at 0.4 g/L (2 mM); ■ %ee of (*S*)-**1** at 500 g/L (2.5 M).

therefore have explored the possibility to decrease the enzyme/substrate ratio, and the results we have obtained are shown in Figure 4. It appeared that the resolution process could be carried out even when the enzyme/substrate ratio was decreased from 100 to 25 U/mmol of *rac*-**1**, the reaction time being then increased from 60 to 300 min for reaching the 50% conversion ratio (ee>99%).

2.2. Gram scale reactor

Using these optimised experimental conditions, a gram scale preparative experiment was conducted at a 2.5 M (500 g/L) concentration in a two-liquid-phase reactor. This was performed using 2 g of *rac*-**1** in the presence of 275 U of crude recombinant *A. niger* epoxide hydrolase (corresponding to 25 mg of crude powder at 11 U/mg) i.e., an enzyme/substrate ratio of 28, dissolved in 1.8 mL plain water and 0.2 mL DMSO. This was stirred for 2 h at 27 °C, and the reaction was quenched by addition of 1 mL of acetonitrile to the reaction medium. Unreacted **1** was extracted with pentane (3×15 mL), whereas diol **2** was further extracted with ethyl acetate (3×15 mL). Both products were purified by flash chromatography, then by bulb-to-bulb distillation, which led to 0.83 g of (*S*)-**1** [99.9% ee; 41.5% yield; $[\alpha]_D^{25}=+46.5$ (*c* 1; THF)], and to 0.95 g of diol (*R*)-**2** [94.5% ee; 43.5% yield; $[\alpha]_D^{25}=+3.4$ (*c* 1; THF)]. This experiment allowed us to determine (a) the turnover frequency (TOF) of the enzyme against **1**, which was

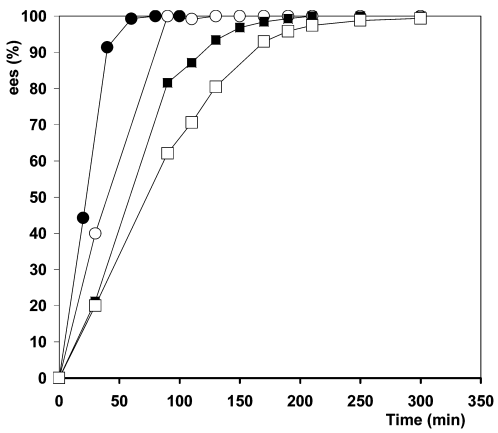


Figure 4. Influence of the enzyme/substrate ratio on the ee of the residual epoxide-**1** as a function of time at an initial concentration of 500 g/L. The enzymatic powder used throughout this study showed an activity of 9.6 U/mg. Enzyme/substrate ratio (U/mmol): ●, 100; ○, 50; ■, 37.5; □, 25.

estimated to be about 1000 h^{-1}; (b) the total turn over number (TON) which was about 21,500 and (c) the space time yield of the reactor which was about 2850 g/L/day. As can be seen, these values are very satisfactory and indicate that the industrial implementation of such a process could be interestingly envisaged (Scheme 3).

As a complement to this process it is to be stressed that, as we have described previously,[8] it is possible to chemically cyclise the obtained chloro-diol (*R*)-**2** into epoxy-alcohol (*R*)-**3**, which can itself be transformed back into epoxide (*S*)-**1** via treatment with triphenylphosphine in carbon tetrachloride (Scheme 3). Both these reactions were shown to occur without loss of stereochemical integrity. Thus, by pooling the 41.5% of the unreacted and the thus obtained (*S*)-**1** epoxide, it is possible to render this BHKR strategy enantioconvergent (i.e., to overcome the 50% yield limitation linked to a classical resolution process), the epoxide (*S*)-**1** of 100% ee being easily obtained by performing a second-round BHKR of the thus obtained highly enantiomerically enriched substrate.

3. Conclusion

The aim of this work was to optimise the experimental conditions of the BHKR of epoxide *rac*-**1**. This epoxide is a key building block for the synthesis of D0870, a triazole drug derivatives known to display efficient activity against infections—as for instance fluconazole-resistant oro-oesophageal candidiasis—often encountered in HIV-infected (and other immunocompromised) patients. By using our commercially available (recombinant) *A. niger* epoxide hydrolase as biocatalyst, we have shown that (a) this enzyme was very active against this particular substrate, (b) this BHKR could be performed at nearly room temperature, in plain water (and 5% DMSO) i.e., using a salt free methodology (c) a two-phase reactor could be conducted at a concentration as high as 2.5 M (i.e., 500 g/L) without apparent substrate and/or product inhibition (d) both the unreacted epoxide (*S*)-**1** and the formed chloro-diol (*R*)-**2** could be obtained in nearly enantiopure form and nearly quantitative yield. Interestingly, both the TOF and the TON, as well as the space time yield of this reaction, were excellent.

Moreover we have previously shown that (*R*)-**2** could be easily transformed chemically into (*S*)-**1** without loss of stereochemical integrity. Thus, in this particular case, it is possible to set up an enantioconvergent process, i.e., to overcome the theoretical 50% yield limitation theoretically linked to a resolution process. This is an additional illustration to the fact that this novel green chemistry type of process should offer very interesting opportunities for industrial preparation of both enantio-enriched epoxides and of their corresponding vicinal diols. Further work is going on in our laboratory in order to scale up this type of process to a multigram scale and to explore other possible applications of this two phase methodology.

4. Experimental

4.1. General

Gas chromatography (GC) analysis were performed using the chiral column Lipodex G (25 m×0.25 mm, Macherey–Nagel, 1 kg/cm^2 helium). NMR spectra were recorded in $CDCl_3$ using a Bruker 250 MHz instrument. The crude powders of the recombinant *A. niger* epoxide used throughout this study respectively showed an activity of 7, 8 and 11 U per mg against styrene oxide (Units are expressed in μmol/min/mg of powder), as measured by our new (sodium periodate based) spectrophotometric assay.[10]

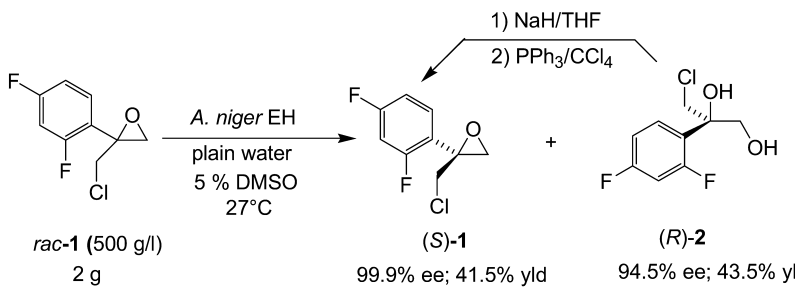
4.1.1. Synthesis of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane 1. This was synthesised following the procedure described by Murakami and Mochizuki.[11] This epoxide was obtained as a low-melting solid. Mp=42 °C. ^{1}H NMR δ: 2.9 (d, *J*=5 Hz, 1H, OCH_2), 3.2 (d, *J*=5 Hz, 1H, OCH_2), 3.7 (d, *J*=12 Hz, 1H, CH_2Cl), 4.1 (d, *J*=12 Hz, 1H, CH_2Cl), 6.9 (m, 2H, ArH), 7.4 (m, 1H, ArH); GC-condition: 100 °C; (*R*)-**1**=28.7 min; (*S*)-**1**=29.7 min.

4.2. Comparative study between the BHKR of *rac*-1 conducted in phosphate buffer solution or in plain water

8 mg of chloro-epoxide **1** dissolved in 2 mL of DMSO were mixed to 18 mL of plain water (or pH 7 phosphate buffer, 0.1 M). To this solution, 0.5 mg of *A. niger* EH powder (7 U/mg solid) dissolved in 100 μL of water were added and the medium was stirred at 27 °C. Aliquots of 400 μL were withdrawn at different time intervals; 600 μL of acetonitrile were added to stop the reaction. The epoxide was extracted with 600 μL of isooctane containing 4-bromo-acetophenone (2 mM) as internal standard and analysed by chiral GC for conversion ratio and ee.

4.3. Optimisation of the substrate concentration

General procedure. These enzymatic reactions were carried



Scheme 3. Optimal biocatalysed hydrolytic kinetic resolution of *rac*-**1**, using a two-phase process at a substrate concentration of 500 g/L (i.e., 2.5 M).

out at 27 °C in microvials containing an adapted final volume of 1000–50 μL as a function of the substrate concentration (0.4–750 g/L). To different substrate emulsions in plain water containing 10% of DMSO a calculated enzyme extract quantity (7 U/mg solid) was added in order to keep constant the enzyme/substrate ratio at 93 U/mmol of *rac*-**1**. The determination of the ee of the residual epoxide after 20, 40 and 60 min of reaction time was carried out as described above using one microvial for each value.

4.4. Optimisation of the enzyme over substrate ratio

This study was carried out at 27 °C in microvials containing a final volume of 50 μL. In each vial 25 mg of chloro-epoxide **1** were mixed at 27 °C to 25 μL of plain water containing 10% of DMSO and different quantity of enzyme powder (7 U/mg solid; enzyme/substrate ratio: 25–100 U/mmol). Very small aliquots were withdrawn at different time intervals and analysed as described above.

4.4.1. Preparative scale resolution of *rac*-1 at 0.4 g/L. 400 mg of chloro-epoxide **1** dissolved in 100 mL of DMSO were mixed to 900 mL of phosphate buffer (0.1 M, pH 7). The biohydrolysis was started by addition of 13 mg of *A. niger* powder (8 U/mg). The medium was stirred and maintained at 27 °C. When the ee of **1** reached about 98% (4 h, 45 min) the reaction was stopped by adding 250 mL of acetonitrile. The medium was saturated with NaCl and then extracted with AcOEt (4×200 mL). After drying over $MgSO_4$ and concentration in vacuum (*S*)-**1** [163 mg, 41% yield, $[\alpha]_D^{26}$=+45 (*c* 1.2; THF)] and (*R*)-**2** [163 mg, 38% yield, $[\alpha]_D^{26}$=+3.6 (*c* 1; THF); ^{1}H NMR δ: 2.2 (dd, *J*=5.4, 7.7 Hz, 1H, OH), 3.3 (s, 1H, OH), 3.8–4.1 (m, 4H, CH_2Cl and CH_2O), 6.7–7.0 (m, 2H, ArH), 7.6–7.75 (m, 1H, ArH)] were separated and purified by flash chromatography then bulb-to-bulb distillation (130 °C, 5 mbar for **1** and 200 °C, 0.1 mbar for **2**). The diol **2** was obtained as a viscous oil.

4.4.2. Synthesis of (*R*)-1-hydroxy-2-(2,4-difluorophenyl)-2,3-epoxypropane 3 from (*R*)-2. To a stirred solution of 73 mg (0.33 mmol) of (*R*)-**2** in dry THF (4 mL) cooled on an ice bath, were added 20 mg (0.5 mmol) of sodium hydride (60% mineral oil dispersion). The resulting suspension was then agitated at 0 °C for 2 h. After addition of water (2 mL), the mixture was extracted twice with ether (5 mL). The combined extracts were washed twice with a saturated NH_4Cl solution (2 mL) then twice with brine (2 mL) and dried over magnesium sulfate. After concentration in vacuum and purification by bulb-to-bulb distillation (0.1 mbar, 200 °C) 59 mg of (*R*)-**3** (96% yield) were isolated. $[\alpha]_D^{26}$=+44.6 (*c* 1; THF); lit.[11] for (*S*)-**3**: $[\alpha]_D$=−42 (*c* 1; THF); GC-condition: 120 °C, (*S*)-**3**=19 min (small peak); (*R*)-**3**=20.2 min. ^{1}H NMR δ: 2.2 (s, 1H, OH), 2.85 (d, *J*=5 Hz, 1H, OCH_2), 3.3 (d, *J*=5 Hz, 1H, OCH_2), 4.0 (m, 2H, CH_2OH), 6.7–7.0 (m, 2H, ArH), 7.3–7.5 (m, 1H, ArH).

4.4.3. Synthesis of (*S*)-1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane 1 from (*R*)-3. 20 mg (0.107 mmol) of (*R*)-**3** obtained previously and 32 mg (0.122 mmol) of triphenylphosphine were dissolved in 1 mL carbon tetrachloride, and heated for 7 h under reflux.[11] After cooling and addition of water (2 mL), the mixture was extracted with methylene chloride (2 mL). The organic phase was washed with brine, dried over magnesium sulfate and evaporated. Injection on chiral GC at 100 °C led to identical retention time (29.7 min) as one recovered after biohydrolysis.

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Preparation of N-unsubstituted β-ketoamides by *Rhodococcus rhodochrous*-catalysed hydration of β-ketonitriles

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Abstract—A varied set of N-unsubstituted β-ketoamides, hardly obtainable or non-accessible by non-enzymatic methods, have been synthesized, with good to excellent yields, by the generally fast hydration of the corresponding β-ketonitriles, catalysed by the bacterium *Rhodococcus rhodochrous* IFO 15564. This bacterium shows nitrile hydratase and amidase activities, the latter being inhibited during its growth phase with diethyl phosphoramidate (DEPA). Optimization of the processes and studies concerning large-scale biotransformations were also carried out. β-Ketoamides exist as keto–enol mixtures whose composition depends on their substituents and varies with solvent polarity.

1. Introduction

β-Ketoamides are versatile intermediates for the synthesis of heterocycles, for instance 3-acyltetramic acids[1] (used in the total synthesis of tirandamycin[2] and other related natural antibiotics), pyrans,[3] alkaloids,[4] lactams and spirolactams,[5] and azetidin-2-ones,[6] as well as several 3-hydroxyisothiazol bioisosteres of glutamic acid and analogs of the AMPA receptor agonist.[7] Recently, some β-ketoamides have been converted into γ-ketoamides, a class of compound related with a wide variety of biologically relevant systems.[8]

In connection with our current interest in the bioreduction of β-ketoamides and their subsequent transformation into non-racemic β- and γ-aminoalcohols,[9] we needed a set of N-unsubstituted β-ketoamides as starting materials. This type of β-ketoamide is hardly accessible by means of their usual preparative methods,[10] mostly directed towards the N-substituted ones. For such a cause, we decided to prepare them via the hydration of the corresponding β-ketonitriles, a commercially or easily available kind of compound. After a series of failed attempts using traditional, non-enzymatic methodologies, we envisaged that enzymatic hydration of β-ketonitriles would be a suitable, environmentally benign way of reaching our objective.

The enzymes involved in the desired biotransformation, i.e. nitrile hydratases (EC 4.2.1.84), are not commercially available, which forced us to use an appropriate whole cell culture. However, the bacterial strains able to hydrolyze nitriles can act according to one or both of the following routes: (i) a nitrile hydratase converts the nitrile into an amide, which is subsequently transformed into a carboxylic acid by an amidase; (ii) a nitrilase directly converts the nitrile into an acid. The presence of amidases and/or nitrilases is a serious drawback for our purposes of stopping the process in the amide stage, and it should be avoided.

A solution would be the use of some bacterial strains (*Rhodococcus* sp. N774, *Pseudomonas chlororaphis* B23, *Rhodococcus rhodochrous* J1) which develop nitrile hydratases almost exclusively when growing under appropriate conditions. Consequently, they have been used for an important industrial biotransformation (Nitto Chemical Industry Co.) leading to acrylamide, as well as for the preparation of other commodity chemicals.[11] One of these strains (*P. chlororaphis* B23) has also been employed for the industrial conversion of adiponitrile to 5-cyanovaleramide (DuPont),[12] as the first step in the manufacture of a new herbicide. Unfortunately, these strains are commercially unavailable.

Apart from these examples, the scarcely reported bacterial transformations of nitriles into amides[13] seem to be related to an accidental, unfavourable steric interaction between the amidase and the corresponding amide, which slows down its further conversion to acid. Thus, several *ortho*-substituted benzonitriles (but not their *meta*- or *para*-isomers) and five membered hetarene-2-carbonitriles were transformed into

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their corresponding amides after short reaction times with *R. rhodochrous* AJ270,[14] another inaccessible strain. In the same way, the hydration carried out with *R. rhodochrous* IFO 15564 of three cyanocarbohydrate derivatives to amides,[15] as well as that of 3-cyanoprop-1-enylboronic acid,[16] can probably be rationalised.

On the other hand, it has recently been demonstrated that diethyl phosphoramidate [$H_2N-P(O)(OEt)_2$; DEPA] behaves as an amidase inhibitor for three nitrile hydrolyzing bacterial strains when exposed to 2-arylpropanenitriles and to 2-arylpropanamides. With *Agrobacterium tumefaciens* strain d3, 2-arylpropanenitriles were enantioselectively converted into 2-arylpropanamides,[17] as the hydration of the nitrile to amide was not inhibited by DEPA;[17b] similar results were observed for the strains *Rhodococcus equi* A4[18a] and *Rhodococcus* sp. MP50.[18b]

The above observations prompted us to undertake the biotransformations of a varied set of β-ketonitriles into their corresponding β-ketoamides. This paper describes our findings with the concourse of the bacterium *R. rhodochrous* IFO 15564 in metabolic resting phase. Since the obtained β-ketoamides are also accepted as substrates by this strain, we have reached our goal by using DEPA as amidase inhibitor. To the best of our knowledge, bacterial hydration of β-ketonitriles has not been previously performed for preparative purposes.

2. Results and discussion

2.1. Choice of the starting β-ketonitriles

Eighteen varied β-ketonitriles, R^1-CO–CHR^2–CN (**1**), were selected for this study (see Table 3). Sixteen of them (**1a**–**r**) show no substituent at α position (i.e., R^2=H). Three of them have R^1 of aliphatic nature (**1a**–**c**), although **1c** includes a hetarene ring in R^1. The following eight (**1d**–**k**) possess aromatic R^1 groups, with or without electron-donor or electron-withdrawing substituents. In five cases (**1m**–**r**), R^1 is heterocyclic, both π-excessive and π-deficient, as well as mono- or bicyclic. Last, two starting materials (**1s**,**t**) are α-substituted, showing their R^1 and R^2 groups having interchanged aliphatic and aromatic nature. Some of them (**1c**,**m**,**n** and, in lesser extent, **1r**) are acid-labile. Such a selection seems to be wide enough to test the versatility of the biotransformation, as well as to compare with the following non-enzymatic reactions.

2.2. Non-enzymatic attempts

A variety of conventional reactions to transform nitriles into their corresponding amides were tried in order to find a general method for a wide range of β-ketonitriles **1** (see Table 1). First, the classical hydration using hydrogen chloride dissolved in concentrated hydrochloric acid was employed,[19] which led to acceptable results with the acid-stable starting material 3-oxo-3-phenylpropanenitrile (benzoylacetonitrile, **1d**; entry 1), but not with acid-labile heterocyclic substrates (entries 2 and 3). Another recent, mild and acid mediated way of converting nitriles into amides implies the use of chlorotrimethylsilane–water.[20] When applied on β-ketonitriles, it worked acceptably on **1d** again (entry 4), but not on the presumably acid-stable pivaloylacetonitrile **1b** (entry 5), and even less on the acid-labile heterocyclic **1m** (entry 6).

It can be predicted that hydration of β-ketonitriles under basic conditions would not work, since the abstraction of the acidic α-hydrogen will prevent a further nucleophilic attack to the cyano group.[21] Indeed, using **1d** as a test substrate, we observed no reaction after heating with potassium hydroxide in *tert*-butyl alcohol (entry 7),[22] and neither with potassium trimethylsilanolate in tetrahydrofuran (entry 8).[23] Last, the use of basic hydrogen peroxide under phase-transfer catalysed conditions (entry 9)[21] converted **1d** into benzoate anion, presumably via a retro-Claisen type reaction, opposite to that used by us for preparing some of the β-ketonitriles employed in this study (see Section 3.2).

Two enzymatic reactions with an acid-stable (entry 10) and an acid-labile β-ketonitrile (entry 11) were also included in Table 1 for comparison. From their results, it can be advanced that the bacterial biotransformation is the method of choice for hydrating β-ketonitriles to β-ketoamides, as will be plain in the sections to come.

2.3. Bacterial strain selection

After checking that no conventional procedure was able to convert the majority of β-ketonitriles into β-ketoamides, we decided to submit them to microbial hydration. As previously stated, except for a few anecdotal cases,[24] the overwhelming majority of known nitrile-converting microorganisms are bacteria. After discarding those exhibiting nitrilase activity, our attention was focused on four of them available at some culture collection, and able to transform nitriles via nitrile hydratase and amidase activities: *Brevibacterium imperiale* B222 (CBS 498.78),[13f] *Rhodococcus* sp. R312 (CBS 717.73),[25,26] *Rhodococcus erythropolis* IFO 12539[27] and *R. rhodochrous* IFO 15564.[15,16,28]

Benzoylacetonitrile (**1d**) was chosen as a reference substrate to evaluate the enzymatic activities of these four strains. Bacteria were grown both in the presence and in the absence of DEPA. Incubations with **1d** were carried out with resting phase bacteria and monitored by TLC. With *B. imperiale* neither benzoylacetamide (**2d**) nor benzoylacetic acid (**3d**) were detected (see Scheme 1). Amidase from *Rhodococcus* sp. R312, after growing this bacterium in the absence of DEPA, was much more active than the corresponding nitrile hydratase as only **1d** and **3d**, but not the ketoamide **2d**, were observed during the biotransformation. The amidase activity of the bacterium clearly decreased if growing in the presence of DEPA, although not enough to prevent a considerable fall in the ketoamide yield.

On the contrary, *R. rhodochrous* has been described to show higher nitrile hydratase than amidase activity. In fact, in our hands both this strain and *R. erythropolis* gave the best results in transforming **1d** into **2d**, especially after growing them in the presence of DEPA. Under certain reaction conditions, they allow the isolation of high yields of the ketoamide **2d**, the ketonitrile **1d** and the ketoacid **3d** being observed only in very small or null amounts. A comparative

Table 1. Non-enzymatic attempts at hydrating[a] R^1-CO–CHR^2–CN (**1**)[b] to R^1-CO–CHR^2–$CONH_2$ (**2**)

Entry	Substrate[c]	R^1	Reagents	Reaction time (h)	**2**, Crude yield (%)/remarks
1	**1d**	Ph	HCl(g)/HCl(aq)	13	70
2	**1m**	2-Furyl	HCl(g)/HCl(aq)	13	13/Notable polymerisation
3	**1c**	(1-Methylpy-rrol-2-yl)methyl	HCl(g)/HCl(aq)	13	0/Full polymerisation
4	**1d**	Ph	Me_3SiCl/H_2O	23	71
5	**1b**	Bu^t	Me_3SiCl/H_2O	39	36
6	**1m**	2-Furyl	Me_3SiCl/H_2O	39	5
7[c]	**1d**	Ph	KOH/Bu^tOH	2	No reaction
8[d]	**1d**	Ph	$Me_3SiO^-K^+$/THF	22	No reaction
9	**1d**	Ph	H_2O_2/NaOH(aq)/$Bu_4N^+Cl^-$	2	Retro-condensation to $PhCO_2H$
10[e]	**1d**	Ph	*R. rhodochrous*/H_2O	0.67	96
11[e]	**1m**	2-Furyl	*R. rhodochrous*/H_2O	1.2	90

[a] The reactions were carried out at room temperature except for entries 7, 8, 10 and 11.
[b] The four substrates selected for this table have R^2=H.
[c] Reaction temperature, 83 °C.
[d] Reaction temperature, 66 °C.
[e] Enzymatic process included for the sake of comparison; reaction temperature, 28 °C.

analysis of the ^{1}H NMR spectra of the crude products allowed us to select *R. rhodochrous* IFO 15564 as the most suitable bacterial strain for the enzymatic hydration of β-ketonitriles to β-ketoamides.

2.4. Optimization of the growth and biotransformation conditions

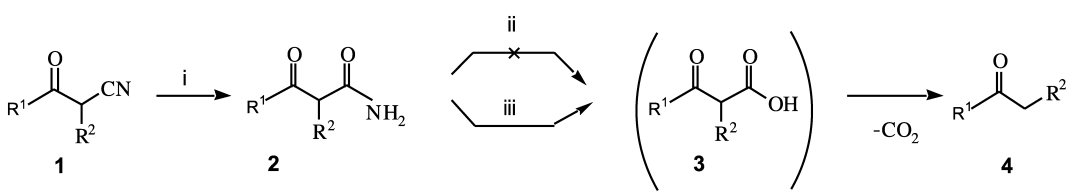
In order to determine the optimal conditions for obtaining the best yield of benzoylacetamide (**2d**) from benzoylacetonitrile (**1d**), as well as to check the amidase inhibitory power of DEPA, we carried out a series of biotransformations (see Table 2). Half of these processes were carried out with *R. rhodochrous* grown in the presence of DEPA, and the other half in the absence of this amidase inhibitor. The percentage values of compounds **1d** and **2d** were deduced from the ^{1}H NMR spectra of the crude reaction products, which were obtained from the cell-free supernatant reaction medium by means of continuous extraction and further solvent elimination. This work-up precludes the possibility of isolating benzoylacetic acid, **3d** (or other β-ketoacids **3** when other substrates were used), since the extractions were carried out at pH 8. No efforts were made for detecting β-ketoacids **3**; however, their undesirable formation was proved: (1) by TLC monitoring of the bioconversions;[29] (2) by the presence in some crude products of the ketones **4**, coming from the decarboxylation of the corresponding **3** during the biotransformation at 28 °C[30] (see Scheme 1).

From the above paragraph, it is clear that, in each process contained in Table 2, the difference $100-\Sigma'$ roughly represents the molar percentage of β-ketoacid coming from the amidase activity of the bacterium. Thus, by comparing both columns of Σ' values, the efficiency of DEPA as amidase inhibitor can be estimated from the $\Delta\Sigma'$ values, ranging from 14% (first row) to 27% (sixth row) of avoided β-ketoacid formation. However, DEPA also shows an unexpected feature as promoter of the nitrile hydratase activity, as deduced from the comparison of both **1d**-columns: the average reaction rate (rows 1 to 4) of the ketonitrile to ketoamide step is ca. 18% higher when the bacterium was grown in the presence of DEPA. Thus, the actual amidase inhibitory power of DEPA is higher than that merely inferred from the $\Delta\Sigma'$ values since, with its mediation, less β-ketoacid is formed just when more β-ketoamide is present in the reaction medium.

Apart from the obvious need of growing *R. rhodochrous* in the presence of DEPA, the ideal reaction conditions for preparing benzoylacetamide (**2d**) must include an adequate balance between the nitrile hydratase concentration (i.e., the absorbance or optical density of the bacterial suspension) and the reaction time. Data of Table 2 show that an A_{650} (absorbance of the bacterial suspension at λ=650 nm) value of 3.0 and a reaction time of 40 min lead to an excellent yield of **2d**. Data of row 6, if compared with those of row 3, clearly prove how longer reaction times favours the formation of β-ketoacid **3d**, which is an evident disadvantage in order to reach a good ketoamide yield.

Among the standard reaction conditions for the other substrates, we adopted the above A_{650} value of 3.0, the biotransformations being stopped after TLC showed the disappearance of the corresponding β-ketonitrile **1**. The use of more concentrated bacterial suspensions causes some problems of entrapping reaction products. Nevertheless, this method or other variations were applied when necessary, as described in the next Sections 2.5 and 2.6.

It has been described that DEPA acts as an amidase inhibitor



Scheme 1. (i) *R. rhodochrous* IFO 15564 (nitrile hydratase activity), aq. phosphate buffer pH 8, 28 °C, 200 rpm; (ii) *R. rhodochrous* IFO 15564 previously grown in the presence of DEPA, aq. phosphate buffer pH 8, 28 °C, 200 rpm; (iii) *R. rhodochrous* IFO 15564 (amidase activity) previously grown in the absence of DEPA, aq. phosphate buffer pH 8, 28 °C, 200 rpm.

Table 2. Molar percentages of recovered substances in *R. rhodochrous*-catalysed hydration of benzoylacetonitrile (**1d**)[a]

Row	A_{650}[b]	Reaction time (min)	*R. rhodochrous* grown in the absence of DEPA			*R. rhodochrous* grown in the presence of 15 mM DEPA		
			1d	**2d**	Σ[c]	**1d**	**2d**	Σ[c]
1	1.0	10	60	22	82	52	44	96
2	1.0	20	48	30	78	38	57	95
3	1.0	30	38	39	77	27	66	94
4	2.0	30	27	53	80	16	79	95
5	3.0	40	4	71	75	n.o.[d]	96	96
6	1.0	240	10	42	52	n.o.[d]	79	79

[a] **1d** (0.70 mmol, 102 mg) in EtOH (1.0 mL) was added to a suspension of *R. rhodochrous* in 0.10 M potassium phosphate buffer (100 mL, pH 8.0); rotary shaking (200 rpm) at 28 °C.
[b] Absorbance (optical density) of the bacterial suspension at 650 nm.
[c] Sum of percentages of **1d** and **2d**.
[d] Not observed by ^{1}H NMR.

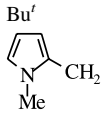
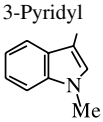
if added to the reaction mixture during the biotransformation,[17,18] which has the drawback of leading to an impure crude reaction product needing chromatographic purification. Thus, our observation is noteworthy that DEPA also works if added only during the growth of *R. rhodochrous*. This was our standard methodology, since it usually allows us to obtain crude β-ketoamides as NMR-pure compounds ready to use, for instance, as starting materials for further fungal bioreductions.

2.5. Preparative biotransformations

Scheme 1 shows the straightforward course of these biotransformations which, for preparative purposes, have to be stopped in the β-ketoamide **2** stage. Table 3 summarizes the preparation of β-ketoamides **2a**–**t**. Standard reaction conditions already were outlined in Section 2.4. A few cases deviated somewhat from the standard conditions and will be briefly discussed in the next paragraphs. Since typical bacterial nitrile hydrolyses were carried out in aqueous buffers containing ca. 0.1% [w(g)/v(mL)] of substrate,[13e] we usually started from 100–130 mg (0.60–0.80 mmol) of a β-ketonitrile **1** and a *R. rhodochrous* suspension in 100 mL of aqueous 0.10 M potassium phosphate buffer at pH 8.0 (A_{650}=3.0). The same results are roughly obtained if the pH varies in the range 6–8. For other details concerning the standard methodology, see Sections 3.3 and 3.4. Other non-standard processes will be discussed in Section 2.6.

All β-ketoamides yields lie in the good to excellent range 71–94%, except for the ketoamide **2q** (60%). Most of the crude products are pure β-ketoamides as their ^{1}H NMR spectra reveal. However, trace amounts of the β-ketonitriles **1** or the ketones **4** are also present in a few cases. Exceptionally, β-ketoamides **2i** and **2q** are contaminated

Table 3. β-Ketoamides **2** obtained by *R. rhodochrous*[a]-catalysed hydration[b] of β-ketonitriles **1**[c]

Compound	R^1	R^2	Reaction time (min)	Yield (%)[d]	Compound	R^1	R^2	Reaction time	Yield (%)[d]
2a	Pr	H	90	87	**2j**	4-Cl–C_6H_4	H	110 min	72
2b	Bu^t	H	25	90	**2k**[e]	3,4-$Cl_2C_6H_3$	H	60 min	71
2c		H	120	91	**2m**	2-Furyl	H	70 min	87
					2n	3-Furyl	H	40 min	87
					2p	2-Thienyl	H	75 min	89
					2q[f]	3-Pyridyl	H	9.5 h	60
2d	Ph	H	40	93	**2r**[g]		H	14 h	75
2e	3-Me–C_6H_4	H	60	93					
2f	4-Me–C_6H_4	H	60	88					
2g	4-MeO–C_6H_4	H	70	94					
2h	3-F_3C–C_6H_4	H	215	89	**2s**[h]	Me	Ph	23.5 h	79
2i[i]	3-Cl–C_6H_4	H	45	85	**2t**	Ph	Et	190 min	77

[a] Grown in sterilized medium M (see Section 3.3) plus 15 mM DEPA, except for **2s**.
[b] In a bacterial suspension (A_{650}=3.0, unless otherwise specified) in aq. 0.10 M phosphate buffer (100 mL), pH 8.0 (28 °C, 200 rpm).
[c] Except for **1i**,**k**,**r**, 100–130 mg (0.60–0.80 mmol) of starting material **1** in ethanol (1.0 mL) were added to the bacterial suspension.
[d] After flash column chromatography purification.
[e] Seventy four milligrams (0.35 mmol) of **1k** and 0.74 mL of ethanol were employed.
[f] The biotransformation was carried out with a bacterial absorbance of 6.7.
[g] Fifty milligrams (0.25 mmol) of **1r** and 0.50 mL of ethanol were employed; the biotransformation was carried out with a bacterial absorbance of 14.0.
[h] Bacteria were grown in sterilized medium N (see Section 3.3) plus 15 mM DEPA; the biotransformation was carried out with a bacterial absorbance of 11.6.
[i] 60 mg (0.33 mmol) of **1i** and 0.60 mL of ethanol were employed; the biotransformation was carried out in the presence of 5 mM DEPA.

by remarkable amounts of DEPA and ketone **4q**, respectively, due to their special reaction conditions (see below). Nevertheless, purification of all these compounds is easily and efficiently accomplished by flash column chromatography (see Section 3.4). For instance, from the crude product obtained after the biotransformation of **1q** (85:15 mixture **2q/4q**; crude yield, 63%), 60% yield of pure nicotinoylacetamide (**2q**) was reached.

All chlorinated β-ketonitriles (**1i**–**k**) and, especially, 3-(1-methylindol-3-yl)-3-oxopropanenitrile (**1r**) are only slightly soluble in the aqueous phosphate buffer, so that their bacterial hydration reactions are very slow. For instance, using **1r** as substrate under standard conditions, only ca. 17% of the corresponding amide **2r** was obtained after 5 days, together with ca. 40% of the remaining substrate (entrapped in the bacterial cake, from which its recovery is difficult) and ca. 40% of 3-acetyl-1-methylindole (**4r**).[31] Since much substrate was wasted, we decided to diminish its amount (only 50 mg, 0.25 mmol), and moreover, to increase the bacterial optical density to an A_{650} value of 14.0, in order to accelerate the biotransformation. Both experimental changes led to 80% crude yield of **2r** (75% after chromatographic purification), in spite of the long time (14 h) required for the disappearance of the substrate.

A good 71% yield of 2,3-dichlorobenzoylacetamide (**2k**) was obtained by merely starting from ca. half of the usual substrate amount. In the case of 3-chlorobenzoylacetamide (**2i**), the best yield (85%) was reached not only by using a reduced amount of **1i**, but also in the presence of 5 mM DEPA during the biotransformation. Under standard conditions, only 54 and 64% of **2k** and **2i**, respectively, could be obtained. In the case of 4-chlorobenzoylacetamide (**2j**), however, the standard methodology led to a good 74% yield, and no relevant improvements were observed by changing it.

The bacterial hydration of nicotinoylacetonitrile, **1q**, is also very slow. With the standard bacterial absorbance (A_{650}=3.0), only ca. 10% of nicotinoylacetamide, **2q**, was obtained after 6 h; most of the crude product was **1q**, together with a significant amount of gummy material. With the purpose of speeding up the bacterial hydration and decreasing the formation of impurities, we decided to increase the bacterial absorbance to an A_{650} value of 6.7. Thus, although some gummy material still appeared, we were able to isolate 60% of **2q** after 9.5 h.

Of α-substituted β-ketonitriles **1s** and **1t**, the latter offered no special problems, but the former failed as substrate under the standard conditions. In fact, after 24 h, TLC does not show noteworthy transformation of **1s**; after several days, some **2s** appeared (^{1}H NMR), but approximately half of the mass was lost and, moreover, the crude product was a complex mixture. After other failed attempts, we observed that the use of a somewhat more complex medium for growing the bacterium (medium N[28c] instead of the standard medium M, see Section 3.3) led to 79% yield of **2s** after a 23.5 h biotransformation. Despite this long reaction time, no β-ketoacid **3s** was observed. Moreover, the yield of **2s** was very similar (76%) after growing the bacterium in the absence of DEPA. These facts seem to prove the existence of some unfavourable steric and/or electronic interaction between the amide **2s** and the amidase.[32]

Finally, a β-ketonitrile not included in Table 3 also deserves some comments. 3-(2-Chlorophenyl)-3-oxopropanenitrile (**1**, R^1=2-Cl–C_6H_4, R^2=H) is an *ortho*-substituted aromatic substrate whose solubility in aqueous media is very low. Nonetheless, it was accepted by *R. rhodochrous* under standard conditions, TLC analysis showing a little amount of β-ketoamide, but not β-ketoacid, formed after 2 h. As the time elapses (10 h), most of the ketonitrile remains, a clear spot of ketoacid emerges, but only a trace of ketoamide is seen. Several variations were established in order to overcome the low solubility and/or to stop the process at the ketoamide stage (use of half amount of starting material, increase of the bacterial absorbance, presence of DEPA during biotransformation, change of the culture medium), some of them even simultaneously. However, the best yield in ketoamide was only 27%, this product being always accompanied by ketonitrile and 2-chloroacetophenone. Thus, we must conclude that this *ortho*-substitution negatively affects the nitrile hydratase activity of *R. rhodochrous*.

2.6. Biotransformations on a larger scale

Apart from the standard reactions used for synthetic purposes, it would be interesting to know to what extent the catalytic potential of the *R. rhodochrous* cells could be exploited. Thus, choosing benzoylacetonitrile (**1d**) as a reference substrate again, we initially carried out several experiments with the bacterium previously grown in the presence of DEPA and suspended (with a reference absorbance A_{650}=1.0) in 100 mL of the usual phosphate buffer. The cells contained in this suspension (resuspended in distilled water) gave ca. 90 mg of dry residue (cell dry weight, CDW) after evaporation at 120 °C.

Starting from 100 mg (0.69 mmol) of **1d**, a very good yield of **2d** was obtained after 2 h, just after **1d** disappeared (Table 4, entry 1). Doubling the amount of substrate, it was necessary to wait for 8 h until disappearance of the substrate (entry 2); moreover, the ketoamide yield strongly fell due to the residual amidase activity. Serious problems arose starting from 250 mg (1.72 mmol) of **1d** (entry 3). TLC monitoring showed that, at the most, only half of the substrate was transformed, approximately. A remarkable amount of β-ketoamide **2d** was formed in the beginning, but it was hydrolyzed to the corresponding acid and disappeared after 2 days. Thus, it seems that an excess of **1d** inhibits the

Table 4. Biotransformations of variable amounts of benzoylacetonitrile (**1d**) with *R. rhodochrous*[a] (A_{650}=1.0)

Entry	Mass of **1d**[b] (mg (mmol))	Reaction time (h)	Yield[c] of **2d** (%)
1	100 (0.69)	1.5	91
2	200 (1.38)	8	62
3	250 (1.72)	48[d]	—[d]

[a] Grown in the presence of 15 mM DEPA and suspended in 100 mL of 0.10 M potassium phosphate buffer, pH 8.0.
[b] 10 μL EtOH/mg **1d** were added to the reaction mixture.
[c] Determined by ^{1}H NMR of the crude reaction product.
[d] See text.

nitrile hydratase. Consequently, it can be estimated that the bacterial mass equivalent to ca. 100 mg of CDW does not accept more than about 150 mg (ca. 1 mmol) of **1d**. Our standard preparative substrate/bacterial mass ratio is notably lower in order to attain the best yields in the shortest reaction times.

With the data of the previous paragraph in mind, we resolved to carry out a series of larger scale experiments, all of them in such conditions that the inhibition by substrate of the nitrile hydratase was avoided. First we decided to recycle the cells in our standard conditions. After each reaction, cells were harvested by centrifugation, resuspended in fresh potassium phosphate buffer, and then exposed to a new amount of substrate **1d**. DEPA was present, as usual, during the growth of the bacterium, but not during the biotransformation. Using this methodology, yields of **2d** progressively diminished with the number of recyclings, so that in the fourth reaction only a very little amount of the substrate was transformed.

In view of the previous unsatisfactory results, we repeated the recycling experiments introducing a change, namely, adding 5 mM DEPA during the phase of reaction. The results are collected in Table 5. The four first ca. 1 h-reactions gave an excellent average yield of 93% of **2d** as the sole product. The fifth reaction is slightly worse, but, even so, it produces a very good yield. Altogether, almost 750 mg of **1d** were transformed by the cellular mass equivalent to 265 mg of CDW, i.e. ca. 1.95 mmol of **1d**/100 mg of CDW, which roughly doubles the previously estimated bioconversion efficiency for simple processes.

Another obvious method of avoiding the substrate inhibition would consist of the addition of the substrate in successive portions on a same bacterial suspension. To such an aim, we established three subroutines, according to the presence of DEPA only during the growth phase, only during the reaction phase, or during both phases. As expected, since this methodology implies a long time contact between the β-ketoamide **2d** and the amidase, the third subroutine was the best by far. To a bacterial suspension (A_{650}=4.0) in the usual phosphate buffer (100 mL) plus 5 mM DEPA, seven average 108 mg portions of **1d** were added in regular intervals of time during 8 h. The overall 755 mg (5.21 mmol) of **1d** were transformed into 672 mg (4.12 mmol, 79%) of **2d**, which was accompanied in the crude reaction product by 0.27 mmol (5%) of unchanged **1d**.[33] From these data, a bioconversion efficiency of 1.16 mmol of **1d**/100 mg of CDW can be deduced. As a corollary of these experiments, the nitrile hydratase and the amidase do not seem to be appreciably inhibited by a moderate excess of β-ketoamide **2d**.

Table 5. Biotransformations[a] (in the presence of DEPA) of benzoylacetonitrile (**1d**) with *R. rhodochrous*[b] recycling[c] (A_{650}=3.0)

Reaction no.	Reaction time (min)	Yield[d] of **2d** (%)
1	55	93
2	50	93
3	55	94
4	60	92
5	270	85

[a] 150 mg (1.03 mmol) of **1d** in 1.5 mL of EtOH were employed in each experiment.
[b] Grown in the presence of 15 mM DEPA and suspended in 100 mL of 0.10 M potassium phosphate buffer (pH 8.0) plus 5 mM DEPA.
[c] After each reaction, the bacterial cake was resuspended in 100 mL of fresh 0.10 M potassium phosphate buffer (pH 8.0) plus 5 mM DEPA, and more **1d**/EtOH was added.
[d] Determined by ^{1}H NMR of the crude reaction product.

From the point of view of the bioconversion efficiency, the recycling method (in the presence of DEPA) is better than that of feeding in portions, but also is more tedious, consumes more DEPA and requires more expensive column chromatography. Therefore, the selection of one or another will depend on the circumstances of each case.

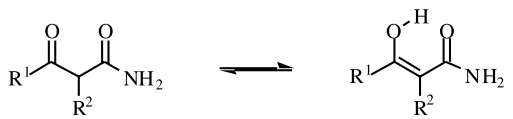
Finally, a further non-standard experiment is noteworthy. Using a 50 mL suspension of *R. rhodochrous* (A_{650}=11) in the usual potassium phosphate buffer plus 5 mM DEPA, 454 mg (3.13 mmol) of **1d** were converted in 485 mg (2.97 mmol, 92% yield) of β-ketoamide **2d** after 2 h. This result is not surprising, since the substrate/bacterial mass ratio is ca. one third lower than its previously estimated maximum value (1 mmol of substrate/100 mg CDW). However, this experiment represents an alternative, fast and efficient way of obtaining notable amounts of β-ketoamide from concentrated suspensions of the bacterium.

2.7. Keto–enol tautomerism in β-ketoamides 2a–t

Keto–enol tautomerism in β-ketoesters and β-diketones is a topic extensively studied from several points of view and by means of a variety of experimental methods.[34,35] This phenomenon, however, has been much less studied for β-ketoamides[34a,36] (Scheme 2). With a few exceptions,[37] it is usual to describe them only as keto forms,[10] despite some of them, in our hands, have shown to exist as a tautomeric mixture where the enol form was the major tautomer.[38] The apparent carelessness in this matter contrasts with the recent recognition of the β-ketoamides' enol forms as important structural elements of tetracyclines and piroxicams.[39]

In view of this situation, we show in Table 6 the percentages of enol forms in the keto–enol equilibria of β-ketoamides **2a–t** (Scheme 2). Data were obtained by ^{1}H NMR spectroscopy [in $CDCl_3$ for all of them and in CD_3OD and/or $(CD_3)_2CO$ for some of them], at 0.01–0.20 molar concentrations, after several days, in order to ensure that the equilibria were reached. As a general rule, however, no time dependence was observed in $CDCl_3$, whereas an almost imperceptible and a strong drop of the enol content in $(CD_3)_2CO$ and in CD_3OD, respectively, were noticed as the time elapses.

For β-ketoesters and β-diketones it is well established that the presence of electron-withdrawing substituents raises the



Scheme 2. Keto–enol tautomerism in β-ketoamides **2**.

Table 6. Percentages[a] of enol tautomers for β-ketoamides **2**[b] in three deuterated solvents[c]

Compound	R^1	$CDCl_3$	CD_3OD	$(CD_3)_2CO$	Compound	R^1	$CDCl_3$	CD_3OD	$(CD_3)_2CO$
2a	Pr	~3			**2j**	4-Cl–C_6H_4	17	23	60
2b	Bu^t	5	[d]	~40	**2k**	3,4-$Cl_2C_6H_3$	30		68
2c	1-(Methylpyrrol-2-yl)methyl	11			**2m**	2-Furyl	~3		
2d	Ph	12		55	**2n**	3-Furyl	~4	7	28
2e	3-Me–C_6H_4	9		51	**2p**	2-Thienyl	~2		16
2f	4-Me–C_6H_4	8	13	46	**2q**	3-Pyridyl	35	42	67
2g	4-MeO–C_6H_4	~2	6		**2r**	1-Methyl-indol-3-yl	0		
2h	3-F_3C–C_6H_4	30		68	**2s**	Me (R^2=Ph)	79	28	65
2i	3-Cl–C_6H_4	23		66	**2t**	Ph (R^2=Et)	0		0

[a] Determined at room temperature by ^1H NMR spectrometry after the equilibria were reached.
[b] Except for **2s**,**t**, R^2=H.
[c] Solute concentrations: 0.01–0.20 mol/L.
[d] This value clearly lies between 5 and 40%, although signal overlapping precludes its exact determination.

enol percentage,[34g,h] since electron withdrawal from the enol ring enhances the electrostatic contribution to the strength of the intramolecular hydrogen bond. Conversely, electron-releasing substituents decrease the enol content.

Such a tendency is clearly reflected in the enol percentages of monosubstituted 3-oxopropanamides **2a**–**r** when dissolved in deuteriochloroform (see Table 6). Since it is usually assumed that phenyl group is a weakly withdrawing one, the enol content of **2d** is somewhat higher than those of the alkyl substituted, weakly releasing **2a**,**b**.[40] If one considers the aryl substituted **2d**–**k** as a whole, it is evident that those bearing one or two further electron-withdrawing groups (**2h**–**k**) exhibit a higher enol percentage than the 'unsubstituted' **2d**, and this percentage increases with the withdrawing strength of the substituents (**2h**,**k**).[41] The opposite tendency is just observed for the aryl substituted **2e**–**g** bearing *meta*- and *para*-substituents that release electronic charge. The case of heterocyclic systems **2m**–**r** is also very illustrative. When the heterocycle is π-excessive, i.e. releases electronic charge (**2m**–**p**,**r**), the enol content is very low, especially for **2r**, whose indole ring is linked at its highly electron rich C-3 position. On the contrary, the presence of a π-deficient, highly electron-withdrawing pyridine ring (**2q**) dramatically increases the percentage of enol tautomer.

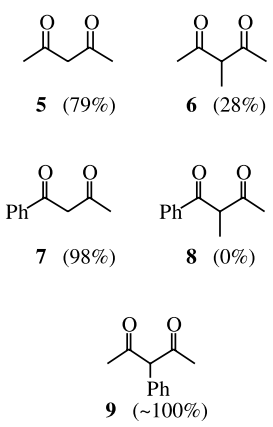


Figure 1. Enol percentages for several selected β-diketones (^1H NMR, $CDCl_3$). (Compounds **5**–**8**: Ref. 34g; Compound **9**: Ref. 43).

α,γ-Disubstituted 3-oxopropanamides **2s**,**t** should be commented independently. Alkyl substitution in the methylene group of β-diketones highly favours the keto tautomer, as shown by comparing diketones **5** and **6** and, especially **7** and **8** (Fig. 1). Although steric effects have been invoked to explain this fact,[34g] the matter is far from being clear, since similar steric hindrance are to be expected in the enol forms of β-diketones **8** and **9**, whose enol percentages differ as dramatically as shown in Figure 1. In any case, a parallelism between similarly substituted β-diketones **7** and **8** and β-ketoamides **2d** and **2t** accounts for the exclusive existence of the latter ketoamide as keto tautomer.[42] On the contrary, α-phenyl substituted β-ketoamide **2s** preponderantly exists as enol tautomer (79%), as it was to be expected after knowing the case of 3-phenylpentane-2,4-dione **9**.[43]

Finally, let us consider the influence of solvent polarity on the keto–enol equilibria of β-ketoamides **2**. For β-diketones and β-ketoesters, it is generally accepted that their tautomeric equilibria are shifted towards their less polar enol forms as the solvent polarity decreases.[35] Our data for β-ketoamides **2** (Table 6) concern three deuterated solvents, whose relative polarities depend on the method employed for evaluating them. For normal, non-deuterated solvents, polarities decrease in the series $MeOH > Me_2CO > CHCl_3$ when measured from spectroscopic data,[44] whereas they differ as $MeOH > CHCl_3 > Me_2CO$ if deduced from equilibria data[45] and from the solvent's anion-solvating and cation-solvating tendencies.[46]

Literature and Table 6 data show that the relationship between solvent polarity and keto–enol equilibria is a very complex problem. In fact, enol percentages deduced from ^1H NMR for pentane-2,4-dione increase as in the former series, i.e. $CD_3OD < (CD_3)_2CO < CDCl_3$,[47] whereas for ethyl acetoacetate they increase by the latter, namely, $CD_3OD < CDCl_3 < (CD_3)_2CO$.[34e] For their part, β-ketoamides **2** also behaves in their own way. Thus, all monosubstituted 3-oxopropanamides for which all three data are collected in Table 6 (**2b**,**f**,**j**,**n**,**q**) show increasing enol percentages in the series $CDCl_3 < CD_3OD \ll (CD_3)_2CO$, whereas the disubstituted **2s** behaves as pentane-2,4-dione. Thus, it seems that several factors, among which the high number of possible hydrogen bonds in N-unsubstituted β-ketoamides will probably play an important role, must be considered in depth in order to clarify this matter, but this study is beyond of the goals of this paper.

3. Experimental

3.1. General

Thin-layer chromatography was performed on precoated TLC plates of Merck silica gel $60F_{254}$, using potassium permanganate as developing reagent. For column chromatography, Merck silica gel 60 (particle size, 40–63 μm) was used. Melting points were taken using a Gallenkamp or a Stuart SMP3 apparatus and are uncorrected. Mass spectra were recorded on a Hewlett–Packard 5897 A (electron impact, 70 eV) and a Hewlett–Packard 1100 HPLC/MS (electrospray) instruments. The C, H, N analyses were performed on a Perkin–Elmer 2400 analyzer. Absorbances (optical densities) were measured at 650 nm on a Perkin–Elmer UV–Vis Lambda20 spectrometer. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC-200 spectrometer (200.13 MHz for ^{1}H and 50.3 MHz for ^{13}C), using the δ scale (ppm) for chemical shifts; calibration was made on the corresponding solvent signal; ^{13}C NMR spectra were edited using DEPT techniques.

3.2. Starting materials 1

Substrates **1b**,**d**–**m**,**p**,**s** are commercially available (Aldrich, Avocado or Lancaster). Substrates **1a**,**c**,**n**,**q**,**r**,**t** were prepared by means of a modified previous method,[48] namely, by reacting acetonitrile (propionitrile for **2t**) with sodium amide (ca. −30 °C), and then with the respective R^1-CO_2Et (same temperature) until the ester disappeared.

3.3. Cultures of *R. rhodochrous* IFO 15564

R. rhodochrous IFO 15564 was maintained at 4 °C on Petri plates containing tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L) and agar (15 g/L), with pH adjusted at 7.0. Sub-culturing was performed every 3 months.

Except for the incubation of **1s**, the bacterium was grown in a culture medium M,[13e] whose components were heat-sterilized (115 °C, 20 min) in two separate groups. Group 1: glucose (15.0 g), yeast extract (1.0 g), KH_2PO_4 (0.50 g), K_2HPO_4 (0.50 g) and distilled water (950 mL), with pH adjusted at 7.2; group 2: ε-caprolactam (5.0 g), $FeSO_4{\cdot}7H_2O$ (0.50 g) and distilled water (50 mL). For the incubation of **1s**, the following medium N[28c] was employed: heat-sterilized (115 °C, 20 min): glucose (15.0 g), peptone (5.0 g), yeast extract (1.0 g), KH_2PO_4 (0.40 g), K_2HPO_4 (1.20 g), $MgSO_4{\cdot}7H_2O$ (0.50 g) and distilled water (950 mL), with pH adjusted at 7.2; filter-sterilized: ε-caprolactam (1.0 g), $FeSO_4{\cdot}7H_2O$ (0.30 g) and distilled water (50 mL).

A loop of solid culture of *R. rhodochrous* IFO 15564, from an agar plate, was sowed on 200–500 mL of sterilized medium M (medium N for the synthesis of **2s**), supplemented with 15 mM DEPA (from a 1.0 M reserve solution in methanol). After growing (rotary shaker, 200 rpm, 28 °C) for 36–42 h (medium M; final A_{650}=2–2.5) or for 60 h (medium N; final $A_{650}\cong$12.5), cells were harvested by centrifugation (5000 rpm, 3 min), washed with 0.10 M KH_2PO_4–K_2HPO_4 buffer pH 8.0 (20 mL), again collected by centrifugation and then resuspended in the same, fresh potassium phosphate buffer, adjusting the absorbance of the bacterial suspension to the value of 3.0, except for the preparation of **2q** (A_{650}=6.7), **2r** (A_{650}=14.0) and **2s** (A_{650}=11.6).

3.4. Standard preparations of β-ketoamides 2

To the appropriate bacterial suspension (100 mL) as described above, 0.60–0.80 mmol of the corresponding β-ketonitrile **1** (0.25–0.35 mmol in the cases of **1i**,**k**,**r**; vide infra) and ca. 1 mL of ethanol were added. In the case of **1i**, 5 mM DEPA (from a 1.0 M reserve solution in methanol) was also incorporated. Incubation (rotary shaker, 200 rpm, 28 °C) was carried out until the substrate disappeared (TLC monitoring, ethyl acetate as eluent; aliquots of 0.5 mL plus one drop of aqueous 1 M HCl, extracted with ethyl acetate), and then the cells were discarded by centrifugation (5000 rpm, 3 min). The supernatant, a slightly basic liquid, was continuously extracted with dichloromethane (8 h). [When concentrated bacterial suspensions were used (in the preparation of **2q**,**r**,**s**), the bacterial cake was washed with distilled water (20 mL), centrifuged again, and the supernatant liquid combined with the previous one before continuous extraction]. After drying the organic phase with anhydrous Na_2SO_4, low pressure elimination of solvent yielded the corresponding, essentially pure (except **2i**,**q**) β-ketoamide **2**. Further purification was accomplished by silica gel flash column chromatography [hexane–ethyl acetate as eluent, except for **2q** (see below)].

In this way, the following β-ketoamides **2** were obtained. To our knowledge, **2c**,**e**–**h**,**j**,**k**,**n**,**p**,**r** are new compounds. Complete NMR spectral data are included for all keto tautomers, whereas only selected resonances are shown for most enol tautomers. For signal assignments, Greek letters are used for the aliphatic chains, and numerical locants for the rings.

3.4.1. 3-Oxohexanamide (2a). From 90 mg (0.80 mmol) of **1a**/1.0 mL EtOH, 91 mg (0.69 mmol, 87%) of **2a** were obtained. Mp 80–81 °C (lit.,[49] 79–81 °C). ^{1}H NMR (keto, $CDCl_3$): 0.87 (t, $3H_\varepsilon$, J=7.4 Hz), 1.56 (sext., $2H_\delta$, J=7.4 Hz), 2.49 (t, $2H_\gamma$, J=7.4 Hz), 3.37 (s, $2H_\alpha$), 6.37 (broad s, 1NH), 7.06 (broad s, 1NH). ^{13}C NMR (keto, $CDCl_3$): 13.3 (C_ε), 16.6 (C_δ), 45.4 (C_γ), 48.7 (C_α), 168.5 (amide C), 206.3 (C_β). MS, *m*/*z* (%): 129 (M^+, 35), 114 (29), 112 (21), 101 (60), 86 (81), 71 (88), 59 (100).

3.4.2. 4,4-Dimethyl-3-oxopentanamide (2b). From 100 mg (0.80 mmol) of **1b**/1.0 mL EtOH, 103 mg (0.72 mmol, 90%) of **2b** were obtained. Mp 93–95 °C (lit., 95[50] and 83–84[7] °C). ^{1}H NMR (keto, $CDCl_3$): 1.15 (s, $9H_\delta$), 3.48 (s, $2H_\alpha$), 5.59 (broad s, 1NH), 7.06 (broad s, 1NH); [enol, $(CD_3)_2CO$]: 5.12 (s, $1H_\alpha$), 14.7 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 25.7 (C_δ), 43.3 (C_α), 45.0 (C_γ), 168.8 (amide C), 211.5 (C_β); (enol, $CDCl_3$): 27.3 (C_δ), 36.2 (C_γ), 85.3 (C_α), 174.7 (amide C), 184.8 (C_β). MS, *m*/*z* (%): 143 (M^+, 13), 115 (30), 111 (11), 86 (79), 59 (100), 57 (51).

3.4.3. 4-(1-Methylpyrrol-2-yl)methyl-3-oxobutanamide (2c). From 102 mg (0.63 mmol) of **1c**/1.0 mL EtOH, 103 mg (0.57 mmol, 91%) of **2c** (oil) were obtained. ^{1}H NMR (keto, $CDCl_3$): 3.42 (s, $2H_\alpha$), 3.47 (s, 3H, N–Me),

3.78 (s, 2H$_\gamma$), 6.0–6.15 (m, 2H, H-3 and H-4), 6.25 (broad s, 1NH), 6.60 (t, 1H, H-5, J=1.9 Hz), 6.76 (broad s, 1NH); (enol, $CDCl_3$): 4.60 (s, 1H$_\alpha$), 13.9 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 33.7 (N–Me), 41.8 (C$_\gamma$), 47.4 (C$_\alpha$), 107.2 and 108.7 (C-3 and C-4), 122.9 (C-5), 123.7 (C-2), 168.1 (amide C), 202.8 (C$_\beta$); (enol, $CDCl_3$): 89.5 (C$_\alpha$), 174.0 and 175.3 (amide C and C$_\beta$). MS, *m*/*z* (%): 180 (M$^+$, 58), 163 (7), 121 (20), 94 (100). Anal. (%) calcd for $C_9H_{12}N_2O_2$ (180.20): C, 59.99; H, 6.71; N, 15.55; found: C, 60.15; H, 6.58; N, 15.51.

3.4.4. 3-Oxo-3-phenylpropanamide (2d). From 101 mg (0.70 mmol) of **1d**/1.0 mL EtOH, 106 mg (0.65 mmol, 93%) of **2d** were obtained. Mp 111.5–113.5 °C (lit.,[10h] 110–111 °C). ^{1}H NMR (keto, $CDCl_3$): 3.98 (s, 2H$_\alpha$), 5.56 (broad s, 1NH), 7.13 (broad s, 1NH), 7.4–7.7 (m, 3H, H-3, H-4 and H-5), 7.95–8.1 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.54 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.8 (C$_\alpha$), 129.2 and 129.5 (C-2, C-3, C-5 and C-6), 134.8 (C-4), 136.7 (C-1), 169.1 (amide C), 196.2 (C$_\beta$); (enol, $CDCl_3$): 88.1 (C$_\alpha$), 171.7 (amide C), 175.2 (C$_\beta$). MS, *m*/*z* (%): 163 (M$^+$, 13), 146 (9), 105 (100), 77 (46).

3.4.5. 3-(3-Methylphenyl)-3-oxopropanamide (2e). From 112 mg (0.70 mmol) of **1e**/1.0 mL EtOH, 116 mg (0.66 mmol, 93%) of **2e** were obtained. Mp 98.5–99.5 °C. ^{1}H NMR (keto, $CDCl_3$): 2.42 (s, 3H, Me), 3.96 (s, 2H$_\alpha$), 5.78 (broad s, 1NH), 7.16 (broad s, 1NH), 7.25–7.5 (m, 2H, H-4 and H-5), 7.75–7.9 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.55 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 21.2 (Me), 45.0 (C$_\alpha$), 125.7 (C-6), 128.6 and 128.9 (C-2 and C-5), 134.8 (C-4), 136.0 (C-1), 138.6 (C-3), 168.4 (amide C), 195.7 (C$_\beta$); (enol, $CDCl_3$): 87.3 (C$_\alpha$). MS, *m*/*z* (%): 177 (M$^+$, 57), 160 (14), 120 (16), 119 (100), 91 (75). Anal. (%) calcd for $C_{10}H_{11}NO_2$ (177.20): C, 67.78; H, 6.26; N, 7.90; found: C, 67.90; H, 6.10; N, 7.69.

3.4.6. 3-(4-Methylphenyl)-3-oxopropanamide (2f). From 111 mg (0.70 mmol) of **1f**/1.0 mL EtOH, 109 mg (0.61 mmol, 88%) of **2f** were obtained. Mp 117–118 °C. ^{1}H NMR (keto, $CDCl_3$): 2.42 (s, 3H, Me), 3.94 (s, 2H$_\alpha$), 5.75 (broad s, 1NH), 7.16 (broad s, 1NH), 7.29 (d, 2H, H-3 and H-5, J=8.0 Hz), 7.89 (d, 2H, H-2 and H-6, J=8.0 Hz); (enol, $CDCl_3$): 5.52 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 21.6 (Me), 44.9 (C$_\alpha$), 128.6 and 129.5 (C-2, C-3, C-5 and C-6), 133.5 (C-1), 145.1 (C-4), 168.5 (amide C), 195.1 (C$_\beta$); (enol, $CDCl_3$): 86.6 (C$_\alpha$). MS, *m*/*z*: 200.1 (M+Na)$^+$. Anal. (%) calcd for $C_{10}H_{11}NO_2$ (177.20): C, 67.78; H, 6.26; N, 7.90; found: C, 67.52; H, 6.26; N, 7.70.

3.4.7. 3-(4-Methoxyphenyl)-3-oxopropanamide (2g). From 112 mg (0.64 mmol) of **1g**/1.0 mL EtOH, 116 mg (0.60 mmol, 94%) of **2g** were obtained. Mp 145–146.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.89 (s, 3H, Me), 3.92 (s, 2H$_\alpha$), 5.56 (broad s, 1NH), 6.96 (d, 2H, H-3 and H-5, J=9.2 Hz), 7.18 (broad s, 1NH), 7.98 (d, 2H, H-2 and H-6, J=9.2 Hz); (enol, CD_3OD): 5.67 (s, 1H$_\alpha$). ^{13}C NMR (keto, $CDCl_3$): 44.6 (C$_\alpha$), 55.5 (Me), 114.0 (C-3 and C-5), 129.0 (C-1), 131.0 (C-2 and C-6), 164.3 (C-4), 168.3 (amide C), 194.0 (C$_\beta$). MS, *m*/*z* (%): 193 (M$^+$, 18), 149 (22), 135 (100), 97 (33), 91 (30), 83 (38), 77 (41), 71 (60), 69 (55), 57 (98), 55 (56). Anal. (%) calcd for $C_{10}H_{11}NO_3$ (193.20): C, 62.17; H, 5.74; N, 7.25; found: C, 62.34; H, 5.66; N, 7.14.

3.4.8. 3-Oxo-3-[(3-trifluoromethyl)phenyl]propanamide (2h). From 128 mg (0.60 mmol) of **1h**/1.0 mL EtOH, 123 mg (0.53 mmol, 88%) of **2h** were obtained. Mp 69–71 °C. ^{1}H NMR (keto, $CDCl_3$): 4.00 (s, 2H$_\alpha$), 5.93 (broad s, 1NH), 6.98 (broad s, 1NH), 7.65–7.8 (m, 1H, H-5), 7.85–7.95 (m, 1H, H-4), 8.2–8.4 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.61 (s, 1H$_\alpha$), 14.3 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.3 (C$_\alpha$), 122.7–136.5 (several aromatic and F_3C peaks), 167.6 (amide C), 194.1 (C$_\beta$); (enol, $CDCl_3$): 88.2 (C$_\alpha$), 169.4 (amide C), 173.9 (C$_\beta$). MS, *m*/*z* (%): 254 (M+Na)$^+$, 232 (M+H$^+$). Anal. (%) calcd for $C_{10}H_8F_3NO_2$ (231.17): C, 51.96; H, 3.49; N, 6.06; found: C, 51.70; H, 3.47; N, 5.78.

3.4.9. 3-(3-Chlorophenyl)-3-oxopropanamide (2i). From 60 mg (0.33 mmol) of **1i**/0.60 mL EtOH, 56 mg (0.28 mmol, 85%) of **2i** were obtained. Mp 136.5–138 °C (lit.,[10h] 135–137 °C). ^{1}H NMR (keto, $CDCl_3$): 3.96 (s, 2H$_\alpha$), 5.65 (broad s, 1NH), 7.02 (broad s, 1NH), 7.4–7.5 (m, 1H, H-5), 7.55–7.65 (m, 1H, H-4), 7.85–7.92 (m, 1H, H-6), 7.96–8.01 (m, 1H, H-2); (enol, $CDCl_3$): 5.55 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.1 (C$_\alpha$), 126.7 (C-6), 128.5 (C-2), 130.2 (C-5), 134.0 (C-4), 135.3 (C-3), 137.5 (C-1), 167.4 (amide C), 194.3 (C$_\beta$); [enol, $(CD_3)_2CO$]: 89.9 (C$_\alpha$), 169.2 (amide C), 175.7 (C$_\beta$). MS, *m*/*z* (%): 222 (M+2+Na)$^+$, 220 (M+Na)$^+$. Anal. (%) calcd for $C_9H_8ClNO_2$ (197.62): C, 54.70; H, 4.08; N, 7.09; found: C, 54.73; H, 3.95; N, 6.93.

3.4.10. 3-(4-Chlorophenyl)-3-oxopropanamide (2j). From 128 mg (0.71 mmol) of **1j**/1.0 mL EtOH, 101 mg (0.51 mmol, 72%) of **2j** were obtained. Mp 143–145 °C (dec.). ^{1}H NMR (keto, $CDCl_3$): 3.94 (s, 2H$_\alpha$), 5.67 (broad s, 1NH), 6.98 (broad s, 1NH), 7.48 (d, 2H, H-3 and H-5, J=8.6 Hz), 7.94 (d, 2H, H-2 and H-6, J=8.6 Hz); (enol, $CDCl_3$): 5.53 (s, 1H$_\alpha$), 7.38 (d, 2H, H-3 and H-5, J=8.6 Hz), 7.69 (d, 2H, H-2 and H-6, J=8.6 Hz), 14.3 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.0 (C$_\alpha$), 129.2 (C-3 and C-5), 129.9 (C-2 and C-6), 134.3 (C-1), 140.8 (C-4), 167.6 (amide C), 194.3 (C$_\beta$); [enol, $(CD_3)_2CO$]: 88.6 (C$_\alpha$), 168.9 (amide C), 175.0 (C$_\beta$). MS, *m*/*z* (%): 199 [(M+2)$^+$, 4], 197 (M$^+$, 14), 141 (31), 139 (100), 111 (37). Anal. (%) calcd for $C_9H_8ClNO_2$ (197.62): C, 54.70; H, 4.08; N, 7.09; found: C,54.45; H, 4.17; N, 6.87.

3.4.11. 3-(3,4-Dichlorophenyl)-3-oxopropanamide (2k). From 74 mg (0.35 mmol) of **1k**/0.74 mL EtOH, 57 mg (0.25 mmol, 71%) of **2k** were obtained. Mp 114–115.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.94 (s, 2H$_\alpha$), 5.83 (broad s, 1NH), 6.93 (broad s, 1NH), 7.59 (d,1H, H-5, J=8.6 Hz), 7.83 (dd, 1H, H-6, J=2.3, 8.6 Hz), 8.09 (d, 1H, H-2, J=2.3 Hz); (enol, $CDCl_3$): 5.53 (s, 1H$_\alpha$), 14.2 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.2 (C$_\alpha$), 127.6–138.9 (six aromatic signals), 167.2 (amide C), 193.2 (C$_\beta$); (enol, $CDCl_3$): 88.0 (C$_\alpha$), 168.9 (amide C), 173.7 (C$_\beta$). MS, *m*/*z* (%): 272 (M+2+K)$^+$, 270 (M+K)$^+$, 256 (M+2+Na)$^+$, 254 (M+Na)$^+$, 232 (M+H)$^+$. Anal. (%) calcd for $C_9H_7Cl_2NO_2$ (232.06): C, 46.58; H, 3.04; N, 6.04; found: C, 46.80; H, 2.89; N, 5.91.

3.4.12. 3-(2-Furyl)-3-oxopropanamide (2m). From 103 mg (0.76 mmol) of **1m**/1.0 mL EtOH, 102 mg (0.66 mmol, 87%) of **2m** were obtained. Mp 142.5–144 °C (lit.,[51]

159 °C). ^{1}H NMR (keto, $CDCl_3$): 3.85 (s, $2H_\alpha$), 5.60 (broad s, 1NH), 7.10 (broad s, 1NH), 6.61 (dd, 1H, H-4, J=1.6, 3.7 Hz), 7.35 (d, 1H, H-3, J=3.7 Hz), 7.67 (d, 1H, H-5, J=1.6 Hz); (enol, $CDCl_3$): 5.52 (s, $1H_\alpha$). ^{13}C NMR (keto, $CDCl_3$): 44.5 (C_α), 112.9 (C-4), 119.6 (C-3), 147.9 (C-5), 151.8 (C-2), 167.4 (amide C), 183.7 (C_β). MS, *m*/*z* (%): 153 (M^+, 67), 110 (28), 97 (19), 95 (100), 71 (23), 57 (31).

3.4.13. 3-(3-Furyl)-3-oxopropanamide (2n). From 108 mg (0.80 mmol) of **1n**/1.0 mL EtOH, 106 mg (0.70 mmol, 87%) of **2n** were obtained. Mp 105.5–107 °C. ^{1}H NMR (keto, $CDCl_3$): 3.75 (s, $2H_\alpha$), 5.61 (broad s, 1NH), 6.80 (dd, 1H, H-4, J=0.8, 2.0 Hz), 7.10 (broad s, 1NH), 7.48 (t, 1H, H-5, J=1.6 Hz), 8.15 (broad s, 1H, H-2); [enol, $(CD_3)_2CO$]: 5.54 (s, $1H_\alpha$), 6.65 (d, 1H, H-4, J=1.0 Hz), 7.61 (t, 1H, H-5, J=1.6 Hz), 7.96 (broad s, 1H, H-2). ^{13}C NMR (keto, CD_3OD): 109.3 (C-4), 128.8 (C-3), 146.1 (C-5), 150.8 (C-2), 171.7 (amide C), 190.5 (C_β); C_α signal is overlapped with the solvent signal. MS, *m*/*z* (%): 153 (M^+, 14), 111 (18), 97 (14), 95 (100), 71 (14), 57 (17). Anal. (%) calcd for $C_7H_7NO_3$ (153.14): C, 54.90; H, 4.61; N, 9.15; found: C, 54.64; H, 4.78; N, 8.97.

3.4.14. 3-Oxo-3-(2-thienyl)propanamide (2p). From 100 mg (0.66 mmol) of **1p**/1.0 mL EtOH, 100 mg (0.59 mmol, 89%) of **2p** were obtained. Mp 125.5–127 °C. ^{1}H NMR (keto, $CDCl_3$): 3.92 (s, $2H_\alpha$), 5.57 (broad s, 1NH), 7.18 (dd, 1H, H-4, J=3.9, 5.1 Hz), 7.20 (broad s, 1NH), 7.76 (dd, 1H, H-5 or H-3, J=1.2, 5.1 Hz), 7.83 (dd, 1H, H-3 or H-5, J=1.2, 3.9 Hz); [enol, $(CD_3)_2CO$]: 5.72 (s, $1H_\alpha$), 7.13 (dd, 1H, H-4, J=3.9, 4.9 Hz), 7.55 (m, 1H, H-5 or H-3), 7.63 (broad d, 1H, H-3 or H-5, J=4.9 Hz). ^{13}C NMR [keto, $(CD_3)_2CO$]: 48.1 (C_α), 129.6 (C-4), 135.0 and 135.9 (C-5 and C-3), 145.3 (C-2), 168.7 (amide C), 188.4 (C_β); [enol, $(CD_3)_2CO$]: 88.0 (C_α). MS, *m*/*z* (%): 169 (M^+, 27), 111 (100). Anal. (%) calcd for $C_7H_7NO_2S$ (169.20): C, 49.69; H, 4.17; N, 8.28; found: C, 49.78; H, 4.12; N, 8.30.

3.4.15. 3-Oxo-3-(3-pyridyl)propanamide (2q). Due to the special acid–base properties of this product, the work-up was slightly different from the standard protocol. After the bioconversion [103 mg (0.71 mmol) of **1p**/1.0 mL EtOH], the supernatant liquid (ca. 100 mL) was concentrated in vacuo until ca. 10 mL, and then extracted with ethyl acetate–methanol 10:1 (5×10 mL), without washing. Usual drying and elimination of solvents led to a 85:15 crude mixture of **2q**: 3-acetylpyridine (**4q**), finally purified by flash column chromatography (eluent, chloroform–hexane–diethyl ether–methanol 3:1:1:0.7), to yield 69 mg (0.42 mmol, 60%) of **2q**. Mp 110.5–112 °C (lit.,[34a] 102–104 °C). ^{1}H NMR (keto, $CDCl_3$): 4.00 (s, $2H_\alpha$), 5.64 (broad s, 1NH), 6.94 (broad s, 1NH), 7.45 (dd, 1H, H-5, J=4.7, 8.2 Hz), 8.29 (dt, 1H, H-4, J=8.2, 2.0 Hz), 8.84 (dd, 1H, H-6, J=1.6, 4.7 Hz), 9.21 (d, 1H, H-2, J=2.3 Hz); (enol, $CDCl_3$): 5.60 (s, $1H_\alpha$), 7.37 (dd, 1H, H-5, J=4.7, 8.0 Hz), 8.06 (dt, 1H, H-4, J=7.8, 2.0 Hz), 8.66 (dd, 1H, H-6, J=1.6, 4.7 Hz), 8.96 (d, 1H, H-2, J=2.0 Hz), 14.2 (s, 1H, OH). ^{13}C NMR (keto, CD_3OD): 125.4 (C-5), 133.6 (C-3), 137.9 (C-4), 150.4 (C-6), 154.2 (C-2), 171.7 (amide C), 194.8 (C_β); C_α signal is overlapped with the solvent signal. MS, *m*/*z* (%): 164 (M^+, 52), 147 (91), 106 (100), 78 (66), 51 (20).

3.4.16. 3-(1-Methylindol-3-yl)-3-oxopropanamide (2r). From 50 mg (0.25 mmol) of **1r**/1.0 mL EtOH, 41 mg (0.19 mmol, 75%) of **2r** were obtained. Mp 179.5–181.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.81 (s, $2H_\alpha$), 3.88 (s, 1H, N–Me), 5.48 (broad s, 1NH), 7.2–7.5 (m, $3H_{Ar}$+1NH), 7.87 (s, 1H, H-2), 8.3–8.45 (m, 1H, H-4). ^{13}C NMR (keto, $CDCl_3$): 33.9 (N–Me), 46.7 (C_α), 110.0 (C-7), 116.1 (C-3), 122.5, 123.3 and 124.0 (C-4, C-5 and C-6), 126.3 (C-3a), 137.2 (C-2), 126.3 (C-7a), 169.1 (amide C), 189.4 (C_β). MS, *m*/*z* (%): 216 (M^+, 26), 158 (100), 130 (12). Anal. (%) calcd for $C_{12}H_{12}N_2O_2$ (216.24): C, 66.65; H, 5.59; N, 12.96; found: C, 66.39; H, 5.55; N, 12.79.

3.4.17. 3-Oxo-2-phenylbutanamide (2s). From 101 mg (0.64 mmol) of **1s**/1.0 mL EtOH, 89 mg (0.50 mmol, 79%) of **2s** were obtained. Mp 122.5–124.5 °C (lit.,[10h] 124–126 °C). ^{1}H NMR (keto, CD_3OD):[52] 2.22 (s, 3H, C_γ), 4.81 (s, $1H_\alpha$), 7.3–7.5 (m, $5H_{Ar}$); (enol, $CDCl_3$): 1.78 (s, 3H, C_γ), 5.07 (broad s, 1NH), 5.30 (broad s, 1NH), 7.2–7.5 (m, $5H_{Ar}$), 14.6 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 29.8 (C_γ), 65.0 (C_α), 170.6 (amide C), 204.8 (C_β); aromatic carbon nuclei masked by the aromatic signals due to the major (79%) enol tautomer; (enol, $CDCl_3$): 19.7 (C_γ), 104.0 (C_α), 129.1, 131.2, 133.5 and 135.5 (aromatic carbon atoms), 172.4 (amide C), 174.6 (C_β). MS, *m*/*z* (%): 177 (M^+, 22), 160 (56), 135 (38), 118 (100), 105 (18), 90 (39), 77 (24).

3.4.18. 2-Benzoylbutanamide (2t). From 116 mg (0.67 mmol) of **1t**/1.0 mL EtOH, 99 mg (0.52 mmol, 77%) of **2t** were obtained. Mp 154–156 °C (lit.,[53] 153–153.5 °C). ^{1}H NMR (keto, $CDCl_3$): 1.00 (t, $3H_\delta$, J=7.4 Hz), 1.9–2.04 (m, $2H_\gamma$), 4.29 (t, $1H_\alpha$, J=7.2 Hz), 5.46 (broad s, 1NH), 6.59 (broad s, 1NH), 7.4–7.7 (m, 3H, H-3, H-4 and H-5), 8.02 (d, 2H, H-2 and H-6, J=7.0 Hz). ^{13}C NMR (keto, CD_3OD): 13.4 (C_δ), 25.2 (C_γ), 58.8 (C_α), 130.5 and 130.8 (C-2, C-3, C-5 and C-6), 135.6 (C-4), 139.0 (C-1), 175.7 (amide C), 199.0 (C_β). MS, *m*/*z* (%): 191 (M^+, 2), 163 (5), 122 (13), 105 (100), 77 (48).

3.5. Determination of keto–enol ratio in β-ketoamides 2

For each compound and solvent, comparison of integral values (^{1}H NMR) was made between those cleaner, non-overlapped signals corresponding to both tautomers. Thus, the most useful signals to this end with samples in $CDCl_3$ were those of H_α protons (**2a**–**e**,**h**,**k**,**m**,**p**), followed by H-2 and H-6 protons (*ortho* to keto or enol group: **2d**,**f**,**g**,**j**) and H-2 protons (**2i**,**n**); for **2s**, the signals due to H_γ protons were selected, whereas an average of integral values of H-2, H-4 and H-6 protons was taken for **2q**.

Using CD_3OD as solvent, H_α signals disappeared as a result of H/D interchange, in such a way that the determination was impossible in several cases (for instance, **2d**,**h**). However, keto–enol percentages were easily deduced from all aromatic or heterocyclic protons (**2f**,**j**,**n**,**p**), from *tert*-butyl protons (**2b**), from H_γ protons (**2s**) and from H-2, H-4 and H-6 protons (**2q**).

β-Ketoamides **2** showed a very slow H_α/D interchange when dissolved in $(CD_3)_2CO$ (except in the case of **2f**). For such a reason, H_α protons could be selected for a freshly prepared sample of **2b**, since the enol percentages remain

almost invariable with time in this solvent. H-2 and H-6 protons were useful for measurements in **2d**–**f**,**j**. The signals selected for the remaining four compounds were as follows: H-2 (**2n**); H-3 and H-5 (**2p**); H-2, H-4 and H-6 (**2q**); H_γ (**2s**).

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31. Ketone **4r** was isolated from the supernatant after extracting **2r** at pH 8 and further continuous extraction (40 °C, 8 h) at pH 6.
32. When grown in this new culture medium N, *R. rhodochrous* has shown to develop amidase activity[28c].
33. We assume that the remaining 16% includes the formed β-ketoacid **3d** and small losses during the work-up.

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38. A clear example is that of β-ketoamide **2s**. Our ^{1}H NMR spectrum ($CDCl_3$) shows that it is a 21:79 keto–enol mixture; however, it has been described as only keto form from ^{1}H NMR ($CDCl_3$).[10g]
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41. Two β-ketoamides analogous to **2**, bearing strong electron-withdrawing substituents [R^1=F_3C, R^2=H; and R^1=$H(CF_2)_4$, R^2=H], have been described to exist only as enol forms in $CDCl_3$[36d].
42. When **2d** and **2t** bear an additional *N*-phenyl substituent, concordant enol percentages of 20 and 0%, respectively, were measured in chloroform using UV techniques[36c].
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52. When freshly dissolved in CD_3OD, the keto–enol ratio is 95:5, and virtually no H_α/D interchange is observed. In $CDCl_3$ (keto): 2.24 (s, H_γ), 4.66 (s, H_α), 5.75 (broad s, 1NH), 6.90 (broad s, 1NH); H_{Ar} masked by the aromatic signals due to the major (79%) enol tautomer.
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Unusual reversal of regioselectivity in antibody-mediated aldol additions with unsymmetrical methyl ketones

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Dedicated to Professor R.A. Lerner (The Scripps Research Institute, La Jolla, USA)

Abstract—A catalytic regio- and enantioselective aldol reaction of various unsymmetrical methyl ketones with para-nitrobenzaldehyde has been developed using aldolase antibodies as the catalysts. It has been found that the sense and level of regioselectivity for the reactions catalysed by antibody 38C2 and 33F12 are highly dependent on the structure of both the donor and the acceptor but in contrast, antibodies 84G3 and 93F3 catalyse the exclusive formation of the linear regioisomer independent of the structure of the reactants examined. The level of enantiocontrol is very high for most reactions. Both linear aldol enantiomers could be accessed through aldol or retro-aldol reactions using the same antibody. Theoretical studies on regioisomeric α- and β-heteroatom substituted enamines derived from unsymmetrical ketones suggest that most of the linear aldol products formed in the presence of antibodies 84G3 and 93F3 must be formed from intermediate enamines which are not the thermodynamically most favourable.

1. Introduction

The development of strategies for the preparation of enantiomerically pure aldol products remains a very important area of research.[1] An extensive number of enantioselective aldol reactions of B, Ti, Si and Zr enolates using stoichiometric amounts of chiral sources have been reported.[2] In addition, catalytic strategies involving preformed enolate equivalents have been very promising.[3] However, the development of direct catalytic asymmetric aldol reactions starting from aldehydes and unmodified ketones remains an exciting challenge. The first reports of chemical catalysts for this process from the groups of Shibasaki,[4] List[5] and Trost[6] have recently appeared. Complementing traditional synthetic strategies, many diverse aldolase enzymes[7] and antibodies[8] have also been involved in direct aldol reactions and have provided efficient routes to elaborated targets including natural products. General methods for the preparation of enantiomerically enriched aldol products derived from unsymmetrical ketones that could lead to two regioisomeric products have not been developed. The simultaneous control of the regio-, diastereo- and enantioselectivity of direct aldol reactions involving unsymmetrical ketones constitutes one of the most demanding challenges in synthetic chemistry. A few direct catalytic aldol reactions with simple unsymmetrical ketones possessing two sites of enolisation were reported in the literature but examples of high levels of regioselectivity are rare when the reaction pathways leading to the regioisomers are too close in energy. We anticipate that in more demanding cases, enzyme or antibody catalysts can accentuate the energy difference in the regioisomeric transition states through multiple interactions and possibly favour the formation of the otherwise minor regioisomer. Unfortunately, the use of natural aldolases is hindered by the fact that enzymes usually show a strict requirement for the donor substrate. Unlike natural enzymes, aldolase antibodies were found to accept a wide range of ketone donor substrates including small aliphatic ketones. We recently described aldolase antibody 84G3, as a highly efficient catalyst for the regio- and enantioselective aldol reaction between *para*-nitrobenzaldehyde and four unmodified unsymmetrical methyl ketones with the new C–C bond formed on the less substituted α-side of these ketones. We also demonstrated the use of 84G3 as an efficient catalyst for the retro-aldol reaction, allowing for the kinetic resolution of the corresponding racemic secondary aldols.[9] With antibodies 84G3, 38C2, 33F12 and 93F3, we have now addressed four issues: (a) a study of the regioselectivity with 2-pentanone and 2-hexanone in the presence of these four antibodies; (b) for antibodies 84G3 and 38C2, the effect on the regioselectivity of the presence of an α- or β-heteroatom such as oxygen, sulfur, chlorine and fluorine on the ketone; (c) the enantioselectivity for both the forward aldol and

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reverse aldol reactions as well as the determination of kinetic parameters for these reactions; (d) theoretical calculations on the tautomeric equilibrium of 'imine–enamines' of imines derived from unsymmetrical methyl ketones.

2. Results and discussion

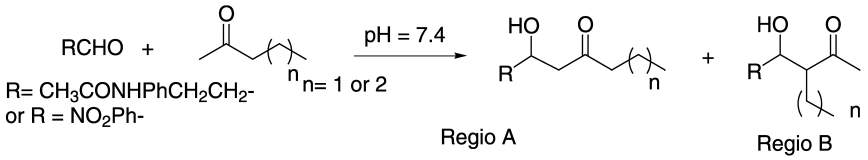
2.1. Regioselectivity of the aldol reaction of 2-pentanone and 2-hexanone with *para*-nitrobenzaldehyde or 3-(4′-acetamidophenyl)propanal (Table 1)

We set out to study the degree of regiocontrol that antibodies 38C2, 33F12, 84G3 and 93F3 could exercise on these reactions. For all experiments, the product assignment and the product distribution were proven unambiguously by comparison of the retention times with independently chemically synthesised standards using high performance liquid chromatography (HPLC). To screen for catalytic activity, we performed the reactions under the following defined conditions: 90 mM of donor, 130 μM of acceptor and 9 mol% antibody in PBS (pH=7.4) at room temperature. Control experiments revealed that, in the absence of antibody, no product formation could be detected under these conditions (entries 1, 6, 11). When higher donor and acceptor concentrations were used, the products were the *syn* and *anti* stereoisomers resulting from an addition of the more substituted carbon of 2-pentanone on the aldehyde. These results suggest that under these conditions, the reactions are under thermodynamic control. The antibody-catalysed reactions were carried out using experimental conditions under which no spontaneous reaction occurs. Under these conditions, we found that the product distribution is a function of the catalyst, the acceptor and the donor. Both antibodies 38C2 and 33F12 are moderate catalysts and afforded a mixture of regioisomeric products. The reaction of 2-pentanone with 3-(4′-acetamidophenyl)-propanal produced predominantly the branched isomer (entries 2 and 3) but in reaction with *para*-nitrobenzaldehyde, 2-pentanone led preferentially to the linear products (entries 7 and 8). The reaction of 2-hexanone with *para*-nitrobenzaldehyde did not produce any detectable amount of aldol products after 69 h in the presence of up to 25 mol% ab38C2 or ab33F12 suggesting that 2-hexanone is not a suitable donor for these two antibodies (entries 12 and 13). Previous reports in the literature revealed that in the presence of ab38C2, butanone reacts with 3-(4′-acetamidophenyl)propanal to give a 94:6 ratio of the two regioisomers with the branched aldol product as the major compound.[10] We found that in contrast to antibodies 38C2 and 33F12, antibodies 84G3 and 93F3 catalysed the exclusive formation of the linear regioisomer independent of the structure of the aldehyde or the all carbon methyl ketone. Indeed, all reactions involving 2-pentanone with *para*-nitrobenzaldehyde or 3-(4′-acetamidophenyl)propanal, and 2-hexanone with *para*-nitrobenzaldehyde afforded the corresponding aldol products resulting from a reaction on the less substituted carbon (entries 4–5, 9–10 and 14–15). In addition, antibodies 84G3 and 93F3 are more efficient catalysts as reflected by the reduced reaction times and the higher conversions. These results suggest that for the all carbon ketones that we have examined, the sense and level of regioselectivity for the reactions catalysed by antibodies 38C2 and 33F12 are highly dependent on the structure of both the donor and the acceptor. In contrast, antibodies 84G3 and 93F3 catalyse the exclusive formation of the linear regioisomer independent of the structure of the reactants examined herein (Table 1).

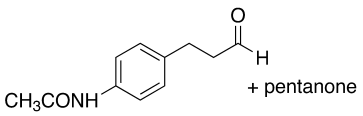
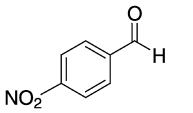
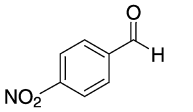
2.2. Effects of α- and β-heteroatom substituents on ketone aldolisation

Next, we focused on defining the regioselectivity in reactions involving unsymmetrical α- or β-heteroatom substituted ketones in the presence of antibodies 84G3 and 39C2. We examined first the aldol reactions between *para*-nitrobenzaldehyde and a series of unsymmetrical ketones possessing an α-heteroatom such as oxygen, sulfur, chlorine or fluorine (Table 2). The presence of the heteroatom α to the ketone greatly accelerates the spontaneous aldol additions when compared to the corresponding all carbon ketones. When performed under our standard conditions (defined as above), these reactions give preferentially the aldol products resulting from an addition at the more substituted carbon. These results suggest that under these conditions, thermodynamic control prevails but, for some of these reactions, the equilibrium constants are probably not sufficiently high to achieve a high degree of regioselectivity. The reactions leading to the products resulting from an addition of the aldehyde to the less substituted carbon of the donor are the disfavoured processes under these conditions. In order to define unambiguously the regioselectivity of these same reactions in the presence of the antibodies, it was critical to find screening conditions that suppress or at least minimize any spontaneous background reaction. This was achieved by lowering the temperature, adjusting the concentration of the different reactants and when necessary increasing the catalyst load. Under optimised conditions, it was found that, in the presence of antibody 84G3, methoxyacetone undergoes the aldol condensation with *para*-nitrobenzaldehyde to afford exclusively the linear regioisomer with a conversion of 76% after 41 h (entry 2). Similarly, the antibody-catalysed reactions of thiomethoxy- and chloroacetone are highly regioselective with the preferential formation of the otherwise disfavoured linear regioisomer resulting from an addition at the less substituted carbon (entries 5 and 8). The conversion for chloroacetone never exceeded 11% suggesting substrate or product inhibition as expected from the presence of a highly reactive electrophilic site that can chemically modify the active site lysine residue essential to the activity of the antibody. The reaction of fluoroacetone is much more efficient with 90% conversion after only 30 min in the presence of 25 mol% ab84G3 but less selective leading to a mixture of regioisomers with nevertheless preferential formation of the linear isomer (entry 11). Finally, hydroxyacetone proved to be the poorest substrate for antibody 84G3 as reflected by both the low conversion and the product distribution. After 15 h in the presence of a stoichiometric amount of ab84G3, only 10% conversion was observed with the formation of a 1/1 mixture of the two regioisomers (entry 14). The regioselectivity of these antibody-catalysed transformations contrast sharply with the results obtained for the uncatalysed reactions or in the presence of antibody 38C2. The product distribution observed for the uncatalysed reactions is in accordance with the reactivity expected from the presence

Table 1. Aldol reactions with 2-pentanone and 2-hexanone



1 n = 1 R= $CH_3CONHPhCH_2CH_2$- **2** n = 1 R= $CH_3CONHPhCH_2CH_2$-
3 n = 1 R = NO_2Ph- **4** n = 1 R = NO_2Ph-
5 n = 2 R= NO_2Ph- **6** n = 1 R = NO_2Ph-

Entry	Acceptor and donor	Conditions	Conversion (%)	Ratio A/B
1	 + pentanone	PBS, 11 days	0	0/0
2		ab38C2, 11 days	33	31/69 (lit.: 27/73)[10]
3		ab33F12, 11 days	37	29/71
4		ab84G3, 11 days	63	100/0
5		ab93F3, 11 days	79	100/0
6	 + pentanone	PBS, 135 h	0	0/0
7		ab38C2, 135 h	59	62/38
8		ab33F12, 135 h	75	60/40
9		ab84G3, 3 h	88	100/0
10		ab93F3, 2.5 h	87	100/0
11	 + hexanone	PBS, 26 h	0	0/0
12		ab38C2, 69h[a]	0	0/0
13		ab33F12, 69h[a]	0	0/0
14		ab84G3, 26 h	66	99/1
15		ab93F3, 12 h	35	100/0

[a] 25 mol% antibody for this experiment.

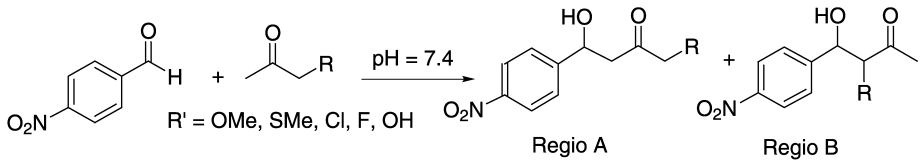
of the α-heteroatom on the donor ketone (entries 1, 4, 7, 10 and 13). Indeed, for chloroacetone and thiomethoxyacetone, it has been reported that the rates of enolisation at the methylene carbon are about 135 times faster than at the methyl carbon.[11] Aldol reactions with hydroxyacetone have also been reported to be highly regioselective providing for an easy access to the synthesis of vicinal diols. In contrast, methoxyacetone and fluoroacetone can react on both the C-1 and C-3 carbon depending on the reaction conditions.[12] Previous reports in the literature have shown that in contrast to antibody 84G3, reactions involving hydroxyacetone as the donor were the most efficient for antibody 38C2 yielding exclusively the *syn*-aldol product (entry 15).[13] Fluoroacetone is also a substrate for antibody 38C2 and combined to 3-(4′-acetamidophenyl)propanal afforded a mixture of three products, 72% of the *syn* branched isomer, 21% of the *trans*-branched isomer and only 7% of the linear regioisomer.[10] We found that ab38C2 afforded exclusively the branched isomer for the aldol reactions of *para*-nitrobenzaldehyde with methoxyacetone and with thiomethoxyacetone (entries 3 and 6). Therefore, for these two donors, it is possible to selectively access the branched and the linear regioisomer by using ab38C2 and ab84G3, respectively. Antibody 84G3 is a unique catalyst as this antibody promotes the preferential or exclusive formation of the less substituted regioisomer except for hydroxyacetone. This unusual reactivity could be regarded as an example, hitherto unknown, of the controlled generation of the less-substituted regioisomer independent of the presence of an α-heteroatom such as O, S, Cl or F under reaction conditions that would normally favour the formation of the more-substituted regioisomer.

For antibody 84G3, we also studied the regioselectivity of the reaction of *para*-nitrobenzaldehyde with three different β-heteroatom substituted ketones (Table 3). Under our standard conditions, the uncatalysed reactions proceeded slowly with the formation of the more substituted aldol product. We found that the presence of antibody 84G3 accelerated all three reactions and afforded exclusively the linear regioisomer. However, the reactions are substantially slower than the reactions involving ketones possessing an α-heteroatom with only poor conversions after extended reaction times despite the use of large amount of antibody. Nevertheless, the regioselectivity is excellent.

2.3. Enantioselectivity of the aldol and retro-aldol products and catalytic efficiency

To probe further the synthetic scope of these antibodies, we studied the enantioselectivity of several forward aldol reactions. The enantiomeric excesses of the products as determined by chiral-phase HPLC (Table 4) were all superior to 94%. All aldol products possess the (*R*)-configuration. To assign the absolute configuration unambiguously, products **5**,

Table 2. Aldol reactions of *para*-nitrobenzaldehyde and α-heteroatom substituted methyl ketones



7 R = OMe
9 R = SMe
11 R = Cl
13 R = F
15 R = OH

8 R = OMe
10 R = SMe
12 R = Cl
14 R = F
16 R = OH

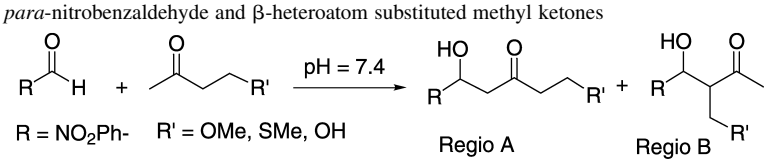
Entry	R	Conditions	Conversion (%)	Ratio A/B
1	OMe	PBS, 0 °C, 41 h	0	0/0
2	OMe	25% ab84G3, 0 °C, 41 h	76	100/0
3	OMe	25% ab38C2, 0 °C, 35 h	35	0/100
4	SMe	PBS, 0 °C, 1h40	9	10/90
5	SMe	25% ab84G3, 0 °C, 1h40	56	98/2
6	SMe	25% ab38C2, 0 °C, 29 h	53	1/99
7	Cl	PBS, 0 °C, 40 h	11	1/99
8	Cl	25% ab84G3, 0 °C, 40 h	11	95/5
10	F	PBS, 0 °C, 0.5 h.	4	30/70
11	F	25% ab84G3, 0 °C, 0.5 h	90	70/30
13	OH	PBS, rt 15 h	2	0/100
14	OH	100% ab84G3, 0 °C, 15 h	10	50/50
15	OH	ab38C2, rt	nd[a]	0/100[a]

[a] Conversion not reported, see Ref. [10].

7, **9**, **19** and **21** were prepared independently by asymmetric synthesis.[14] For compounds **3**, **13** and **17**, the absolute configuration has been attributed by analogy. We then studied the utility of antibody 84G3 in the kinetic resolution of racemic linear regioisomers. The racemic aldols were treated with ab84G3 (4 mol%) in aqueous buffer. Analysis by HPLC indicated that in each case the retro-aldolisation reactions halted at approximately 50% conversion. The catalyst was highly enantioselective and provided the recovered (*S*)-aldols with ee values typically greater than 91%. Both aldol enantiomers could, therefore, be accessed through aldol or retro-aldol reactions using the same antibody 84G3.

The results of the kinetic studies of four aldolisation reactions and four retro-aldol reactions are provided (Table 5). The kinetic parameters are reported per antibody site assuming that both sites of the antibody are active. All the forward aldol reactions with *para*-nitrobenzaldehyde and ketones performed under pseudo-first-order conditions showed Michaelis–Menten kinetics. The determination of k_{cat}/k_{uncat} was not possible because in the uncatalysed reactions, the formation of the linear regioisomer was negligible under our assay conditions. The data revealed that the introduction of a heteroatom within the ketone leads to an increase in the K_M values (entry 1 versus entries 2 and 3). Addition of an extra methylene group increases even further the K_M and results in a slower reaction rate (entry 4). The catalytic turnovers achieved by antibody 84G3 for the

Table 3. Aldol reactions of *para*-nitrobenzaldehyde and β-heteroatom substituted methyl ketones

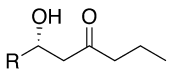
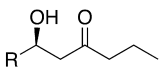
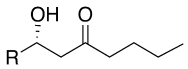
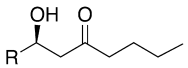
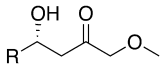
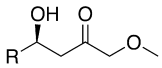
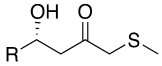
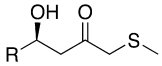
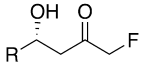
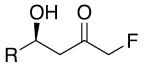
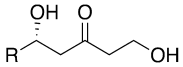
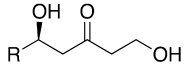
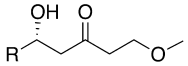
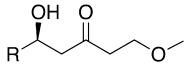
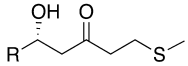
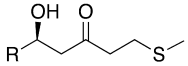


17 R = OH, R' = NO_2Ph
19 R = OMe, R' = NO_2Ph
21 R = SMe, R' = NO_2Ph

18 R = OH, R' = NO_2Ph
20 R = OMe, R' = NO_2Ph
22 R = SMe, R' = NO_2Ph

Entry	R	Conditions	Conversion (%)	Ratio A/B
1	OH	PBS, 0 °C, 77 h	7	0/100
2	OH	25% ab84G3, 0 °C, 41 h	40	100/0
3	OMe	PBS, rt, 20 h	1	0/100
4	OMe	100% ab84G3, 0 °C, 20 h	11	100/0
5	SMe	PBS, rt, 22 h	9	0/100
6	SMe	100% ab84G3, 0 °C, 22 h	41	95/5

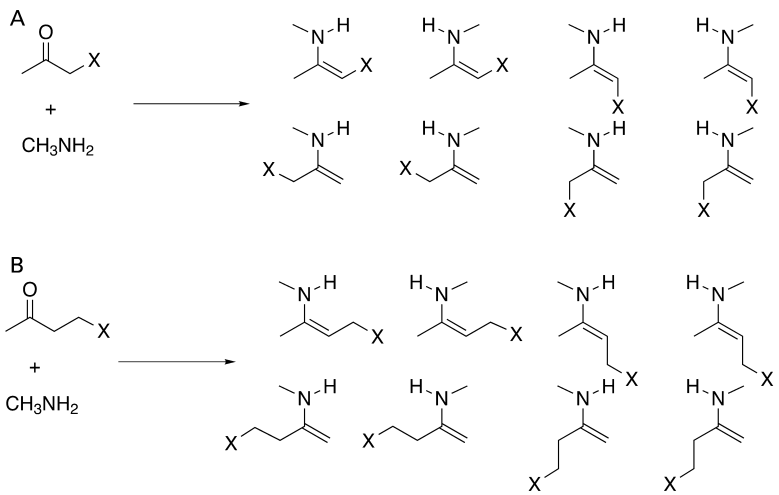
Table 4. ee Values for antibody-catalysed aldol and retro-aldol reactions with R=NO_2Ph–

Product aldol	ee (%)	Recovered product Retro-aldol	Conversion (%) ee (%)
 (*R*)-**3**	94	 (*S*)-**3**	55 97
 (*R*)-**5**	98	 (*S*)-**5**	50 94
 (*R*)-**7**	98	 (*S*)-**7**	47 97
 (*R*)-**9**	97	 (*S*)-**9**	50 96
 (*R*)-**13**	99	 (*S*)-**13**	50 94
 (*R*)-**17**	95	 (*S*)-**17**	55 91
 (*R*)-**19**	nd	 (*S*)-**19**	49 95
 (*R*)-**21**	96	 (*S*)-**21**	54 99

retro-aldol reactions were higher than those of the corresponding aldol reactions by 2.5 to 10-fold depending on the substrate (entries 5–8). The catalytic proficiency compares favourably with the efficiency of aldolase antibodies with other retro-aldol reactions.

2.4. Theoretical studies on regioisomeric α- and β-heteroatom substituted enamines derived from unsymmetrical ketones

To gain a better understanding of the unique reactivity of ab84G3, we carried out a computational study to assess the effect of various heteroatoms on the stability of regioisomeric enamines of unsymmetrical ketones. Lien et al. previously carried out similar computational studies on the substituent effects on imine–enamine tautomers of α-substituted acetaldimines $XCH_2CH{=}NH$ (X=H, BH_2, CH_3, NH_2, OH, F, Cl, CN, NO).[15] The geometries of α- and β-heteroatom substituted regioisomeric enamines derived from a series of unsymmetrical ketones (Scheme 1) were computed using hybrid density functional theory (B3LYP)[16] and the 6–31G* basis set[17] as implemented in Gaussian 98.[18] All gas phase minima were characterised by frequency analysis. Reported electronic energies include zero point energy corrections scaled by 0.9806.[19] The optimised geometries of four enamines derived from hydroxy, thiomethoxy, chloro, and fluoro acetones plus methylamine are shown in Figure 1. These include four conformations of each enamine with the substituent on the double bond, and four conformations with the substituent on the allylic carbon. The intramolecular hydrogen-bonded species are generally more stable than those lacking hydrogen-bonds, and the more substituted enamines are more stable than those with allylic substituents except for the enamine derived from fluoroacetone. The more substituted enamine is more stable by 1.1 kcal/mol for the hydroxy, 0.9 kcal/mol for the thiomethoxy and 1.1 kcal/mol for the chloro group when compared to the other regioisomer. In contrast, the allylic fluoride is more stable by only 0.2 kcal/mol. The optimised geometries of four enamines of butanone plus methylamine and five enamines derived from hydroxy butanones plus methylamine are shown in Figure 2. These include four conformations of each enamine with the substituent on the double bond, and four conformations with the substituent on the allylic carbon. The substituted enamine of butanone is more stable by 0.9 kcal/mol and the hydrogen bonded allylic enamine of hydroxy butanone is more stable by 1.5 kcal/mol when compared to the corresponding less substituted regioisomer. Antibody



Scheme 1. (A) Various conformations of regioisomeric enamines derived from the reaction of α-heteroatom substituted propanone with methyl amine. (B) Various conformations of regioisomeric enamines derived from the reaction of β-heteroatom substituted butanone with methyl amine.

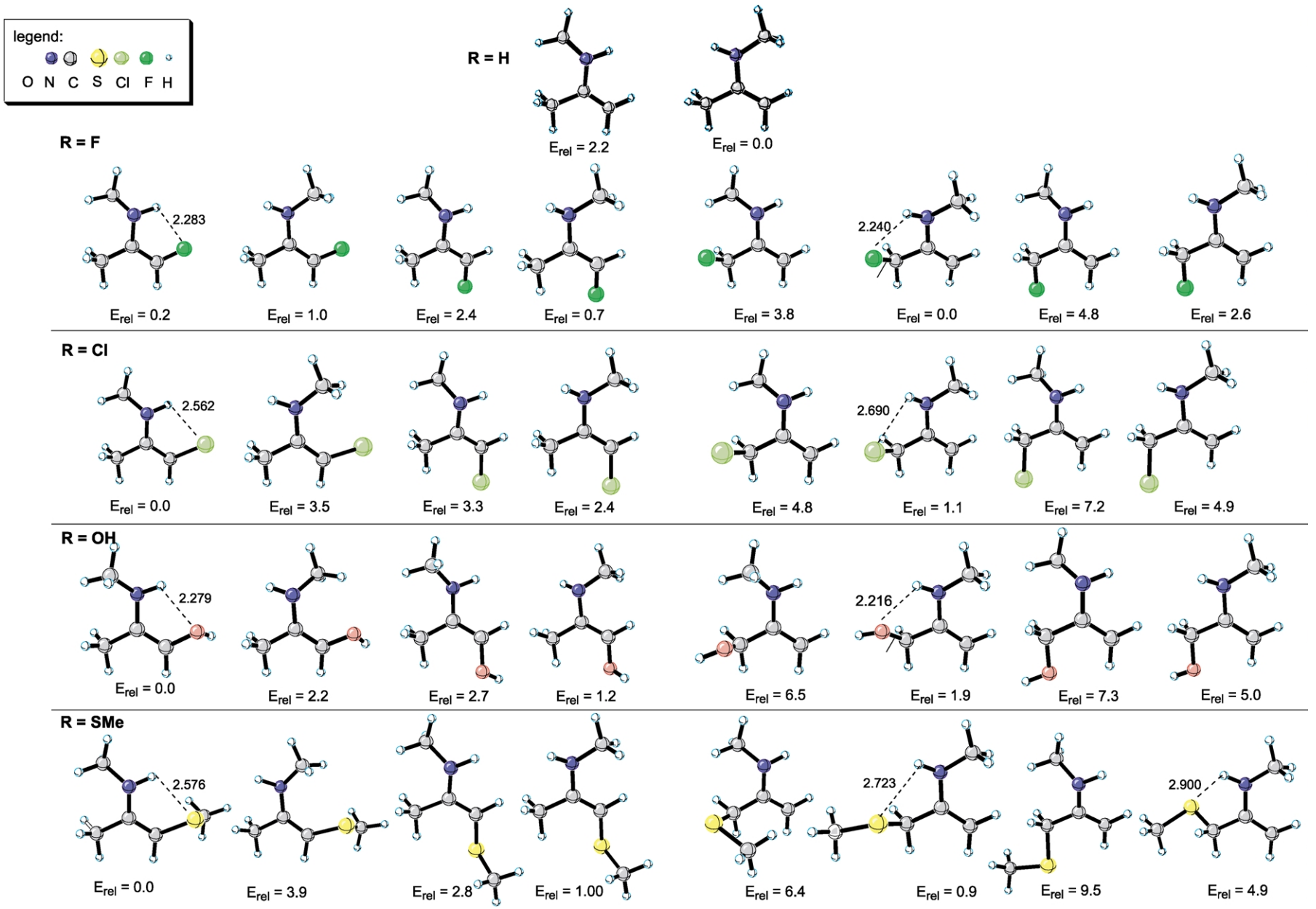


Figure 1. Optimized geometries of the various conformations of regioisomeric enamines derived from substituted methyl ketone. Relative energies are reported in kcal/mol and distances are reported in Angstroms.

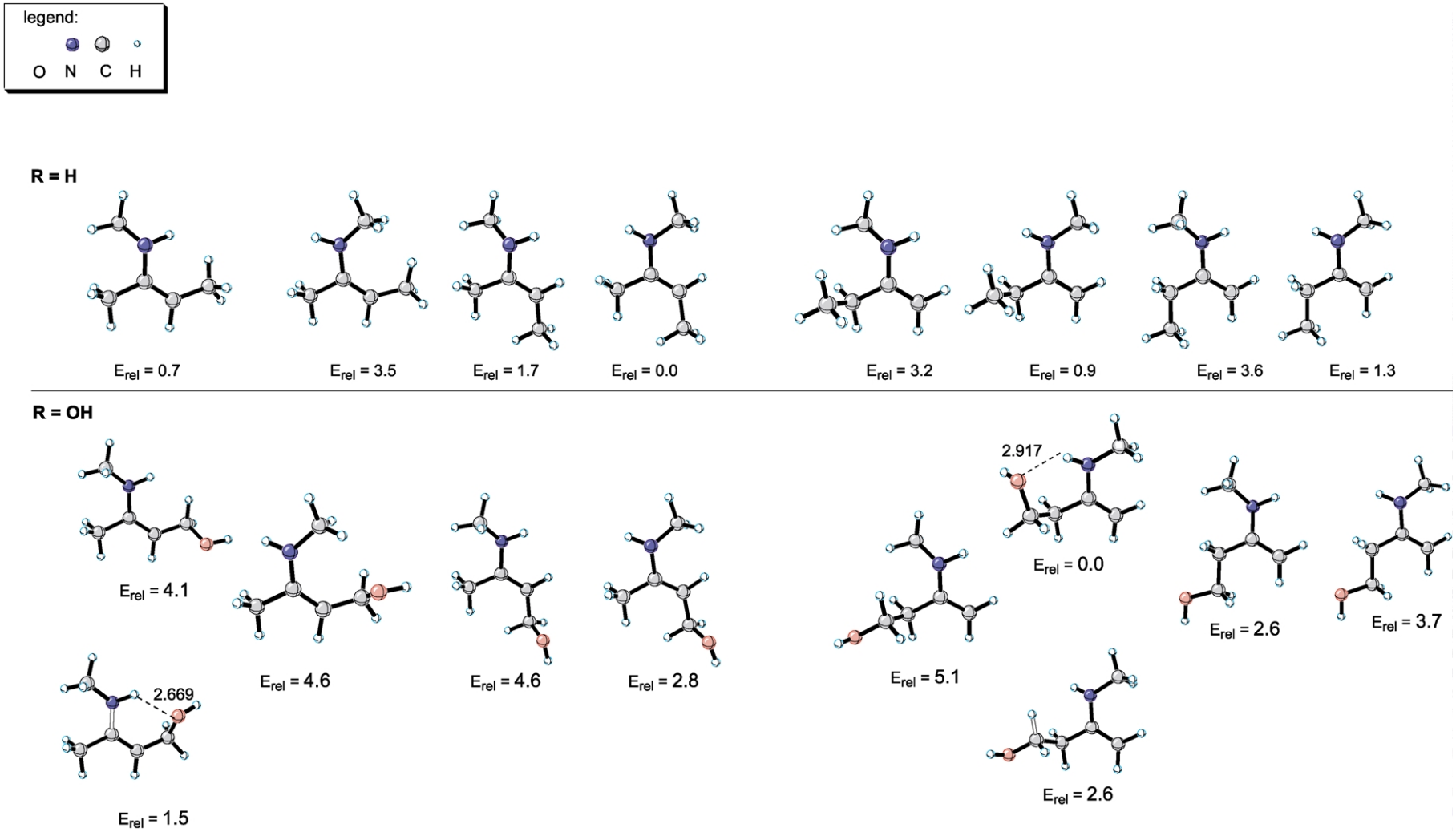


Figure 2. Optimized geometries of the various conformations of regioisomeric enamines derived from butanone and hydroxy substituted butanone. Relative energies are reported in kcal/mol and distances are reported in Angstroms.

Table 5. Kinetic parameters for selected aldol and retro-aldol reactions

Entry	Substrate	K_{cat} (min^{-1})	K_M (μM)	K_{cat}/K_{uncat}	$(K_{cat}/K_M)K_{uncat}$ (M^{-1})
1	2-Pentanone[a]	0.030	28	–	–
2	Methoxyacetone[a]	0.040	390	–	–
3	Thiomethoxyacetone[a]	0.089	217	–	–
4	4-Thiomethoxybutanone[a]	0.008	383	–	–
5	(±)-**3**	0.18	79	1.2×10^5	1.5×10^9
6	(±)-**7**	0.099	170	5.5×10^4	3.2×10^8
7	(±)-**9**	0.810	69	4.3×10^5	6.2×10^9
8	(±)-**21**	0.035	263	1.9×10^4	7.4×10^7

[a] With *p*-nitrobenzaldehyde.

84G3 is able to overcome the thermodynamic preferences shown by these calculations. That is, many of the observed products must be formed from intermediate enamines, which are not the thermodynamically most favourable.

3. Conclusion

Two major issues were under consideration. First was the critical issue as to whether aldolase antibodies 84G3 and 38C2 could differentiate the two reactive sites of unsymmetrical methyl ketones. Secondly, the possibility of controlling simultaneously the regio- and enantioselectivity of aldol reactions with a variety of unsymmetrical methyl ketones had to be determined. We have identified important differences between antibodies 38C2 and 84G3. As an overall trend, antibody 38C2 favours the formation of the branched regioisomer resulting from a reaction on the more substituted carbon whereas antibody 84G3 leads preferentially to the formation of the linear isomer. Therefore, the ab84G3-catalysed aldols and retro-aldolisations provide a rapid entry to enantioenriched linear aldol products that are difficult to access with other catalysts. Both enantiomers are accessible by using both the forward aldol and retro-aldol reactions. The retro-aldolisations are more efficient than the synthetic aldols as reflected by the higher rate constants. The unique reactivity of antibody 84G3 is quite remarkable since, to our knowledge, no known catalysts display similar level of regio- and enantiocontrol in the presence of the ketones used for this study. This unusual reactivity further demonstrates that, through a combination of immunological diversity and basic chemical principles, efficient catalysts that display new reactivity can be created. Further work is in progress to address the question of whether L-proline is capable of catalysing the aldol reactions of *para*-nitrobenzaldehyde and various unsymmetrical methyl ketones and assesses the degree of regio- and stereocontrol exercised by this catalyst. This study aims to compare the synthetic value of the antibody-with the L-proline-catalysed reactions.

4. Experimental

4.1. General

All reactions were carried out under an argon atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Dry tetrahydrofuran (THF) was obtained by distillation over sodium and benzophenone, dry methylene chloride (CH_2Cl_2) was obtained by distillation over calcium hydride. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Commercially available reagents were used without further purification, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on Merck aluminum foil backed sheets precoated with Kieselgel 60F-254 using UV light as visualizing agent and an ethanolic solution of potassium permanganate and heat as developing agent. Merck Silica gel C60 (40–60 μM) was used for flash column chromatography. NMR spectra were recorded on a Bruker DPX-400 or Bruker AMX-500 spectrometer and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain multiplicities: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; b, broad. The coupling constants *J* are given in hertz. IR spectra were recorded on a Perkin–Elmer Paragon 1000 FT-IR spectrometer. Mass spectra (*m*/*z*) and HRMS were recorded on Micromass GCT using Chemical Ionisation (NH_3, CI), Electronic Impact (EI+) or Field Ionization (FI). Microanalyses were performed by 'Elemental Microanalysis Limited', Devon. Melting points were determined in a capillary and are uncorrected.

4.2. Synthesis of racemic aldol products. Path A: direct cross aldolizations with LDA

To a solution of LDA, prepared by dropwise addition of *n*-butyllithium (1.1 mmol) to a solution of diisopropylamine (1.2 mmol) in dry THF (2 mL) at 0 °C, was added a solution of ketone (1 mmol) in THF (2 mL) at −78 °C. After the resulting mixture was stirred at −78 °C for 30 min, the aldehyde (1 mmol) in THF (1 mL) was added. The reaction was quenched after 3 h at −78 °C by addition of saturated NH_4Cl. The mixture was extracted three times with AcOEt, the combined organic layers were dried over $MgSO_4$ and solvents were removed in vacuo. Product ratios were determined by crude ^{1}H NMR spectroscopy before purification by flash column chromatography.

4.2.1. 1-(4′-Nitrophenyl)-1-hydroxy-3-hexanone 3. Crude ratio regio/*syn*/*anti*=78/13/9. Purification by column chromatography over silica (hexane/AcOEt: 80/20), followed by recrystallization from AcOEt yielded **3** as a white solid (38% pure and 18% in mixture with **4** (*syn* and *anti*). R_f 0.16 (hexane/AcOEt, 80/20); mp 59 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, *J*=8.6 Hz, 2H), 7.52 (d, *J*=8.6 Hz, 2H), 5.25 (m, 1H), 3.50 (bs, 1), 2.80 (m, 2H), 2.43 (t, *J*=7.4 Hz, 2H), 1.6 (tq, *J*=7.4, 7.4 Hz, 2H), 0.90 (t, *J*=7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.0, 150.2, 147.2, 126.4,

123.7, 69.0, 50.5, 45.4, 17.0, 13.6; IR (neat) ν_{max} 3516, 1702, 1515, 1346, 1088; Anal. calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.82; H, 6.36; N, 5.98; HRMS (EI+): M^+ 237.1001 (expected), 239.1009 (observed).

4.2.2. 1-(4′-Nitrophenyl)-1-hydroxy-3-heptanone 5. Crude ratio regio/*syn*/*anti*=78/13/9. Purification by column chromatography over silica (AcOEt/hexane: 30/70) afforded **5** as a colorless oil (26% pure and 23% in mixture with **6** (*syn* and *anti*). R_f 0.28 (AcOEt/hexane: 30/70); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J=8.8 Hz, 2H), 7.52 (d, J=8.8 Hz, 2H), 5.25 (m, 1H), 3.80 (d, J=2.4 Hz, 1H), 2.81 (m, 2H), 2.44 (t, J=7.6 Hz, 2H), 1.55 (m, 2H), 1.28 (m, 2H), 0.88 (t, J=7.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.1, 150.2, 147.2, 126.4, 123.7, 69.0, 50.5, 43.3, 25.5, 22.2, 13.8; IR (neat) ν_{max} 3466, 2959, 1709, 1520, 1347, 1081; HRMS (EI+): M^+ 251.1158 (expected), 251.1162 (observed).

4.2.3. 8-(4′-Acetamidophenyl)-6-hydroxy-4-octanone 1. Crude ratio regio/*syn*/*anti*=85/8/7. Purification by column chromatography over silica (Et_2O/AcOEt: 80/20), followed by recrystallization from hexane/DCM afforded **1** as a white solid (48%); mp 96 °C. R_f 0.22 (Et_2O/AcOEt: 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (bs, 1H), 7.39 (d, J=8.0 Hz, 2H), 7.12 (d, J=8.0 Hz, 2H), 4.02 (m, 1H), 3.30 (d, J=2.8 Hz, 1H), 2.76 (m, 1H), 2.63 (m, 1H), 2.55 (m, 2H), 2.39 (t, J=7.2 Hz, 2H), 2.14 (s, 3H), 1.77 (m, 1H), 1.59 (m, 1H), 1.59 (tq, J=7.2, 7.2 Hz, 2H), 0.91 (t, J=7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.5, 168.5, 137.9, 135.7, 128.9, 120.0, 66.7, 48.9, 45.5, 38.0, 31.1, 24.4, 17.0, 13.6; IR (neat) ν_{max} 3304, 2962 and 2932, 1705, 1667, 1603, 1538 and 1515; HRMS (EI+): M^+ 277.1678 (expected), 277.1672 (observed).

4.2.4. 4-(4′-Nitrophenyl)-1,4-dihydroxy-2-butanone 15. 2.2 equiv. of diisopropylamine and 2.1 equivalent of *n*-BuLi were used. Purification by column chromatography over silica (DCM/AcOEt: 80/20) afforded **15** as a colourless oil (25%). R_f 0.12 (DCM/AcOEt: 80/20); ^{1}H NMR (400 MHz, CD_3OD) δ 8.21 (d, J=8.4 Hz, 2H), 7.63 (d, J=8.4 Hz, 2H), 5.28 (dd, J=8.9, 4.2 Hz, 1H), 4.26 (d, J=18.7 Hz, 1H), 4.22 (d, J=18.7 Hz, 1H), 2.88 (dd, J=15.8, 8.9 Hz, 1H), 2.75 (dd, J=15.8, 4.2 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 208.5, 152.2, 147.5, 126.8, 123.4, 68.9, 68.5, 47.7; IR (neat) ν_{max} 3413, 2913, 1703, 1512, 1348, 1035; HRMS (FI): M· 225.0637 (expected), 225.0638 (observed).

4.2.5. 4-(4′-Nitrophenyl)-4-hydroxy-1-methoxy-2-butanone 7 and 4-(4′-nitrophenyl)-4-hydroxy-3-methoxy-2-butanone 8 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=25/50/25. Purification by column chromatography over silica (AcOEt/hexane: 40/60) and (DCM/Et_2O: 90/10) afforded **7** as a white solid (8%) and *syn*-**8** and *anti*-**8** as pale yellow oils (6 and 23%, respectively).

Analytical data for **7**. R_f 0.20 (AcOEt/hexane: 40/60); mp 78 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.21 (d, J=7.0 Hz, 2H), 7.55 (d, J=7.0 Hz, 2H), 5.31 (t, J=5.0 Hz, 1H), 4.05 (d, J=13.8 Hz, 1H), 4.01 (d, J=13.8 Hz, 1H), 3.48 (s, 1H), 3.42 (s, 3H), 2.90 (d, J=5.0 Hz, 2H); ^{13}C NMR (125 MHz $CDCl_3$) δ 208.5, 149.9, 147.2, 126.3, 123.7, 77.7, 68.7, 59.3, 47.2; IR (neat) ν_{max} 3436, 2936, 1726, 1519, 1348, 1088; Anal. calcd for $C_{11}H_{13}NO_5$: C, 55.23; H, 5.48; N, 5.86. Found: C, 55.01; H, 5.57; N, 5.83; HRMS (FI): M· 239.0794 (expected), 239.0789 (observed).

Analytical data for *syn*-**8**. R_f 0.25 (AcOEt/hexane: 40/60); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, J=8.7 Hz, 2H), 7.57 (d, J=8.7 Hz, 2H), 5.06 (dd, J=6.8 and 4.1 Hz, 1H), 3.77 (d, J=4.1 Hz, 1H), 3.37 (s, 3H), 3.15 (d, J=6.8 Hz, 1H), 2.20 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.8, 147.5, 147.2, 127.1, 123.5, 89.8, 73.1, 59.6, 27.5; IR (neat) ν_{max} 3511, 1722, 1518, 1350, 1111; HRMS (FI): M· 239.0794 (expected), 239.0793 (observed).

Analytical data for *anti*-**8**. R_f 0.29 (AcOEt/hexane: 40/60); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, J=8.7 Hz, 2H), 7.57 (d, J=8.7 Hz, 2H), 5.03 (dd, J=6.2, 3.7 Hz, 1H), 3.71 (d, J=6.2 Hz, 1H), 3.33 (s, 3H), 3.14 (d, J=3.7 Hz, 1H), 2.17 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 210.4, 147.1, 128.1, 123.9, 90.0, 73.7, 60.1, 27.9; IR (neat) ν_{max} 3398, 1708, 1517, 1347, 1108; HRMS (FI): M· 239.0794 (expected), 239.0792 (observed).

4.2.6. 4-(4′-Nitrophenyl)-4-hydroxy-1-methylsulfanyl-2-butanone 9. Crude ratio regio/*syn*/*anti*=35/20/45. Purification by column chromatography over silica (CH_2Cl_2/Et_2O: 98/2), followed by recrystallization from AcOEt afforded **9** as a pale yellow solid (10%). R_f 0.19 (DCM/Et_2O: 98/2); mp 86 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, J=8.6 Hz, 2H), 7.57 (d, J=8.6 Hz, 2H), 5.28 (t, J=6.4 Hz, 1H), 3.57 (s, 1H), 3.19 (s, 2H), 3.05 (d, J=6.4 Hz, 2H), 2.05 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.7, 149.9, 147.3, 126.5, 123.7, 69.3, 48.0, 43.4, 15.6; IR (neat) ν_{max} 3468, 2921, 1706, 1519, 1348, 1063; Anal. calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49. Found: C, 51.96; H, 5.19; N, 5.49.

4.2.7. 1-(4′-Nitrophenyl)-1,5-dihydroxy-3-pentanone 17 and 4-(4′-nitrophenyl)-4-hydroxy-3-hydroxymethyl-3-butanone 18. Crude ratio regio/*syn*/*anti*=40/23/37. Purification by column chromatography over silica (AcOEt) followed by (toluene/AcOEt: 40/60) or recrystallization from AcOEt afforded **17** and **18-D2** as pale yellow solids (22 and 9%, respectively) and **18-D1** as a pale yellow oil (7%).

Analytical data for **17**: mp 63 °C. R_f 0.35 (AcOEt); ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.22 (d, J=8.9 Hz, 2H), 7.55 (d, J=8.9 Hz, 2H), 5.32 (m, 1H), 3.91 (dt, J=6.0, 5.4 Hz, 2H), 3.56 (d, J=3.4 Hz, 1H), 2.89 (m, 2H), 2.74 (t, J=5.4 Hz, 2H), 2.33 (t, J=6.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.6, 149.8, 147.3, 126.4, 123.8, 68.9, 57.6, 51.4, 45.4; IR (neat) ν_{max} 3388, 2897, 1710, 1605, 1518, 1348, 1056; Anal. calcd for $C_{11}H_{13}NO_5$: C, 55.23; H, 5.48; N, 5.86. Found: C, 55.24; H, 5.51; N, 5.82; HRMS (FI): M· 239.0794 (expected), 239.0800 (observed).

Analytical data for **18-D1**. R_f 0.31 (toluene/AcOEt: 40/60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J=8.8 Hz, 2H), 7.64 (d, J=8.8 Hz, 2H), 4.98 (d, J=8.6 Hz, 1H), 3.62 (dd, 1H, J=10.8, 8.6 Hz), 3.38 (dd, J=10.8, 4.4 Hz, 1H), 3.07 (ddd, J=8.6, 8.6, 4.4 Hz, 1H), 2.28 (s, 3H); ^{13}C NMR (125 MHz,

$CDCl_3$) δ 211.6, 151.0, 147.9, 127.8, 123.5, 72.0, 62.3, 60.7, 30.9; IR (neat) ν_{max} 3426, 2897, 1710, 1606, 1520, 1348, 1063, 1004; HRMS (CI+): M+NH_4^+ 257.1137 (expected), 257.1132 (observed).

Analytical data for **18**-**D2**: deduced from a 13/87 mixture of D1/D2. R_f=0.21 (toluene/AcOEt: 40/60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, J=8.4 Hz, 2H), 7.60 (d, J=8.4 Hz, 2H), 5.04 (d, J=7.6 Hz, 1H), 3.97 (dd, J=11.2, 7.6 Hz, 1H), 3.88 (dd, J=11.2, 4.4 Hz, 1H), 3.17 (ddd, J=7.6, 7.6, 4.4 Hz, 1H), 2.07 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 210.7, 151.1, 147.7, 127.8, 123.4, 71.6, 62.2, 60.4, 30.9; IR (neat) ν_{max} 3425, 2897, 1710, 1606, 1521, 1348, 1063, 1004; HRMS (CI+): M+NH_4^+ 257.1137 (expected), 257.1132 (observed).

4.2.8. 1-(4′-Nitrophenyl)-1-hydroxy-5-methoxy-3-pentanone 19 and 4-(4′-nitrophenyl)-4-hydroxy-3-methoxymethyl-2-butanone 20 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=34/33/33. Purification by column chromatography over silica (DCM/AcOEt: 80/20) followed by (DCM) afforded **19** as a colourless oil (20%) and **20**-*syn* and **20**-*anti* as pale yellow oils (20 and 23%, respectively).

Analytical data for **19**. R_f 0.27 (DCM/AcOEt: 80/20); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (d, J=8.8 Hz, 2H), 7.55 (d, J=8.8 Hz, 2H), 5.29 (m, 1H), 3.67 (m, 3H), 3.35 (s, 3H), 2.88 (d, J=7.2 Hz, 2H), 2.71 and 2.72 (2t, J=6.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.7, 150.4, 147.8, 126.9, 124.2, 69.4, 67.9, 59.4, 52.1, 43.9; IR (neat) ν_{max} 3470, 2857, 1710, 1516, 1345, 1106; HRMS (FI): M$^{\cdot}$ 253.0950 (expected), 253.0955 (observed).

Analytical data for *anti*-**20**. R_f 0.16 (DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, J=8.6, 2H), 7.52 (d, J=8.6 Hz, 2H), 5.24 (dd, J=4.4, 3.0 Hz, 1H), 3.94 (d, J=3.0 Hz, 1H), 3.66 (dd, 1H, J=9.5, 5.2 Hz), 3.59 (dd, J=9.5, 6.2 Hz, 1H), 3.29 (s, 3H), 3.06 (m, 1H), 2.15 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.4, 149.1, 147.3, 126.9, 123.6, 72.6, 70.3, 59.3, 58.3, 31.2; IR (neat) ν_{max} 3424, 2928, 1711, 1521, 1343, 1111; HRMS (FI): M$^{\cdot}$ 253.0950 (expected), 253.0956 (observed).

Analytical data for *anti*-**20**: Analyses are deduced from a 10/90 mixture of *syn*/*anti*. R_f 0.16 (DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, J=8.6 Hz, 2H), 7.51 (d, J=8.6 Hz, 2H), 5.12 (d, J=7.2 Hz, 1H), 3.72 (bs, 1H), 3.49 (dd, J=9.4, 4.2 Hz, 1H), 3.34 (dd, J=9.4, 6.4 Hz, 1H), 3.24 (s, 3H), 3.04 (ddd, J=7.2, 6.4, 4.2 Hz, 1H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.0, 149.2, 147.4, 127.1, 123.7, 71.9, 70.9, 59.2, 59.0, 31.4; IR (neat) ν_{max} 3420, 2929, 2897, 1711, 1521, 1348, 1118; HRMS (CI+): M+NH_4^+ 271.1290 (expected), 271.1294 (observed).

4.2.9. 1-(4′-Nitrophenyl)-1-hydroxy-5-methylsulfanyl-3-pentanone 21 and 4-(4′-nitrophenyl)-4-hydroxy-3-methylsulfanyl-3-butanone 22 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=74/13/13. Purification by column chromatography over silica (hexane/Et_2O: 40/60) afforded **21** as a yellow solid (40%) and **22**-*syn* and **22**-*anti* as pale yellow oils (3.4 and 5%, respectively).

Analytical data for **21**. R_f 0.21 (hexane/Et_2O: 40/60); mp 47 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, J=7.9 Hz, 2H), 7.56 (d, J=7.9 Hz, 2H), 5.31 (m, 1H), 3.48 (d, J=3.3 Hz, 1H), 2.87 (d, J=6.5 Hz, 2H), 2.77 (m, 4H), 2.12 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 208.7, 149.7, 147.3, 126.3, 123.7, 68.8, 51.0, 43.0, 27.6, 15.7; IR (neat) ν_{max} 3423, 2919, 1710, 1603, 1519, 1347, 1077; HRMS (FI): M$^{\cdot}$ 269.0722 (expected), 269.0719 (observed).

Analytical data for *syn*-**22**. R_f 0.29 (hexane/Et_2O: 40/60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, J=8.7 Hz, 2H), 7.55 (d, J=8.7 Hz, 2H), 5.14 (d, J=4.4 Hz, 1H), 3.31 (s, 1H), 3.13 (ddd, J=9.6, 4.4, 4.4 Hz, 1H), 2.81 (dd, J=13.4, 9.6 Hz, 1H), 2.66 (dd, J=13.4, 4.4 Hz, 1H), 2.24 (s, 3H), 2.00 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 211.8, 148.8, 147.9, 127.5, 124.2, 73.1, 58.1, 32.9, 31.5, 16.9; IR (neat) ν_{max} 3429, 2919, 1708, 1519, 1347; HRMS (CI+): M+NH_4^+ 287.1066 (expected), 287.1064 (observed).

Analytical data for *anti*-**22**: Analyses are deduced from a 68/32 mixture of *syn*/*anti*. R_f 0.25 (hexane/Et_2O: 40/60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J=9.0 Hz, 2H), 7.53 (d, J=9.0 Hz, 2H), 5.06 (d, J=6.0 Hz, 1H), 3.45 (s, 1H), 3.14 (m, 1H), 2.72 (dd, J=13.0, 8.0 Hz, 1H), 2.63 (dd, J=13.0, 6.4 Hz, 1H), 2.17 (s, 3H), 2.10 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 212.3, 149.7, 148.8, 127.4, 124.3, 74.2, 58.0, 33.9, 33.2, 16.9; IR (neat) ν_{max} 3430, 2919, 1709, 1520, 1348; HRMS (CI+): M+NH_4^+ 287.1066 (expected), 287.1062 (observed).

4.3. Synthesis of racemic aldols. Path B: direct cross aldolisations with NaOH

A 1% solution of sodium hydroxide in water (5.5 mL, 1.4 mmol) was added to the ketone (132 mmol) at RT. The aldehyde (6.6 mmol) was then added. After 3 h at RT, the layers were separated and the aqueous layer was extracted three times with AcOEt, the combined organic layers were washed with saturated NH_4Cl, dried over $MgSO_4$ and solvents were removed in vacuo. Product ratios were determined by crude ^{1}H NMR spectroscopy before purification by flash column chromatography.

4.3.1. 3-[Hydroxy-(4′-nitrophenyl)-methyl]-2-pentanone 4 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=16/35/49. Purification by column chromatography over silica (hexane/AcOEt: 80/20) followed by semi-preparative HPLC (C8 column) afforded *syn*-**4** as a colourless oil and *anti*-**4** as a white solid (total yield as a mixture with *anti*-**4**, 60%).

Analytical data for *syn*-**4**. R_f 0.16 (hexane/AcOEt: 80/20); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (d, J=8.9 Hz, 2H), 7.54 (d, J=8.9 Hz, 2H), 5.11 (d, J=4.5 Hz, 1H), 3.25 (bs, 1H), 2.84 (ddd, J=9.0, 9.0, 4.5 Hz, 1H), 2.19 (s, 3H), 1.74 (m, 1H), 1.59 (m, 1H), 0.86 (t, J=7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 213.4, 149.4, 147.5, 127.2, 123.8, 72.6, 59.8, 32.0, 19.7, 12.4; IR (neat) ν_{max} 3409, 2967, 1702, 1521, 1348, 1044; HRMS (CI+): M+NH_4^+ 255.1345 (expected), 255.1347 (observed).

Analytical data for *anti*-**4**. R_f 0.16 (hexane/AcOEt: 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J=8.8 Hz, 2H), 7.52 (d, J=8.8 Hz, 2H), 4.93 (d, J=6.0 Hz, 1H), 3.26 (bs, 1H),

2.88 (ddd, J=8.0, 6.0, 6.0 Hz, 1H), 2.14 (s, 3H), 1.63 (m, 1H), 1.52 (m, 1H), 0.93 (t, J=7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 213.7, 150.1, 147.7, 127.3, 124.0, 74.3, 60.1, 32.4, 22.7, 11.7; IR (neat) ν_{max} 3401, 2922, 1702, 1522, 1347; HRMS (CI+): M+NH_4^+ 255.1345 (expected), 255.1343 (observed).

4.3.2. 3-(Hydroxy-(4′-nitrophenyl)-methyl)-2-hexanone 6 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=20/33/47. Purification by column chromatography over silica (toluene/AcOEt: 95/5) afforded *syn*-**6** and *anti*-**6** as colourless oils (22 and 49%, respectively).

Analytical data for *syn*-**6**. R_f 0.22 (toluene/AcOEt: 95/5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, J=8.8 Hz, 2H), 7.52 (d, J=8.8 Hz, 2H), 5.09 (d, J=4.2 Hz, 1H), 3.32 (bs, 1H), 2.88 (ddd, J=8.8, 4.2 and 4.2, 1H), 2.17 (s, 3H), 1.70–1.60 (m, 1H), 1.51–1.42 (m, 1H), 1.34–1.20 (m, 1H), 1.17–1.04 (m, 1H), 0.82 (t, J=7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.3, 149.2, 147.3, 126.9, 123.5, 72.5, 58.1, 31.6, 28.4, 21.1, 14.2; IR (neat) ν_{max} 3456, 1703, 1521, 1348; HRMS (CI+): M+NH_4^+ 269.1501 (expected), 269.1498 (observed).

Analytical data for *anti*-**6**. R_f 0.16 (toluene/AcOEt: 95/5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, J=8.7 Hz, 2H), 7.50 (d, J=8.7 Hz, 2H), 4.88 (d, J=6.8 Hz, 1H), 3.43 (bs, 1H), 2.92 (ddd, J=8.7, 6.8, 5.3 Hz, 1H), 2.13 (s, 3H), 1.56 (m, 1H), 1.28 (m, 3H), 0.86 (t, J=8.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.5, 150.0, 147.4, 127.1, 123.7, 74.4, 58.3, 32.1, 31.4, 20.4, 14.0; IR (neat) ν_{max} 3444, 1711, 1523, 1348; HRMS (FI): M$^{\cdot}$ 251.1158 (expected), 251.1164 (observed).

4.4. Synthesis of racemic aldols. Path C: direct cross aldolisations in presence of PBS pH=7.4

The ketone (1.45 mmol) was added to a solution of 4-nitrobenzaldehyde (140 mg, 0.95 mmol) in DMF (15 mL). PBS pH=7.4 (20 mL) was then added and the resulting solution was stirred at RT or at reflux for 48 h. The mixture was extracted three times with AcOEt, dried over $MgSO_4$ and solvents were removed in vacuo. Product ratios were determined, when possible, by crude ^{1}H NMR spectroscopy before purification by flash chromatography.

4.4.1. 4-(4′-Nitrophenyl)-3,4-dihydroxy-2-butanone 16 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=3/48/49. Purification by column chromatography over silica (DCM/AcOEt: 80/20) and recrystallization from AcOEt afforded a mixture of *syn*-**16** and *anti*-**16** as a yellow solid (40%). Analyses deduced from a mixture of 79% of *syn* and 21% of *anti*). R_f 0.25 (DCM/AcOEt: 80/20); ^{1}H NMR (400 MHz, CD_3OD) *Syn* δ 8.23 (d, J=8.5 Hz, 2H), 7.71 (d, J=8.5 Hz, 2H), 5.26 (d, J=2.6 Hz, 1H), 4.30 (d, J=2.6 Hz, 1H), 2.34 (s, 3H); *Anti* δ 8.22 (d, J=8.5 Hz, 2H), 7.66 (d, J=8.4 Hz, 2H), 4.94 (d, J=6.0 Hz, 1H), 4.23 (d, J=6.0 Hz, 1H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) *Syn* δ 211.9, 151.4, 149.0, 129.1, 124.5, 82.4, 74.9, 27.4; *Anti* δ 212.1, 150.8, 149.2, 129.7, 124.4, 82.3, 75.8, 28.1; IR (neat) ν_{max} 3422, 1715, 1606, 1518, 1348, 1108, 1064; HRMS of the mixture (CI+): M+NH_4^+ 243.0981 (expected), 243.0985 (observed).

4.4.2. 4-(4′-Nitrophenyl)-4-hydroxy-3-methylsulfanyl-2-butanone 10 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=5/55/40. Purification by column chromatography over silica (CH_2Cl_2/Et_2O: 98/2), followed by recrystallization from AcOEt afforded *syn*-**10** and *anti*-**10** as pale yellow solids (8 and 16%, respectively).

Analytical data for *syn*-**10**. R_f 0.35 (CH_2Cl_2/Et_2O: 98/2); mp 73 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J=8.7 Hz, 2 H), 7.58 (d, J=8.7 Hz, 2 H), 5.16 (d, J=7.8 Hz, 1 H), 3.51 (bs, 1 H), 3.45 (d, J=7.8 Hz, 1 H), 2.22 (s, 3 H), 2.06 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.8, 147.8, 147.6, 127.9, 123.5, 69.1, 59.9, 29.1, 12.3; IR (neat) ν_{max} 3478, 1701, 1520, 1348, 1064; Anal. calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49. Found: C, 51.70; H, 5.13; N, 5.47.

Analytical data for *anti*-**10**. R_f 0.16 (CH_2Cl_2/Et_2O: 98/2); mp 129 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J=8.8 Hz, 2 H), 7.59 (d, J=8.8 Hz, 2 H), 5.12 (d, J=9.2 Hz, 1 H), 3.42 (d, J=9.2 Hz, 1 H), 3.20 (bs, 1 H), 2.41 (s, 3 H), 1.93 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.5, 147.7, 147.6, 127.8, 123.5, 72.1, 58.7, 28.6, 13.0; IR (neat) ν_{max} 3386, 1698, 1510, 1343, 1034; Anal. calcd for $C_{11}H_{13}NO_4S$: C, 51.75; H, 5.13; N, 5.49; O, 25.07. Found: C, 51.84; H, 5.14; N, 5.36; O, 25.13.

4.4.3. 4-(4′-Nitrophenyl)-3-chloro-4-hydroxy-2-butanone 12 (*syn* and *anti*). Crude ratio regio/*syn*/*anti*=1/59/40. Purification by column chromatography over silica (DCM/Et_2O: 98/2) afforded **12** as a colourless oil (43%) in 3:2 ratio of *syn*:*anti*. Analyses deduced from a mixture of 60% of *syn* and 40% of *anti*. R_f 0.20 (DCM/Et_2O: 98/2); ^{1}H NMR (400 MHz, $CDCl_3$) *syn* δ8.25 (d, J=8.3 Hz, 2H), 7.59 (d, J=8.3 Hz, 2H), 5.45 (dd, J=4.0, 4.0 Hz, 1H), 4.44 (d, J=4.0 Hz, 1H), 3.20 (d, J=4.0 Hz, 1H), 2.40 (s, 3H); *anti* δ 8.25 (d, J=8.4 Hz, 2H), 7.61 (d, J=8.4 Hz, 2H), 5.16 (dd, J=8.0, 4.2 Hz, 1H), 4.28 (d, J=8.0 Hz, 1H), 3.35 (d, J=4.2 Hz, 1H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) *syn* δ 203.3, 147.8, 146.1, 127.4, 123.6, 72.0, 67.1, 28.3; *anti* δ 203.3, 148.0, 145.8, 128.1, 123.6, 73.8, 63.4, 28.0; IR (neat) ν_{max} 3441, 1718, 1606, 1521, 1349; HRMS of the mixture (CI+): M+NH_4^+ 261.0642 (expected), 261.0638 (observed).

4.4.4. 4-(4′-Nitrophenyl)-3-fluoro-4-hydroxy-2-butanone 14 (*syn* and *anti*). Purification by column chromatography (two fold) over silica (hexane/Et_2O: 60/40, followed by pentane/Et_2O: 50/50) afforded **14** as a pale yellow solid in 3:4 ratio of *syn*:*anti* (15%). Analyses deduced from a mixture of 43% of *syn* and 57% of *anti*. R_f 0.24 (pentane/Et_2O: 50/50); mp 55 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *syn* δ8.26 (d, J=8.8 Hz, 2H), 7.61 (d, J=8.8 Hz, 2H), 5.31 (ddd, J=24.8, 6.2, 2.2 Hz, 1H), 4.86 (dd, J=48, 2.2 Hz, 1H), 2.84 (d, J=6.2 Hz, 1H), 2.34 (d, J=4.9 Hz, 3H); *anti* δ 8.24 (d, J=8.8 Hz, 2H), 7.57 (d, J=8.8 Hz, 2H), 5.18 (ddd, J=12.7, 5.4, 2.4 Hz, 1H), 4.82 (dd, J=48.9, 5.4 Hz, 1H), 3.30 (d, J=2.4 Hz, 1H), 2.23 (d, J=6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) *syn* δ 207.4, 148.0, 144.7, 127.3, 123.8, 96.3 (d, J=195.0 Hz), 72.9 (d, J=18.0 Hz), 27.6; *anti* δ 207.1, 147.9, 145.8, 127.9, 123.6, 95.6 (d, J=192.0 Hz), 72.6 (d, J=21.3 Hz), 27.4; ^{19}F NMR (235 MHz, $CDCl_3$) *syn* δ −195.3 (ddq, J=48, 24.8, 4.9 Hz); *anti* δ −204.7 (ddq, J=48.9, 12.7, 6.2 Hz); IR

(neat) ν_{max} 3438, 1720, 1607, 1520, 1348; HRMS of the mixture (CI+): M+NH_4^+ 245.0938 (expected), 245.0932 (observed).

4.4.5. 6-(4′-Acetamidophenyl)-3-ethyl-4-hydroxy-2-hexanone 2 (*syn* and *anti*). A DCM solution (2 mL) of a mixture of trimethyl-(1-propyl-vinyloxy)-silane and trimethyl-(1-methyl-but-1-enyloxy)-silane (75 mg, 0.47 mmol) was added dropwise into a mixture of aldehyde (100 mg, 0.52 mmol) and $TiCl_4$ (57 μL, 0.52 mmol) in 4 mL of DCM at −78 °C. After stirring the mixture for 1.5 h at −78 °C, sat. NH_4Cl solution was added. The aqueous phase was extracted three times with AcOEt, the combined organic layers were washed with brine and dried over Na_2SO_4. Purification by column chromatography (Et_2O/AcOEt: 80/20), afforded **2** as a D1/D2: 50/50 mixture. Analytical data of the mixture. R_f 0.20 (Et_2O/AcOEt: 80/20); ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J*=8.2 Hz, 2H), 7.32 (bs, 1H), 7.13 (d, *J*=8.2 Hz, 2H), 3.80 (dt, *J*=9.2, 4 Hz, 0.5H, D1), 3.73 (ddd, *J*=8.8, 5.2, 3.6 Hz, 0.5H, D2), 2.81 (m, 1H), 2.63 (m, 1H), 2.52 (m, 1H), 2.19 (s, 1.5H, D2), 2.18 (s, 1.5H, D1), 2.16 (s, 3H,), 1.70 (m, 4H,), 0.92 (t, *J*=7.2 Hz, 1.5H, D1), 0.90 (t, *J*=7.2 Hz, 1.5H, D2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 214.7 (D2), 213.7 (D1), 168.3, 137.8 (D1), 137.7 (D2), 135.7, 128.9, 120.0, 71.2 (D2), 70.6 (D1), 59.0 (D1), 58.8 (D2), 37.8 (D2), 36.2 (D2), 31.7 (D2), 31.6 (D1), 31.5 (D1), 31.4 (D2), 24.5, 22.2 (D2), 19.8 (D1), 12.4 (D1), 11.7 (D2); IR (neat) ν_{max} 3308, 2953, 2939, 1708, 1665, 1601, 1543, 1522; HRMS of the mixture (FI): $M^{\cdot}$ 277.1678 (expected), 277.1679 (observed).

4.4.6. 4-(*tert*-Butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-2-butanone. Imidazole (2.9 g, 43.4 mmol) and TBSCl (3.2 g, 21.2 mmol) was added to a solution of 4-(4′-nitrophenyl)-4-hydroxy-2-butanone (1.8 g, 8.8 mmol) in 40 mL of DMF at room temperature. The reaction was stirred overnight, quenched with water, extracted with DCM and the combined organic layers were washed with brine and dried over $MgSO_4$. Removal of solvents and purification by chromatography over silica (hexane/DCM: 50/50) afforded a white solid (92%). R_f 0.26 (hexane/DCM: 50/50); mp 36 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 8.19 (d, *J*=9.0 Hz, 2H), 7.52 (d, *J*=9.0 Hz, 2H), 5.28 (dd, *J*=8.3, 4.3 Hz, 1H), 2.94 (dd, *J*=15.8, 8.3 Hz, 1H), 2.58 (dd, *J*=15.8, 4.3 Hz, 1H), 2.16 (s, 3H), 0.86 (s, 9H), 0.04 (s, 3H), −0.14 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.0, 151.9, 147.2, 126.6, 123.7, 70.7, 53.8, 31.7, 25.6, 18.0, −4.8, −5.2; IR (neat) ν_{max} 2930, 1720, 1523, 1347, 1090; HRMS (CI+): M+H^+ 324.1631 (expected), 324.1643 (observed).

4.4.7. 1-Fluoro-4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-2-butanone. Trimethylsilyl triflate (436 μL, 1.9 mmol) was added to a solution of 4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-2-butanone (300 mg, 0.95 mmol) and lutidine (432 μL, 3.7 mmol) in DCM (3 mL) at −78 °C. The reaction mixture was stirred for 2 h at −78 °C, 30 min at 0 °C and then diluted with cold DCM and washed with cold sat. $NaHCO_3$. The organic layer was separated and the aqueous layer was extracted three times with cold DCM. The combined organic layers were washed with cold sat. NaCl, dried over Na_2SO_4 and concentrated to give the corresponding silyl enol ether which was used in the next step without purification. To a solution of the residue in CH_3CN (3 mL) was added at RT Selectfluor (397 mg, 1.1 mmol) in dry acetonitrile (15 mL). The reaction mixture was stirred at RT for 1 h. The mixture was then diluted with EtOAc, washed with $NaHCO_3$ and extracted with EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. Removal of the solvents in vacuo and purification by column chromatography over silica (DCM/hexane: 60/40, followed by hexane/Et_2O: 80/20) afforded a white solid (35% overall yield). R_f 0.15 (hexane/Et_2O: 80/20); mp 64 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, *J*=8.6 Hz, 2H), 7.54 (d, *J*=8.6 Hz, 2H), 5.34 (dd, *J*=8.3, 4.1 Hz, 1H), 4.86 (dd, *J*=47.2, 16.0 Hz, 1H), 4.81 (dd, *J*=47.2, 16.0 Hz, 1H), 3.02 (ddd, *J*=15.8, 4.1, 2.5 Hz, 1H), 2.65 (ddd, *J*=15.8, 8.3, 2.4 Hz, 1H), 0.86 (s, 9H), 0.05 (s, 3H), −0.14 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.7 (d, *J*=19 Hz), 151.3, 147.4, 126.6, 123.8, 85.4 (d, *J*=185 Hz), 70.2, 48.6, 25.6, 18.0, −4.8, −5.3; ^{19}F NMR (235 MHz, $CDCl_3$) δ −227.0 (tt, *J*=47.2, 2.4 Hz); IR (neat) ν_{max} 2956, 1734, 1642, 1517, 1344, 1258; HRMS (CI+): M+NH_4^+ 359.1824 (expected), 359.1811 (observed).

4.4.8. 1-Fluoro-4-hydroxy-4-(4-nitrophenyl)-butan-2-one 13. To a solution of 1-fluoro-4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-2-butanone (69 mg, 0.2 mmol) in dry DCM (2 mL) was added at −78 °C $BF_3.Et_2O$ (56 μL, 0.44 mmol). The mixture was stirred at −78 °C for 1.5 h and at 0 °C for 5 h. The reaction was quenched by addition of water and extracted with DCM. The combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed in vacuo. Purification by column chromatography over silica (hexane/EtOAc: 65:35) afforded **13** as a white solid (66%). R_f0.16 (hexane/EtOAc: 65/35); mp 76 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J*=8.4 Hz, 2H), 7.57 (d, *J*=8.4 Hz, 2H), 5.37 (dd, *J*=8.5, 3.8 Hz, 1H), 4.86 (d, *J*=47.1 Hz, 2H), 3.22 (bs, 1H), 3.02 (ddd, *J*=18.0, 8.5, 2.4 Hz, 1H), 2.98 (ddd, *J*=18.0, 3.8, 2.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.4 (d, *J*=20 Hz), 149.6, 147.5, 126.4, 124.0, 85.0 (d, *J*=184 Hz), 68.4, 47.0; ^{19}F NMR (235 MHz, $CDCl_3$) δ −228.4 (tt, *J*=47.1 Hz, 2.4); IR (neat) ν_{max} 3423, 2921, 1733, 1642, 1520, 1343; HRMS (CI+): M+NH_4^+ 245.0938 (expected), 245.0933 (observed).

4.4.9. 1-Chloro-4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-butan-2-one. Trimethylsilyl triflate (336 μL, 1.9 mmol) was added to a solution of 4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-2-butanone (300 mg, 0.95 mmol) and lutidine (432 μL, 3.8 mmol) in DCM (3 mL) at −78 °C. The reaction mixture was stirred 2 h at −78 °C, 30 min at 0 °C and then diluted with cold DCM and washed with cold sat. $NaHCO_3$. The organic layer was separated and the aqueous layer was extracted three times with cold DCM. The combined organic layer was washed with cold sat. NaCl, dried over Na_2SO_4 and concentrated to give the corresponding silyl enol ether which was used in the next step without purification. To a solution of the residue in CH_3CN (3 mL) was successively added at 0 °C, *N*-chlorosuccinimide (120 mg, 0.9 mmol 160 mg) and water (0.5 mL). The mixture was warmed to RT and stirred for 3 h. Then water was added and the solution was extracted with AcOEt, washed with brine and dried over Na_2SO_4. Removal of solvents and purification by chromatography over silica (hexane/Et_2O: 80/20) afforded a

white solid (150 mg, 35% for both steps). R_f 0.13 (hexane/Et_2O: 80/20); mp 54 °C; ^1H NMR (400 MHz, $CDCl_3$) δ8.22 (d, J=8.6 Hz, 2H), 7.54 (d, J=8.6 Hz, 2H) 5.30 (dd, J=8.5, 4.1 Hz, 1H), 4.15 (d, J=15.8 Hz, 1H), 4.11 (d, J=15.8 Hz, 1H), 3.06 (dd, J=15.6, 8.5 Hz, 1H), 2.70 (dd, J=15.6, 4.1 Hz, 1H), 0.86 (s, 9H), 0.04 (s, 3H), −0.15 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.5, 151.0, 147.4, 126.5, 123.8, 70.8, 50.0, 49.4, 25.6, 17.9, −4.8, −5.4; IR (neat) ν_{max} 2990, 1738, 1523, 1348, 1254, 1093; HRMS (CI+): M+H$^+$ 358.1241 (expected), 358.1235 (observed).

4.4.10. 1-Chloro-4-hydroxy-4-(4-nitrophenyl)-butan-2-one 11. To a solution of 1-chloro-4-(*tert*-butyl-dimethyl-silanyloxy)-4-(4′-nitrophenyl)-butan-2-one (79 mg, 0.22 mmol) in dry DCM (3 mL) was added at −78 °C $BF_3.Et_2O$ (61 μL, 0.48 mmol). The mixture was stirred at −78 °C for 1.5 h, at 0 °C for 1.5 h and at RT for 4 h. The reaction was quenched by addition of water and extracted with DCM. The combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed in vacuo. Purification by column chromatography over silica (hexane/EtOAc: 65/35) afforded **11** as a white solid (67%). R_f 0.21 (hexane/EtOAc: 65/35); mp 94 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J=8.4 Hz, 2H), 7.57 (d, J=8.4 Hz, 2H), 5.34 (m, 1H), 4.14 (s, 2H), 3.15 (d, J=3.6 Hz, 1H), 3.03 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.1, 149.4, 147.5, 126.4, 123.9, 68.9, 48.4, 48.2; IR (neat) ν_{max} 3390, 2912, 1726, 1601, 1509, 1344; HRMS (CI+): M+H$^+$ 261.0642 (expected), 261.0645 (observed).

4.5. Antibody assays

All antibody-catalyzed reactions were performed in phosphate buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4). All antibody-catalyzed reactions and background reactions were monitored by high-pressure liquid chromatography (HPLC; Waters HPLC system (626 Pump, 600S Controller, 996 Photodiode Array Detector, Millenium^{32} Software) using a Nova-pak Waters column (C-18, 60 Å pore size, 4 μm particle size, 3.9×150 mm) and acetonitrile/water or methanol/-water mixtures (containing 0.1% trifluoroacetic acid) as eluents at a flow rate of 1.0 mL/min. For antibody assays run for significant periods of time, a control assay was performed to rule out the possibility of catalysis due to the presence of microflora.

4.6. Michaelis–Menten kinetics

Product formation or percent conversion of antibody-catalyzed reaction mixtures was monitored by HPLC. The points were determined experimentally and the best fit value of V_{max} and K_m were obtained by fitting the v_i versus $[S]_0$ data to hyperbolic saturation curves by weighted non-linear regression. All data are reported per antibody active site. An IgG antibody possesses 2 active sites per MW of ~150,000 g/mol.

4.7. Determination of enantiomeric excesses: forward aldol reaction

10 μL of a 66 mM solution of *p*-nitrobenzaldehyde in acetonitrile was added to 2 mL of antibody (32 μM in PBS). Reactions were initiated by addition of 100 μL of ketone (65 mM) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) containing 10% of acetonitrile. The reactions were monitored by RP-HPLC and, after a minimum of 8% conversion was reached, the unreacted aldol was isolated by semi-preparative reversed-phase HPLC, Hypersil ODS column, 5 μm particle size, 7×250 mm, flow rate 2.0 mL/min. The fractions were freeze-dried, the residue was redissolved in 500 μL of dichloromethane/hexane (50/50) and the ee was determined by normal-phase HPLC using a Chiralcel OD or OJ Daicel column.

4.8. Determination of enantiomeric excesses: retro-aldol reaction

10 μL of a racemic stock solution of aldol (80 mM in acetonitrile) was added to 1 mL of antibody (32 μM in PBS, pH 7.4). The reactions were monitored by RP-HPLC. After reaching 50% conversion, the unreacted aldol was isolated by semi-preparative reverse-phase HPLC, Hypersil ODS column, 5 μm particle size, 7×250 mm, flow rate 2.0 mL/min. The fractions were freeze-dried, the residue was redissolved in 200 μL of dichloromethane/hexane (50/50) and the ee was determined by normal-phase HPLC using a Chiralcel OD or OJ Daicel column.

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Preparative asymmetric reduction of ketones in a biphasic medium with an (*S*)-alcohol dehydrogenase under in situ-cofactor-recycling with a formate dehydrogenase

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Abstract—The substrate range of a novel recombinant (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* is described. In addition, an enzyme-compatible biphasic reaction medium for the asymmetric biocatalytic reduction of ketones with in situ-cofactor regeneration has been developed. Thus, reductions of poorly water soluble ketones in the presence of the alcohol dehydrogenase from *R. erythropolis* and a formate dehydrogenase from *Candida boidinii* can be carried out at higher substrate concentrations of 10–200 mM. The resulting (*S*)-alcohols were formed with moderate to good conversion rates, and with up to >99% ee.

1. Introduction

The enzymatic asymmetric reduction with an in situ-NADH-cofactor regeneration using a formate dehydrogenase (FDH) from *Candida boidinii* (Scheme 1, part (a)) is an interesting technology for the production of optically active alcohols which are valuable specialty chemicals.[1] A related process, namely the enzymatic reductive amination by means of an enzyme-coupled in situ-cofactor regeneration according to Scheme 1, part (b) has been shown to be technically feasible, and is a powerful tool for the preparation of enantiomerically pure α-amino acids. This process runs on the ton scale in the industrial synthesis of L-*tert*-leucine (Scheme 1, part (b)).[2] The extension of this attractive enzymatic concept[3,4] towards the large scale synthesis of optically active alcohols[5–7] is highly desirable but still some drawbacks exist.

Among the main limitations are the lacks of isolated NAD-dependent ADHs commercially available on a technical scale, and the lack of suitable reaction media, which guarantee a high ketone solubility. Thus, reactions are usually carried out at a substrate concentration in the range of 5–10 mM or below due to the low solubility of hydrophobic ketones in water. The presence of an organic solvent could improve the solubility of poorly water-soluble ketones, but generally causes significant enzyme deactivation. In particular, this is known for the formate dehydrogenase from *C. boidinii*[8] which is sensitive to organic solvents.[9] The best solution so far is represented by a continuous process with an enzyme-membrane reactor.[10] Albeit good space–time yields in the range of 60–104 g/(L d) can be obtained,[10a,b] the reaction is limited by the solubility of the ketone in water, which is often below 5–10 mM. Thus, the development of alternative reaction concepts for the asymmetric reduction with isolated enzymes still is a challenge, as well as the access to NAD-dependent ADHs, which cover a broad substrate range. They preferably should be cloned and overexpressed in *Escherichia coli* under conditions showing a high production potential.

In this contribution, we report the investigation of the synthetic scope of the recently developed (*S*)-ADH from *Rhodococcus erythropolis* overexpressed in *E. coli*, as well as the first example of an asymmetric enzymatic reduction of poorly water soluble ketones including an in situ-recycling of the cofactor NADH with a FDH, which runs in

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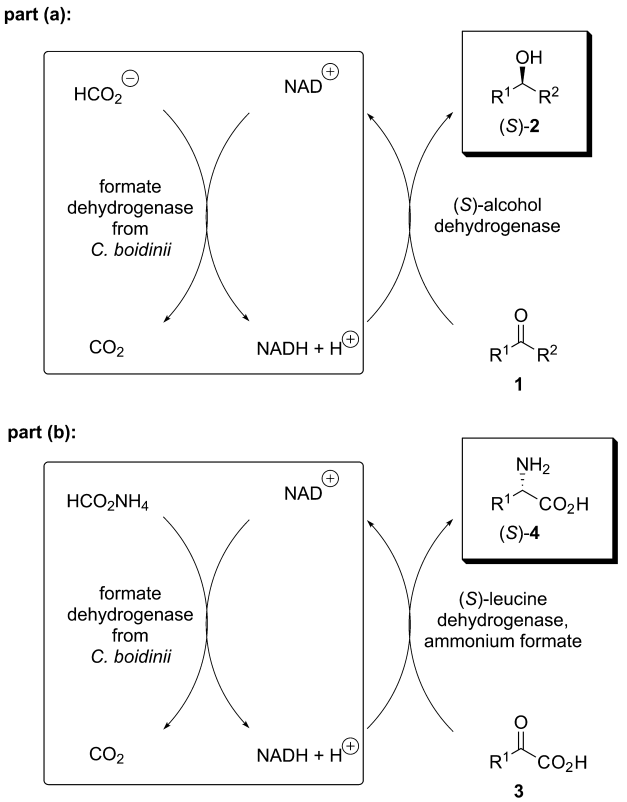


Scheme 1. Production of chiral compounds with NAD-dependent dehydrogenase coupled with formate dehydrogenase for NADH regeneration. (a) Chiral alcohols with an (*S*)-specific ADH; (b) L-amino acids via reductive amination catalyzed by an amino acid dehydrogenase.

the 'direct' presence of an organic solvent at high substrate concentrations.[11]

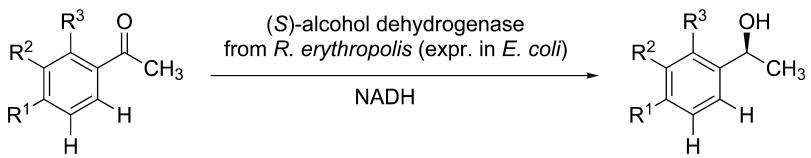
2. Results and discussion

2.1. The scope of a new (*S*)-alcohol dehydrogenase from *R. erythropolis*

We recently developed an *E. coli* expression system for the NAD-dependent (*S*)-alcohol dehydrogenase from *R. erythropolis*,[3k] since previous studies[12] revealed that this enzyme possess a potential for general applicability. This new alcohol dehydrogenase (ADH) isolated from *R. erythropolis* shows a tetrameric structure, it has a molecular weight of 36,206 kDa per subunit, and belongs to the group of zinc-containing medium-chain ADHs.[3k,11a] The developed expression in *E. coli* is efficient and has a high production potential. Thus, this *E. coli* strain should be suited to make the desired enzyme available on a larger scale. With this efficient ADH in hand,[3k,11a] we carried out a detailed substrate screening of various ketones in order to evaluate its general utility.

In general, a broad range of substances represent suitable substrates, and a graphical survey of the photometer-based investigation of the substrate range is presented in Tables 1–3.[13] Acetophenone was used as a reference substrate, and its activity was defined as 100%. With respect to a good preparative applicability, an activity, which is at least in the range of 50–100% is desirable. To start with the aromatic ketone substrates bearing one substituent (Table 1), acetophenones with an interesting variety of substitution pattern are well accepted. Although the acceptance of any type of substituent in the *para*-position is evident (entries 2–9), the type of substituent significantly determine the degree of activity. Any *p*-halogenated acetophenone derivatives (entries 2–4), as well as *p*-methyl- and *p*-methoxysubstituted analogues (entries 5, 8), gave satisfactory to excellent activities in the range of 194–1333% compared to acetophenone as a reference substrate. Notably, the *p*-bromo- and *p*-chloro-derivatives led to the highest activities whereas, the corresponding fluoro-derivative showed a lower activity. In contrast, very low activities of only 19 and 17% were found for *p*-hydroxyacetophenone and *p*-aminoacetophenone (entries 7, 9). An activity of 469% was observed for *p*-(*n*-butyl)acetophenone (entry 6). These latter examples might indicate that electronic properties of the substituent rather than its steric effects are determining the suitability of a substrate. The +I- or −I effect does not appear to have a significant impact. However, the non-satisfactory results for the *p*-hydroxy and *p*-amino-substituted acetophenone indicate that nucleophilicity of the substituent and the ability to form hydrogen bond interactions might play a significant role.

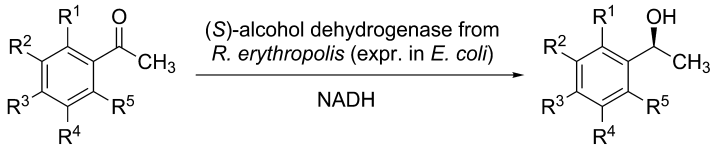
Table 1. Enzymatic activities for monosubstituted acetophenones (determined by photometrical assay)



Entry[a]	R^1	R^2	R^3	Activity (%)	Entry[a]	R^1	R^2	R^3	Activity (%)
1	H	H	H	100	10	H	Cl	H	2384
2	F	H	H	194	11	H	OH	H	47
3	Cl	H	H	1198	12	H	OCH_3	H	394
4	Br	H	H	1333	13	H	NH_2	H	44
5	CH_3	H	H	640	14	H	H	Cl	85
6	n-C_4H_9	H	H	469	15	H	H	CH_3	16
7	OH	H	H	19	16	H	H	OH	9
8	OCH_3	H	H	231	17	H	H	OCH_3	9
9	NH_2	H	H	17					

[a] The photometrical assay is described in Section 3.

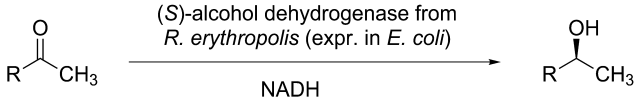
Table 2. Enzymatic activities for di- and trisubstituted acetophenones (determined by photometrical assay)



Entry[a]	R^1	R^2	R^3	R^4	R^5	Activity (%)
1	H	OCH_3	OCH_3	H	H	48
2	H	OCH_3	OCH_3	OCH_3	H	21
3	OH	H	OH	H	H	6
4	OCH_3	H	OCH_3	H	H	7
5	F	H	F	H	H	305

[a] The photometrical assay is described in Section 3.

Table 3. Enzymatic activities for aliphatic ketones (determined by photometrical assay)



Entry[a]	R	Activity (%)	Entry[a]	R	Activity (%)
1	n-C_4H_9	1096	5	CH_2OPh	4180
2	n-C_5H_{11}	3328	6	$CH_2C(O)OCH_3$	134
3	n-C_8H_{17}	2521	7	$CH_2C(O)OC_2H_5$	1020
4	CH_2Cl	119			

[a] The photometrical assay is described in Section 3.

In addition, the influence of the *o*-, *m*-, and *p*-position on the activity was studied. In general, acetophenone derivatives with substituents in *o*- or *m*-position represent suitable substrates, too. However, it appears to be a general tendency that *o*-substituted acetophenones gave less satisfactory results compared with their *m*- or *p*-substituted analogues. For example, the *p*-chloroacetophenone and the *m*-analogue gave high activities of 1198 and 2384%, respectively, whereas an activity of only 85% was detected for *o*-chloroacetophenone (entries 3, 10, 14). A comparable tendency can be found for other types of substituents, for example, methyl- and methoxy-substituents (see entries 5, 8, 15, 17).

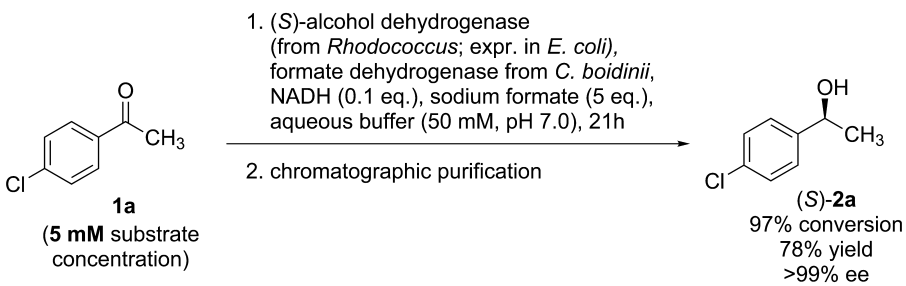
Acetophenones bearing two substituents can also represent suitable substrates in dependence on the substitution pattern (Table 2). For example, 2,4-difluoroacetopheonone gave an activity of 305% whereas a remarkably decreased activity of 7% only was determined when using a 2,4-dimethoxy-acetophenone (entries 4, 5). A significant decrease of the activity was achieved when using acetophenones bearing three substituents. Thus, trimethoxyacetophenone gave a low activity of 21% (entry 2).

In addition, several types of 2-alkanones also serve as good substrates (Table 3). High activities were observed in particular when using longer-chain alkanones, for example, 2-hexanone, 2-heptanone, and 2-decanone (entries 1–3). Furthermore, β-keto esters are good substrates, in particular when bearing an ethoxy group (entry 7). The presence of such an ethyl ester group gave noticeably higher results compared to the methyl ester group (entries 6, 7). The study of ester substrates is ongoing. Further good substrates are substituted acetone derivatives, in particular phenoxy-acetone with an activity of 4180% (entry 5). It should be added at this stage, that in general substrates bearing an aceto functionality are good substrates.

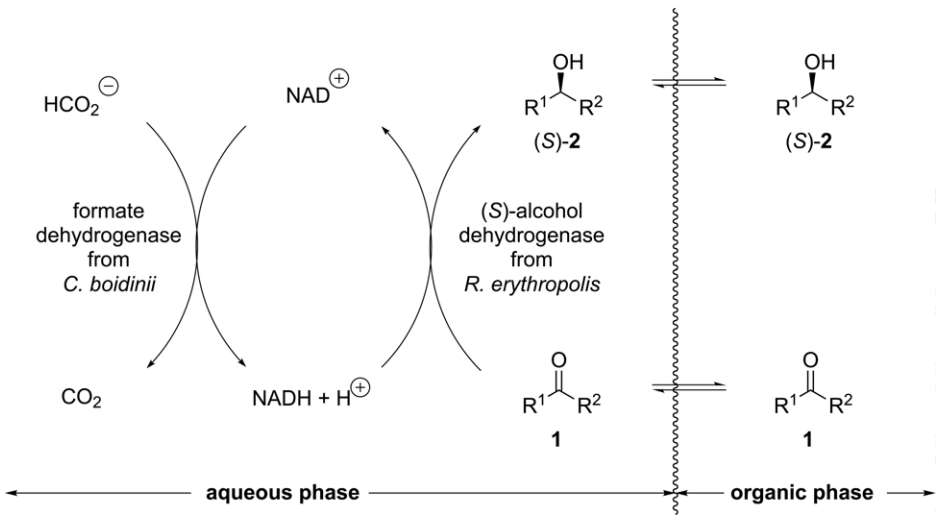
Thus, this new recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* shows both a broad substrate acceptance and high specific activities.[14] Subsequently, the preparative potential of this (*S*)-alcohol dehydrogenase was studied. An initial reduction on a preparative scale with formate and a formate dehydrogenase for cofactor-regeneration was carried out using *p*-chloroacetophenone, **1a**, as a substrate in homogeneous aqueous solution at low substrate concentration (5 mM). According to these standard reaction conditions the product (*S*)-**2a** was obtained with a conversion rate of 97% after a reaction time of 21 h. The subsequent chromatographic purification gave the product, (*S*)-**2a**, in 78% yield, and with an enantioselectivity of >99% ee (Scheme 2).

2.2. Discovery of a new reaction medium

Albeit this reduction works well in principle, the main problem of these standard conditions was the low substrate



Scheme 2. Enzymatic reduction in aqueous medium at low substrate concentration (10 mM).

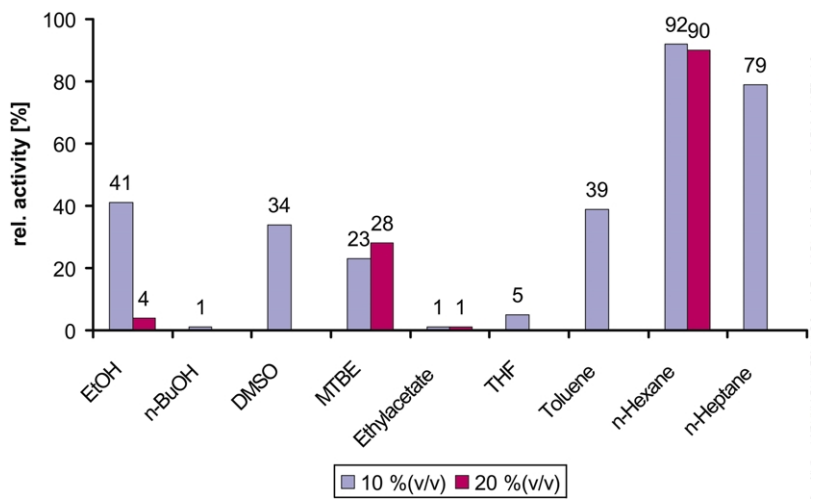


Scheme 3. Concept of an enzymatic reduction in biphasic media.

concentration of the hydrophobic ketones leading to non-satisfactory volumetric productivities (Scheme 2). Thus, the next key step was the development of a suitable aqueous-organic solvent reaction medium according to Scheme 3 which fulfils the following criteria: (i) both enzymes, namely the recombinant (*S*)-ADH and FDH from *C. boidinii* and mutants thereof, remain stable in the presence of the organic solvent; (ii) good solubility of poorly water-soluble ketones which led to high substrate concentrations of up to 200 mM; (iii) simple reaction protocol for lab scale applications, which allows a flexible and fast preparation of chiral alcohols; (iv) robust process with potential for technical scale applications in the future.

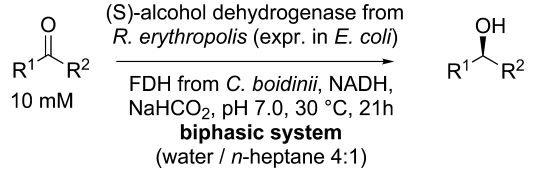
As mentioned above, the stability of the formate dehydrogenase (FDH) from *C. boidinii* as the most sensitive enzyme in this system turned out to be in particular a critical issue. We focused on this enzyme (or mutants thereof) for cofactor-regeneration due to several advantages such as favorable economic data, the large scale availability, and a proven technical applicability.[8] A screening among polar solvents showed a strong deactivation of the FDH (mutant C23S, C262A)[8] even in the presence of only 10% (v/v) of the organic solvent component (Scheme 4).[9] In addition, typical solvents for a biphasic medium failed. For example, MTBE led to a remarkable decrease of the activity after 2 days. A rapid loss of activity was found when ethylacetate was used. In this case a (nearly) complete deactivation was observed within 6 h only. Non-polar aromatic hydrocarbons, for example, toluene, deactivate FDH remarkably, too. However, in the presence of 10% (v/v) of *n*-hexane a long-term stabilization was achieved (Scheme 4). Even after 66–69 h, an activity of 92 and 90% remained for aqueous solutions containing 10% (v/v) and 20% (v/v), respectively, of *n*-hexane. Notably, other aliphatic hydrocarbons, for example, *n*-heptane also showed a positive effect (with 79% activity after ca. 3 days when using 10% (v/v)), and the stability was maintained at increased amount of the organic solvent of up to 60% (v/v). For this study, we have chosen *n*-hexane and in particular *n*-heptane as an organic solvent component. The high degree of stability independent of the amount of organic solvent guarantees sufficient flexibility with respect to the fine-tuning of the reaction later in the process development step.

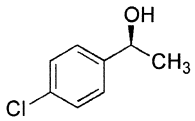
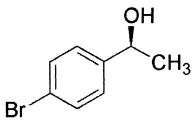
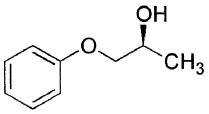
In addition, the (*S*)-selective alcohol dehydrogenase from *R.*



Scheme 4. Stability of the FDH in aqueous-organic media (the activities were measured after ca. 3 days for *n*-hexane, 2 days for *n*-heptane, and 2 days or less for the other solvents).

Table 4. Reduction in a biphasic medium with different substrates

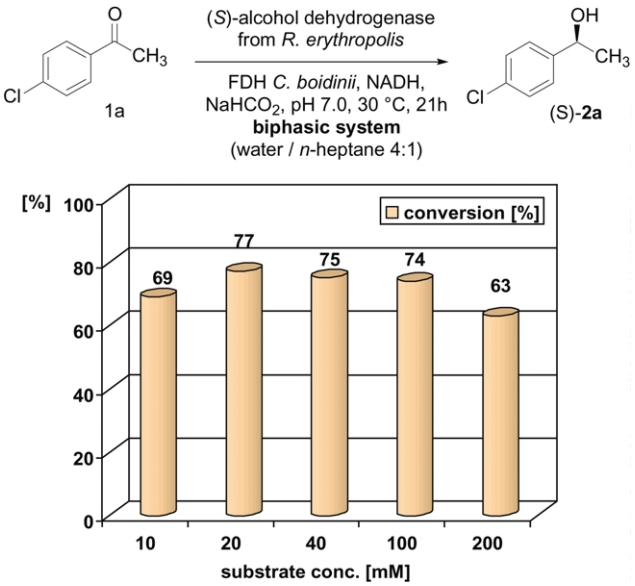


Entry	Product	Conversion (%)	ee (%)[a]
1		69	>99
2		65	97
3		>95	>99

[a] The enantiomeric excess (ee) was determined by GC chiral chromatography.

erythropolis turned out to be stable under the same conditions. In particular, the stability in heptane and hexane—which turned out to be very compatible with the FDH—is high. The activity of the (*S*)-ADH within 48 h remains nearly unchanged in the presence of 20% (v/v) as well as 60% (v/v) of *n*-heptane indicating a good stability in the biphasic medium consisting of an aqueous phase and heptane. Thus, a suitable solvent system was found in which both enzymes remain stable.

With this enzyme-compatible reaction medium in hand, preparative conversions were carried out as a next step. Good conversion rates accompanied by excellent enantioselectivities were obtained with a variety of aromatic ketone substrates. Selected examples are shown in Table 4. In the presence of the (*S*)-ADH from *R. erythropolis*, *p*-chloroacetophenone was converted into the optically active (*S*)-enantiomer, (*S*)-**2a**, with >99% ee with a conversion of 69% (entry 1). A conversion of 65%, and a slightly lower

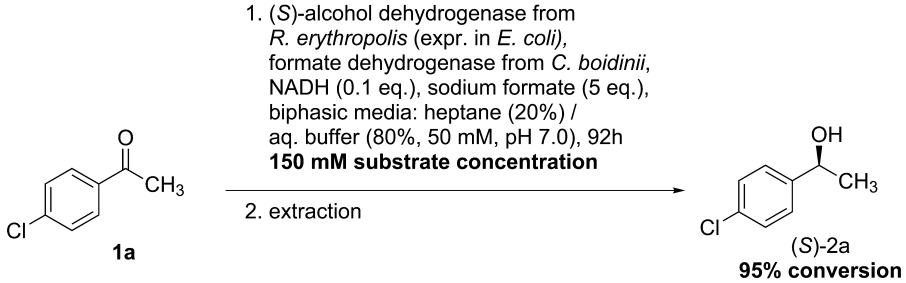


Scheme 5. Conversions of *p*-chloroacetophenone at increased substrate concentrations.

enantioselectivity of 97% ee was observed when using *p*-bromoacetophenone (entry 2). The asymmetric reduction of phenoxyacetone proceeded quantitatively under formation of intermediate (*S*)-**2c** with an enantioselectivity of >99% ee (entry 3).

These studies, which proved the applicability of the new reaction system for preparative conversions were followed by further investigation of the new reaction media at higher substrate concentrations in order to reach high volumetric productivities. As a model reaction, the reduction of *p*-chloroacetophenone, **1a**, in the presence of the (*S*)-ADH from *R. erythropolis*[3k] was investigated. Increased substrate concentrations from 10 mM (69% conversion) up to 100 mM led to comparable conversion rates (Scheme 5). At 20 and 40 mM, slightly improved conversion rates of 77 and 75%, respectively, were obtained. Even at 100 mM, a good conversion of 74% was still achieved. An analogous reaction with a subsequent chromatographical purification of the crude product gave the desired product (*S*)-**2a** with a yield of 67%. A further increase of the substrate concentration up to 200 mM gave a still satisfactory conversion rate of 63%. Thus, higher volumetric productivities can be obtained with this new reaction medium compared to the previous ADH/FDH-coupled reductions in pure aqueous medium.

The reaction conditions were further optimized, for



Scheme 6. Quantitative conversion in enzymatic reduction of *p*-chloroacetophenone in a biphasic medium.

example, with respect to the preferred enzyme/substrate ratio for a high conversion of >90%. Using improved reaction conditions (pH 7.0, 30 °C, (*S*)-ADH and FDH amount of 60 U/mmol of substrate), a conversion of 95% was observed at a substrate concentration of 150 mM (Scheme 6).

In conclusion, the substrate range of a novel recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* was studied, and a practical and efficient reaction medium for the reduction of ketones via enzymatic in situ-cofactor-regeneration was found. The conversions proceed at high substrate concentrations in the 'direct' presence of an organic solvent. The general applicability of this reaction system as well as the simple access to optically active alcohols on a lab scale are further advantages. Currently, further process development is in progress.

3. Experimental

The measurements of the enzymatic activity were carried out using the spectrophotometer JASCO V-530 UV/VIS. The solution of the (*S*)-alcohol dehydrogenase from *R. erythropolis* (overexpressed in *E. coli*) was prepared according to Refs. 3k and 11a. The solution of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*) was prepared according to Ref. 8. The ketones, cofactors, sodium formate and the solvents are commercially available and were used in the activity tests and preparative conversions without further purification.

3.1. Measurement of enzyme activity (typical protocol; according to Tables 1–3)

The NAD-dependent ADH activity was determined spectrophotometrically measuring the oxidation of NADH at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}) in the presence of the ketone. The activity was measured at 30 °C in a cuvette (1 mL) containing 1.45 mM of the ketone in 100 mM sodium phosphate buffer (pH 6.0), and 0.25 mM of NADH. The reaction was started by the addition of the recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* (10 μL of the (*S*)-ADH solution described below as a crude extract or partially purified by ion exchange chromatography, which was diluted by a factor of 100). One unit of ADH activity was defined as the amount of enzyme that converted 1 μmol of NADH per minute. The activity tests described in entries, 1–5, 8, 10, 14 in Table 1, and in all entries in Table 3, are based on the use of an (*S*)-ADH solution with an activity of 13.4 U/mL (for acetophenone); the activity tests described in entries, 6, 7, 9, 11–13, 15–17 in Table 1, and in all entries in Table 2, are based on the use of an (*S*)-ADH solution with an activity of 43.3 U/mL (for acetophenone).

3.2. Enzymatic reduction in aqueous media (according to Scheme 2)

At a reaction temperature of 30 °C, 10 U of the (*S*)-alcohol dehydrogenase, and 10 U of the formate dehydrogenase was added to a solution of 0.5 mmol *p*-chloroacetophenones (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in 100 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the aqueous phase was extracted with 3×100 mL of methyl *tert*-butyl ether. The collected organic phases were analyzed with respect to the conversion rate (97% according to HPLC), dried over magnesium sulfate, and evaporated in vacuo. After chromatographical purification (eluent: ethyl acetate/*n*-hexane 25:75) of the resulting crude product, the desired isolated product (*S*)-**2a** was obtained in 78% yield (64.0 mg), and with an enantioselectivity of >99% ee (determined by HPLC).

3.3. Measurement of the enzymatic activity of the formate dehydrogenase (FDH) from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*; see also Ref. 8) after storage in aqueous organic solvent mixtures

At first, 100 μL of a formate dehydrogenase solution (formate dehydrogenase (FDH) from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*; see also Ref. 8) were dissolved in 10 mL of a solvent mixture containing 10–20% (v/v) of an organic solvent (an overview about the investigated solvents is given in Scheme 4). The samples were stored for a duration of up to 3 days, and regularly samples of 100 μL are taken from the solution. These samples were analyzed with respect to the enzymatic activity by spectrophotometrically measuring the reduction of NAD^+ to NADH at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}) with sodium formate (spectrophotometer: JASCO V-530 UV/VIS). The activity was measured at 30 °C in a cuvette (1 mL) containing 500 μL of a sodium formate solution (0.8 M; pH 8.2; 0.1 M phosphate buffer), and 500 μL of a NAD^+-solution (0.4 mM). The reaction was started by the addition of the enzyme solution (10 μL of the 100 μL solution). One unit of FDH activity was defined as the amount of enzyme that converted 1 μmol of NAD^+ per minute. The resulting enzymatic activities of the FDH in dependency of the storage time in the presence of organic solvents are described in Scheme 4.

3.4. Enzymatic reduction with different types of substrates (according to Table 4)

A general procedure is as follows: At a reaction temperature of 30 °C, 10 U of the (*S*)-alcohol dehydrogenase from recombinant *R. erythropolis* (see also Ref. 3k), and 10 U of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; expressed in *E. coli*; see also Ref. 8) are added to a solution of 0.5 mmol of the ketone component (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in a solvent mixture, consisting of 10 mL of *n*-heptane, and 40 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the organic phase was separated and the aqueous phase is extracted with 3×50 mL of methyl *tert*-butyl ether. The collected organic phases are dried over magnesium sulfate, and after filtration and evaporation of the volatile components in vacuo the resulting oily crude product was analyzed with respect to the conversion rate (via NMR, HPLC). The ee was determined by chiral GC chromatography using a chiral column 'CP-Chirasil-DEX CB' (length: 25 cm; diameter: 25 μm; 1.3 mL/min; gas: He) from the company Chrompack.

3.5. Enzymatic reduction at higher substrate concentrations (according to Scheme 5) exemplified for the reaction with a substrate concentration of 100 mM

At a reaction temperature of 30 °C, 10 U of the recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* (see also Ref. 3k), and 10 U of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; expression in *E. coli*; see also Ref. 8) were added to a solution of 0.5 mmol of *p*-chloroacetophenone (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in a solvent mixture, consisting of 1 mL of *n*-heptane, and 4 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the organic phase was separated and the aqueous phase was extracted with 3×5 mL of methyl *tert*-butyl ether. The collected organic phases were dried over magnesium sulfate, and after filtration and evaporation of the volatile components in vacuo the resulting oily crude product was analyzed with respect to the conversion rate (via NMR, HPLC).

In addition, for the asymmetric reduction at a substrate concentration of 100 mM the product (*S*)-**2a** has been purified from the crude product and isolated via TLC-chromatography (eluent: ethylacetate/*n*-hexane (25:75); thin-layer-chromatography plate: Merck TLC plate 20×20 cm, silica gel 60 F254; R_f-value: 0.21; yield: 67%).

Acknowledgements

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13. The determination of enzyme activities were done using a cell-free crude extract of *E. coli* with the recombinant (*S*)-ADH enzyme from *R. erythropolis*. In addition, activity studies were also done with a (partially) purified form of the enzyme. The presence of other dehydrogenases causing a background activity was not observed in the experimental investigation. The specific activities of this (*S*)-ADH from *R. erythropolis* expressed in *E. coli* is in the range of 100–1000 U/mg of purified protein depending of the structure to be converted, see also Ref. 3k. For comparison, horse liver alcohol dehydrogenase as one of the most widely applied (*S*)-alcohol dehydrogenase so far shows an activity in the range of 1–3 U/mg only, see Ref. 1a.
14. It is to be noticed that several differences in substrate range were observed in comparison with the corresponding wild-type alcohol dehydrogenases (from crude extracts), see also Ref. 11a.

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Synthesis of chiral ADMET polymers containing repeating D-*chiro*-inositol units derived from a biocatalytically prepared diene diol

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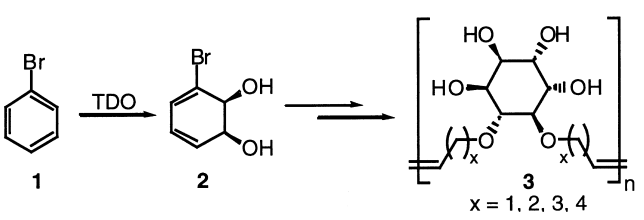
Abstract—Several chiral hydroxylated polymers have been prepared, via ADMET techniques, from the diene diol derived from bromobenzene, obtained by means of whole-cell fermentation with *Escherichia coli* (JM109 pDTG601).

Chiral polymers[1–3] are used in a number of interesting applications: in catalysis for asymmetric induction in organic synthesis,[1,2,4] in chiral separations,[5] and in ferroelectric and nonlinear optical applications.[6] Achiral monomers can be induced to form polymers with a helical conformation by means of 'helix-sense-selective' polymerization with either a chiral initiator or catalyst.[3] These include poly(chloral), poly(isocyanates), poly(isocyanides), and poly(triarylmethylmethacrylates). Other polymers, obtained, for example, by cyclopolymerization of diolefins,[7] diisocyanides,[8] vinyl ethers,[9–12] bis(styrenes),[1,2,11,12] dimethyacylates,[13–16] and diepoxides,[17] derive their optical activity from chirality in the main chain or in side chains. Synthetic peptide-like polymers are also included in this group.[18,19] The introduction of metal catalysts by Ziegler and Natta in the late 1940s provided an approach to making chiral polymers on an industrial scale.[20,21] Development of methods for the preparation chiral polymers remains a challenging field, as radical, anionic, and cationic methods are generally neither stereo- nor enantioselective.[22–24]

We have become interested in polyhydroxylated polymers, or high oligomers, such as **3**, derived from the monomers of established configuration via acyclic diene metathesis (ADMET) of vicinally bis-alkylated olefinic side chains.[25,26] The corresponding monomers are easily obtained from diene diol **2**, the product of the whole-cell fermentation of bromobenzene with a *E. coli* JM109 pDTG601A,[27] recombinant organism expressing toluene dioxygenase (TDO), Scheme 1. The central inositol unit can readily be synthesized in any of the nine possible configurations, as we have demonstrated previously.[28–31] It is also possible to approach the monomer synthesis from protected synthons—in such cases the number of isomeric possibilities in intermediates of this type would approach the theoretical limit of 64. As described in recent papers on combinatorial possibilities of oligoinositols,[32,33] the number of possible isomers increases exponentially with higher oligomers once secondary and tertiary structures develop through hydrogen-bonded forms and β-turns initiated by the 1,2 *trans* connectivity in **3**.[29–31,34] We were interested primarily in generating relatively low molecular weight polymers and investigating physical and chemical properties of these compounds in anticipation of further cross-linking and testing of these compounds as materials for chiral separations.

To test our approach to this problem, bis-allyl ether **8**, Scheme 2, was prepared and subjected to polymerization with Grubbs' first-generation catalyst. The bis-ether was generated by first protecting the *cis*-diol of the diene diol **2** as the acetonide followed by epoxidation of the more electron-rich double bond to afford epoxide **4**. The $BF_3{\cdot}Et_2O$-catalyzed epoxide opening of epoxide **4** with allyl alcohol led to a low yield of ether **5** at the expense of



Scheme 1. Chemoenzymatic approach to polyhydroxylated chiral polymers (D-*chiro*-inositol configuration shown).

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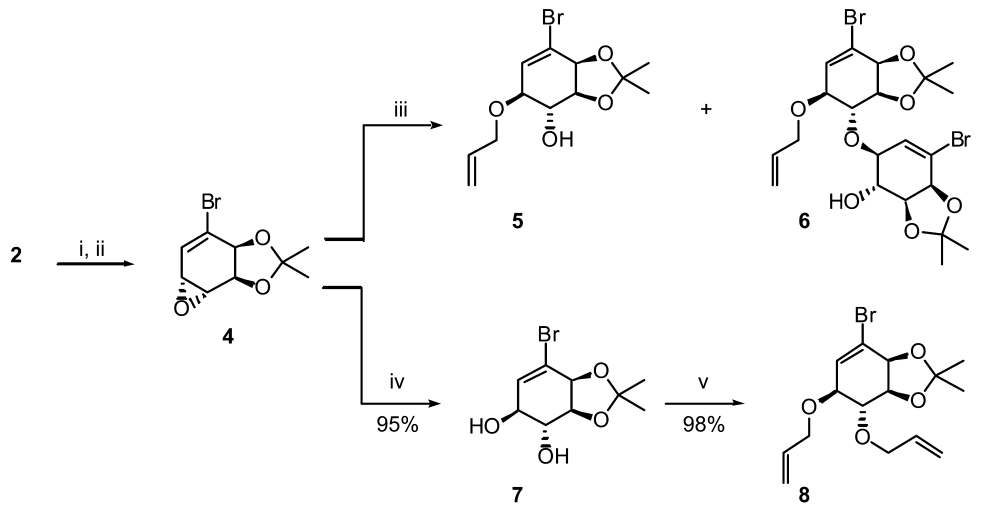
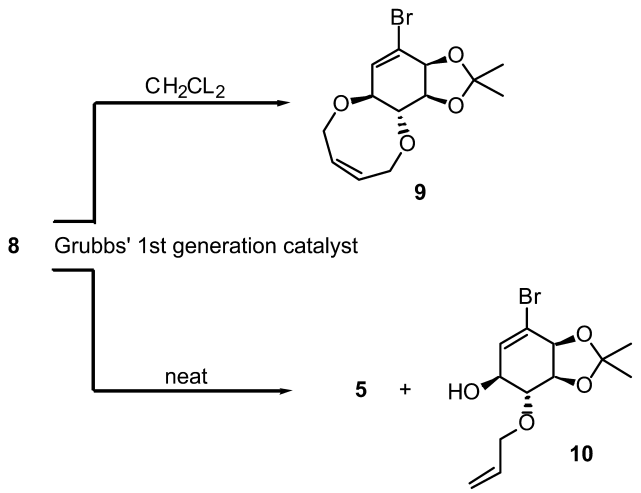


Scheme 2. Synthesis of monomer bis-allyl ether **8**. Key: (i) DMP, *p*TsOH; (ii) *m*CPBA, CH_2Cl_2; (iii) $BF_3{\cdot}Et_2O$, allyl alcohol; (iv) Amberlyst resin; (v) DMF; NaH, allyl bromide.

generating the conduritol dimer **6**. The problem was circumvented by treating epoxide **4** first with Amberlyst resin to afford *trans* diol **7**, which was alkylated with allylbromide to afford allyl ether **8** in 98% yield (Scheme 2).

Allyl ether **8** was subjected to ADMET conditions with Grubbs' first-generation catalyst under several conditions. In dilute methylene chloride, no polymerization occurred, and only the cyclic dioxocane **9** formed (Scheme 3). When the reaction was conducted neat, again no polymer formed,



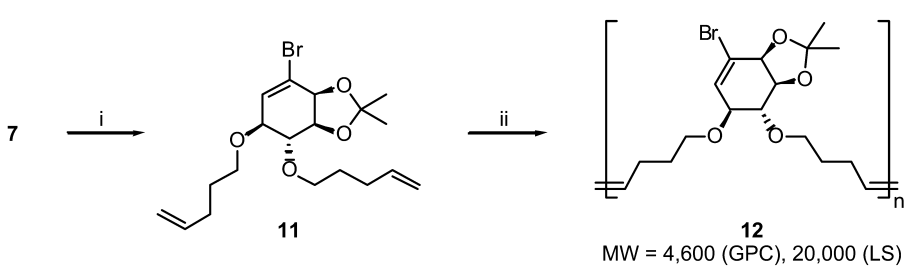
Scheme 3. Polymerization of **8** with Grubbs' first-generation catalyst.

and a mixture of de-allylated products **5** and **10**, along with starting material, was isolated.

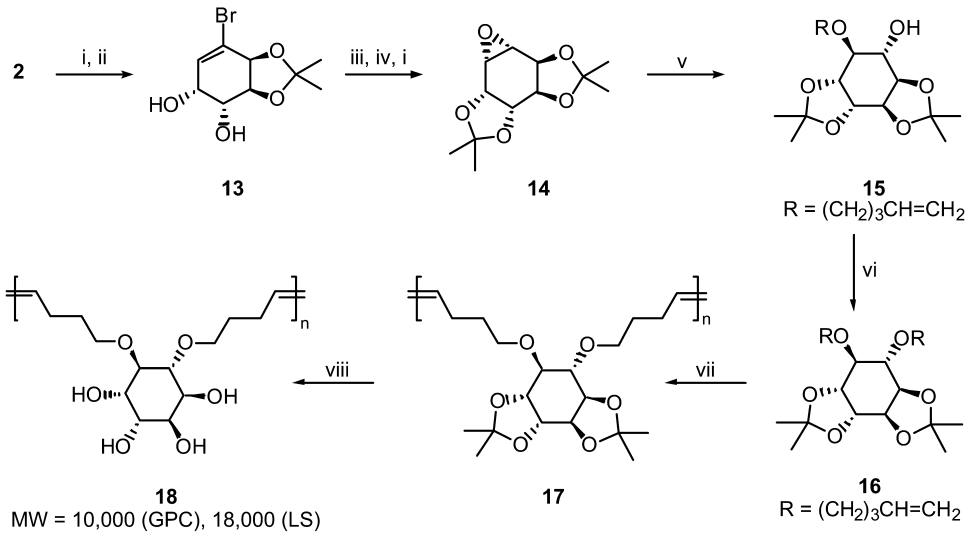
Diol **7** was alkylated with 5-bromopentene to furnish bis-alkylated ether **11**, which polymerized with Grubbs' first-generation catalyst. The result was polymer **12** having an estimated molecular weight of 4600. When monomer **11** was polymerized with Grubbs' second-generation catalyst, a polymer with an average molecular weight of 20,000 was obtained, determined by light-scattering (LS) analysis (Scheme 4).

The synthesis of the fully hydroxylated polymer was carried out as outlined in Scheme 5.

In the approach to polyhydroxylated polymer **18**, the *cis* diol in **2** was protected, and the more electron rich double bond was converted to *cis* diol **13**. Reduction under radical conditions with tri-*n*-butyltinhydride and subsequent epoxidation with *m*CPBA followed by protection of the *cis* diol provided epoxide **14**. $BF_3{\cdot}Et_2O$-catalyzed epoxide opening with 4-penten-1-ol gave alcohol **15**, which was alkylated with 5-bromopentene to furnish the desired monomer **16**. Exposure of this material to Grubbs' first generation of catalyst (neat) gave polymer **17** whose deprotection under acidic condition gave the fully hydroxylated polymer **18**, with a molecular weight estimated at ~10,000 via GPC analysis, as a viscous oil. Light scattering analysis of polymer **18** estimated the average molecular weight of the polymer at 18,000.



Scheme 4. Preparation and polymerization of pentenyl ether **11**. Key: (i) 5-bromopentene, DMF, NaH; (ii) Grubbs' catalyst (1st generation), neat, or Grubbs' catalyst (2nd generation), neat.



Scheme 5. Approach to fully hydroxylated chiral polymers. Key: (i) DMP, *p*TsOH; (ii) acetone, H_2O, NMO, OsO_4; (iii) Bu_3SnH, AIBN, PhH; (iv) *m*CPBA, CH_2Cl_2; (v) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 4-penten-1-ol; (vi) DMF, NaH, 5-bromopentene; (vii) Grubbs' first-generation catalyst; (viii) THF–TFA–H_2O, 4:1:1.

1. Conclusions

New homochiral polymeric materials have been prepared from metabolites of type **2**. Further exploitation of the properties of these hydroxylated polymers in the area of chiral separation and catalyst development are ongoing and will be reported in due course.

2. Experimental

2.1. General

All commercial chemicals and solvents are reagent grade and were used without further purification unless otherwise specified. Standard techniques for the exclusion of moisture were used in all reactions. Reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60F-254, Silicycle) and visualized with UV light, iodine vapors, or 5% phosphomolybdic acid in 95% ethanol. Final compounds were typically purified by flash chromatography on silica gel (230–400 mesh). All ^{1}H- and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using a Varian 300 spectrometer. Chemical shifts are reported relative to TMS, $CDCl_3$, or DMSO-d_6. Coupling constants are measured in Hz. Infrared spectra, recorded on a Perkin–Elmer FT-IR, are reported as wavenumbers (cm^{-1}). High-resolution mass spectra were performed at the University of Florida, elemental analyses at Atlantic Microlab, Inc. Optical rotations were recorded on a Perkin–Elmer 241 digital polarimeter (10^{-1} deg cm^2 g^{-1}). Melting points were obtained on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Gel permeation chromatography (GPC) was performed on two 300 mm Polymer Laboratories 5 μm mixed-C columns with a Rainin SD-300 pump, a Hewlett–Packard 1047-A RI detector, a TC-45 Eppendorf column heater set to 35 °C and a Waters U6K injector.

2.1.1. 5-(5-Allyloxy-7-bromo-2,2-dimethyl-3a,4,5,7a-tetrahydro-benzo[1,3]dioxol-4-yloxy)-7-bromo-2,2-dimethyl-3a,4,5,7a-tetrahydro-benzo[1,3]dioxol-4-ol (6). Bromoepoxide **4** (0.5 g) was added to a flame-dried 50 mL round-bottomed flask containing a stirred solution of allyl alcohol (0.56 g, 9.66 mmol) in freshly distilled methylene chloride (5 mL) under argon atmosphere. The reaction mixture was cooled to −78 °C for 20 min. $BF_3{\cdot}Et_2O$ (10 mol%) was added, and the reaction mixture was stirred overnight. The reaction was quenched with water (10 mL), and the mixture was extracted with methylene chloride (3×10 mL). The organic layers were combined and washed with 5% $NaHCO_3$ (10 mL), dried ($MgSO_4$) and concentrated under reduced pressure to afford a reddish residue. The products were purified by flash chromatography (hexanes–EtOAc, 4:1) to yield dimer **6** as a white solid (356 mg, 63%) with trace amount of alcohol **5** (8 mg, 3%).

2.1.2. 5-Allyloxy-7-bromo-2,2-dimethyl-3a,4,5,7a-tetrahydro-benzo[1,3]dioxol-4-ol (5). R_f=0.48 (hexanes–EtOAc, 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.22 (m, 2H), 5.28 (ddd, J=17.2, 1.75, 1.46 Hz, 1H), 5.16 (ddd, J=10.5, 1.75, 1.46 Hz, 1H), 4.94 (bs, 1H), 4.70 (ddd, J=6.42, 1.75, 1.46 Hz, 1H), 4.25 (ddt, J=13.1, 5.26, 1.46 Hz, 1H), 4.16 (dd, J=9.64, 6.42 Hz, 1H), 3.98 (ddt, J=12.8, 5.84, 1.46 Hz, 1H), 3.60 (t, J=9.64 Hz, 1H), 1.55 (s, 3H), 1.43 (s, 3H).

Compound **6**: R_f=0.65 (hexanes–EtOAc, 3:2); mp=176–177 °C; $[\alpha]_D^{31}$=+47.35 (c, 0.665, $CHCl_3$); IR (KBr) 3498, 2981, 1645, 1457, 1381, 1214, 1046, 998, 870, 513 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.33 (d, J=1.71 Hz, 1H), 6.28 (d, J=1.71 Hz, 1H), 6.18–5.85 (m, 1H), 5.39–5.24 (m, 2H), 4.72 (d, J=6.84 Hz, 1H), 4.47 (d, J=6.84 Hz, 1H), 4.46 (s, 1H), 4.28–4.12 (m, 3H), 4.45–4.00 (m, 1H), 3.90 (d, J=8.30 Hz, 1H), 3.86–3.80 (m, 1H), 3.67 (m, 1H), 3.53 (t, J=8.7 Hz, 1H), 1.58 (s, 3H), 1.57 (s, 3H), 1.42 (s, 3H), 1.41 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 135.1, 133.5, 132.8, 118.8, 118.1, 117.9, 111.5, 111.1, 84.4, 84,1, 78.7, 77.6, 77.20, 77.1, 74.1, 74.0, 71.2, 28.2, 27.7, 25.9, 25.7. HRMS (FAB) calcd for

$C_{21}H_{29}O_7Br_2$: 551.0280. Found: 551.0045. Anal. calcd for $C_{21}H_{28}O_7Br_2$: C, 45.67; H, 5.11. Found: C, 45.92; H, 5.28.

2.1.3. 4,5-*bis*-Allyloxy-7-bromo-2,2-dimethyl-3a,4,5,7a-tetrahydro-benzo[1,3]dioxole (8). Into a 50 mL oven-dried round-bottomed flask, NaH (2.41 g, 45 mmol) was suspended in anhydrous DMF (20 mL) under argon atmosphere. Bromodiol **7** (2.41 g, 9.1 mmol) was added, and the reaction mixture was stirred for 1 h at room temperature. Allylbromide (5.46 g, 45 mmol) dissolved in 10 mL DMF was added dropwise to the reaction mixture. The reaction proceeded for 8 h after the addition of allylbromide. The reaction was quenched with water, and the mixture was extracted with diethyl ether (3×25 mL). The product was purified by flash chromatography (hexanes–EtOAc, 9:1). The fractions containing bis-allylether **8** were combined and concentrated to yield a yellowish oil, which was further purified by distillation at high temperature at under reduced pressure (150 °C, 0.2 mm Hg) using a Kugelrohr apparatus to afford bis-allylether **8** as a colorless oil (3.14 g, 98%). R_f=0.44 (hexanes–EtOAc, 6:1); $[\alpha]_D^{27}$=36.85 (*c* 1.1, $CDCl_3$); IR (film) 3078, 2987, 1646, 1456, 1381, 1246, 1218, 1073, 869, 794 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 6.21 (d, *J*=2.19 Hz, 1H), 6.02–5.84 (m, 2H), 5.33 (dt, *J*=1.47, 1.71 Hz, 1H), 5.28 (dt, *J*=1.47, 1.71 Hz, 1H), 5.24–5.14 (m, 2H), 4.65 (dd, *J*=1.22, 6.35 Hz, 1H), 4.40–4.25 (m, 2H), 4.24–4.19 (dd, *J*=6.35, 8.55 Hz, 1H), 4.16 (t, *J*=1.46 Hz, 1H), 4.14 (t, *J*=1.46 Hz, 1H), 3.90–3.84 (ddd, *J*=1.22, 0.98, 7.82 Hz, 1H), 3.52 (t, *J*=8.05 Hz, 1H), 1.55 (s, 3H), 1.41 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 135.2, 134.5, 133.6, 119.0, 117.4, 117.4, 110.8, 79.2, 78.0, 77.9, 77.65, 73.5, 71.7, 28.2, 26.3; HRMS (EI) *m/z* calcd for $C_{14}H_{18}O_4Br$ $[M-CH_3]^+$: 329.0388. Found: 329.0271. Anal. calcd for $C_{15}H_{21}O_4Br$: C, 52.19; H, 6.13. Found C, 52.35; H, 6.23.

2.1.4. ADMET polymerization of bis-allylether 8: 4-bromo-2,2-dimethyl-3a,5a,7,10,11a,11b-hexahydro-1,3,6,11-tetraoxa-cycloocta[*e*]indene (9). Into an oven-dried 10 mL round-bottomed flask, bis-allylether **8** (76 mg, 0.2 mmol) was dissolved in freshly distilled methylene chloride (5 mL) at room temperature under argon atmosphere. Grubbs' first-generation catalyst (9.4 mg, 0.01 mmol) was then added, and the reaction mixture was stirred under argon for 24 h. The reaction was quenched with ethyl vinyl ether (0.2 mL), and the mixture concentrated under reduced pressure. The residue was purified via flash chromatography to yield the cyclic dioxocane **9** as a colorless oil (25 mg, 36%). R_f=0.45 (hexanes–EtOAc, 4:1); ^1H NMR (300 MHz, $CDCl_3$) δ 6.17 (d, *J*=2.1 Hz), 5.85–5.65 (m, 2H), 4.70–5.58 (m, 2H), 4.46–4.25 (m, 3H), 4.17 (dd, *J*=6.4, 9.0 Hz, 1H), 3.91 (td, *J*=8.47, 1.46 Hz, 1H), 3.47 (t, *J*=8.76 Hz, 1H), 1.56 (s, 3H), 1.42 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 134.9, 130.1, 129.1, 118.2, 111.0, 81.5, 78.8, 77.4, 77.3, 69.5, 67.4, 28.4, 26.1; HRMS (EI) calcd for $C_{13}H_{17}BrO_4$: 316.0310. Found: 316.0309.

2.1.5. 7-Bromo-2,2-dimethyl-4,5-bis-pent-4-enyloxy-3a,4,5,7a-tetrahydro-benzo[1,3]dioxole (11). To a solution of bromodiol **7** (0.73 g, 2.75 mmol) in anhydrous DMF (10 mL) cooled in an ice bath, NaH (0.66 g, 22 mmol) was added under argon atmosphere. The reaction mixture was allowed to stir for 1 h. 5-Bromopentene (3.28 g, 22 mmol) dissolved in DMF (10 mL) was then added dropwise, and the reaction mixture was stirred for 6 h. The reaction was quenched with water (5 mL) and extracted with diethyl ether (3×25 mL). The ethereal extracts were combined, dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography (hexanes–EtOAc, 9:1). Fractions containing monomer **11** were combined and concentrated to yield a yellowish oil, which was distilled under reduced pressure (155 °C, 0.2 mm Hg) using a Kugelrohr apparatus to furnish monomer **11** as a colorless oil (0.35 g, 32%). ^1H NMR (300 MHz, $CDCl_3$) δ 6.2 (d, *J*=3.05 Hz), 5.92–5.74 (m, 2H), 5.05 (*J*=2.05, 1.54 Hz, 1H), 5.03–4.92 (m, 3H), 4.63 (dd, *J*=1.03, 6.41 Hz, 1H), 4.21–4.12 (dd, *J*=6.41, 8.46 Hz, 1H), 3.86–3.67 (m, 3H), 3.65–3.51 (m, *J*=2.56, 5.64, 6.41, 6.67 Hz, 2H), 3.39 (t, *J*=8.1 Hz), 2.20–2.08 (m, 4H), 1.77–1.63 (m, 4H), 1.54 (s, 3H), 1.41 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.5, 138.2, 133.8, 118.8, 115.2, 114.8, 110.7, 80.0, 79.0, 78.0, 77.5, 72.3, 70.0, 30.4, 30.8, 29.5, 29.3, 28.3, 26.3; HRMS (EI) *m/z* calcd for $C_{19}H_{29}O_4Br$: 400.1249 Found: 400.1256.

2.1.6. ADMET polymerization of monomer 11. Freshly distilled monomer **11** (1.68 g, 4.19 mmol) was placed in a 10 mL round-bottomed flask equipped with a magnetic stir bar. The monomer was degassed by means of freeze-pump-thaw cycles under high vacuum ($<10^{-4}$ Torr). Grubbs' second-generation catalyst (35 mg, 1%) was then added under argon atmosphere. A few drops of dry $CDCl_3$ were added to initiate the polymerization process. After the addition of the catalyst, very slow to moderate bubbling of ethylene was observed. The bubbling reaction mixture was then exposed to intermittent vacuum until the viscosity increased, followed by exposure to high vacuum to remove ethylene, which is continuously generated during the course of the polymerization. The reaction was carried out at room temperature until increased viscosity prevented stirring, after which time the temperature was raised to 55 °C over a period of 5 days until a very high viscosity was obtained or bubbling ceased. The reaction mixture was then cooled to room temperature, and the reaction quenched by exposure to air. The viscous residue was dissolved in ethyl acetate and passed through a short column of silica to furnish a brown viscous oil (1.58 g), whose average molecular weight was estimated to be 20,000 by light-scattering analysis. $[\alpha]_D^{27}$=−12.1 (*c* 1.1, CH_3OH).

2.1.7. 2,2,7,7-Tetramethyl-5-pent-4-enyloxy-hexahydro-benzo[1,2-d;3,4-d′]bis[1,3]dioxol-4-ol (15). The diacetonide-protected epoxide **14** (1.0 g, 4.13 mmol) and 4-penten-1-ol (0.64 mL, 6.19 mmol) were dissolved in freshly distilled methylene chloride (15 mL), and the mixture cooled in an ice bath for 15 min. $BF_3{\cdot}Et_2O$ (10 mol%) was added, and the reaction was allowed to proceed overnight. The reaction was quenched with water, and the mixture was extracted with methylene chloride (3×25 mL). The organic layers were combined, dried (Na_2SO_4) and concentrated. The product was purified by flash chromatography (hexanes–EtOAc, 5:1) to yield alcohol **15** (0.794 g, 59%) as a colorless semi-solid material. R_f=0.25 (hexane–EtOAc, 5:1), $[\alpha]_D^{27}$=−29.4 (*c* 1.1, CH_3OH); IR (film) 3459, 2986, 1380, 1260, 1067, 1025 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.92–5.74 (m, 1H), 5.10–4.92 (m, 2H), 4.28–4.12 (m, 4H), 3.96–3.84 (m, 1H), 3.70–3.50 (m, 2H), 3.30–3.20 (m,

1H), 2.93 (s, exchanged with D_2O, 1H), 2.22–2.10 (m, 2H), 1.80–1.65 (m, 2H), 1.51 (s, 6H), 1.36 (s, 3H), 1.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.42, 115.11, 110.3, 110.0, 80.0, 79.3, 78.5, 76.9, 76.8, 71.5, 70,9, 30.5, 29.2, 27.9, 27.8, 25.4, 25.2; HRMS (EI) *m/z* calcd for $C_{16}H_{25}O_6$ $[M-CH_3]^+$: 313.1651. Found: 313.1649.

2.1.8. 2,2,7,7-Tetramethyl-4,5-bis-pent-4-enyloxy-hexahydro-benzo[1,2-d;3,4-d′]bis[1,3]dioxole (16). In a 10 mL round-bottomed flask NaH (169 mg, 4.2 mmol) was suspended in anhydrous DMF (5 mL). Alcohol **15** (138 mg, 0.42 mmol) dissolved in DMF (5 mL) was then added dropwise to the reaction flask and allowed to stir at room temperature for 45 min. 5-Bromopentene (0.62 mL, 4.2 mmol) was then added, and the reaction was allowed to proceed for 5 h. The reaction was quenched with H_2O (5 mL) and extracted with Et_2O (3×10 mL). The ethereal portions were combined, washed with saturated NaCl (10 mL), H_2O (10 mL), dried ($MgSO_4$), and concentrated. The product was purified by flash chromatography (hexanes–EtOAc, 9:1) to yield a thick red oil (96 mg, 93%). R_f=0.68 (hexane–EtOAc, 5:1), ^1H NMR (300 MHz, $CDCl_3$) δ 5.90–5.72 (m, 2H), 5.08–5.02 (m, 1H), 5.02–4.90 (m, 3H), 4.50–4.32 (m, 1H), 4.24–4.10 (m, 4H), 3.80–3.66 (m, 4H), 3.36–3.26 (m, 2H), 2.20–2.06 (m, 4H), 1.76–1.63 (m, 4H), 1.49 (s, 6H), 1.40 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.6, 114.8, 114.7, 112.3, 109.6, 80.3, 79.4, 77.3, 76.7, 75.6, 74.2, 71.7, 70.9, 30.4, 29.9, 29.5, 29.3, 28.0, 26.5, 25.6, 24.3.

2.1.9. ADMET polymerization of diene 16 to generate polymer 17. Monomer **16** (240 mg, 0.61 mmol) was placed in a 10 mL round-bottomed flask equipped with a stir bar and was degassed through several freeze-pump-thaw cycles under high vacuum ($<10^4$ Torr). Grubbs' first-generation catalyst (5 mg) was then added under argon atmosphere. After the addition of Grubb's catalyst, very slow to moderate bubbling of ethylene was observed. The bubbling reaction was then exposed to intermittent vacuum until the viscosity increased, followed by exposure to high vacuum to remove the ethylene, which was continuously generated during the course of the polymerization. The reaction was maintained at room temperature until the increase in viscosity prevented stirring. At this point, the reaction temperature was slowly raised to 45 °C over a period of 5 days until bubbling ceased. The reaction mixture was then cooled to room temperature, and quenched by exposure to air. The viscous residue was dissolved in ethyl acetate and passed through a short column of silica to furnish polymer **17** as a brown viscous oil. ^1H NMR (300 MHz, $CDCl_3$) δ 5.50–5.40 (b, 2H), 4.25–4.15 (b, 4H), 3.80–3.65 (m, 4H), 3.31 (s, 2H), 2.20–1.80 (b, 4H), 1.75–1.55 (m, 4H), 1.50 (s, 6H), 1.33 (s, 6H).

2.1.10. Deprotection of polymer 17. Polymer **17** was dissolved in 5 mL of a 4:1:1 mixture of THF, TFA and H_2O. The reaction was stirred at room temperature for 4 h. The reaction mixture was diluted with brine (3 mL) and extracted with ethyl acetate (4×10 mL). The organic fractions were combined, dried (Na_2SO_4), and concentrated. After the solvent was removed, the residue was introduced onto a silica-gel column and eluted with ethyl acetate to arrive at fully hydroxylated polymer **18**, whose molecular weight was estimated at 10,000 via GPC analysis. Light scattering analysis of polymer **18** estimated the average molecular weight of the polymer to be at around 18,000. $[\alpha]_D^{27}$=25.1 (c 2.1, CH_3OH).

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Chemo-enzymatic preparation of chiral 3-aminopyrrolidine derivatives

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Abstract—A new simple method for the enantioselective enzymatic hydrolysis of N-protected D-asparagine esters suitable for the use on the preparative scale is presented. Due to major obstacles observed under conventional reaction conditions—racemization of the retained ester and a strong enzyme inactivation—a comparatively low pH together with an organic co-solvent had to be employed. Under these conditions, nearly all tested proteases demonstrated good activity and excellent enantioselectivity giving access to the corresponding D-esters and L-asparagines in high optical purities (>95% ee) and good chemical yields (>40%). From the unnatural D-asparagine derivative, sequential cyclization, selective deprotection and reduction yielded efficiently benzyl protected (*R*)-3-aminopyrrolidine, a homo-chiral building block utilized in numerous drug candidates.

1. Introduction

Natural amino acids as well as their antipodes provide the source of chirality in the production of many enantiopure compounds and their efficient preparation is a continuing source of interest. The preparation of D-asparagine, for example, is conducted principally by the resolution of its racemate (or close derivatives) via chromatography,[1] diastereomeric salt separation[2] or industrially via whole cell biotransformation[3] (there are no asymmetric syntheses described).

Besides these classical resolution methods there exist further known enzymatic routes to enantiomerically pure D-asparagine derivatives: the common enzymatic hydrolysis of D,L-5-substitued hydantoins using a D-hydantoin hydrolase and a *N*-carbamoyl-D-amino acid hydrolase.[4] In addition, the newly discovered D-aminoacylase from the genera *Sebekia* and *Amycolatopsis* might be applied as catalysts for the enzymatic preparation of D-asparagine[5] though the purified enzymes have a specific activity towards *N*-acetyl-D-asparagine of only 1.4% and 19%, respectively, as compared to *N*-acetyl-D-methionine (100%).

However, surprisingly, the most conventional method, the enzymatic hydrolysis of racemic asparagine ester derivatives had remained unexplored to the best of our knowledge. Herein we describe the identification of active and selective hydrolases for the resolution of N-protected D,L-asparagine esters and the development of a scalable biotransformation under technically relevant conditions (Scheme 1).

2. Results and discussion

2.1. Screening of enzymatic hydrolysis activity and selectivity

The substrate esters were prepared from the racemic N-protected D,L-asparagines **3** and **4** by esterification with thionylchloride of the respective alcohol. All proteases tested displayed a reasonable activity with racemic ester **1a** in a well plate assay based on the color change of a pH-indicator. Re-evaluation of the four cheapest enzymes in a pH-stat experiment revealed only moderate selectivites (Table 1) when stopped after approximately 50% conversion. A repetition of these experiments at a higher conversion degree of approximately 55% did not result in an increased enantiomeric purity for the remaining ester **1a** (Table 1) as one would expect according to Sih and Girdaukas.[6]

2.2. pH-dependent racemization

As such modest enantioselectivities of proteases towards an amino acid ester substrate would be unusual, and based on the fact the ee-values for **1a** tended to be lower with prolonged reaction time (Fig. 1) we suspected racemization

Keywords: Resolution; D-asparagine; L-asparagine; Enzyme; Protease; Enantioselective; Hydrolysis; Biotransformation.
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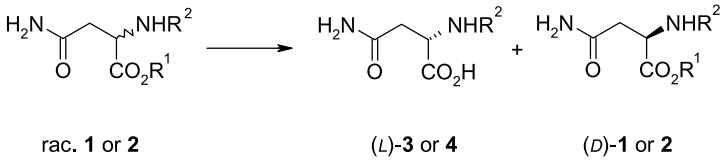


compound	R^1	R^2
1 a	Me	Cbz
1 b	Et	Cbz
1 c	Bu	Cbz
2 b	Et	Bz
2 d	Bn	Bz
3	-	Cbz
4	-	Bz

Scheme 1. Resolution of different N-protected D,L-asparagine esters.

Table 1. Screening for the resolution of *N*-benzyloxycarbonyl-D,L-asparagine methyl ester rac. **1a** with different proteases

Protease[a]	Conversion (%)	Reaction time (h)	ee of **1a** (%)	~*E*
ALC	50	0.6	82	25
NUE	50	1.0	79	20
PZ	50	1.2	77	18
SP539	50	20	57	6
ALC	55	5	75	9.5
NUE	54	10	63	6
PZ	55	9	74	9

[a] The abbreviations are given in Section 4.2. Reaction conditions were similar to General procedure (Section 4.4), except for (i) a lower substrate concentration of 30 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

of the remaining optical enriched ester to be a possible explanation.

Reducing the pH of the reaction resulted indeed in higher enantiomeric excess of **1a** as was demonstrated by the protease Alcalase, but, as expected, at the cost of enzymatic activity (data not shown). A pH of 6.5 was chosen as a suitable compromise between the stability of the remaining ester **1a** with respect to racemization and sufficient enzymatic activity. In addition, one has to avoid also acidic conditions during the work up of the enantiomerically pure ester **1a**, because we recognized a slow racemization of analytical samples at pH~2. The cheap bulk protease Alcalase and several other microbial proteases turned out to be highly enantioselective catalysts for the synthesis of enantiopure remaining methyl ester **1a** (Table 2).

After verifying the former working thesis that the proteases are highly enantioselective catalysts under reaction conditions preventing the racemization of the optically pure

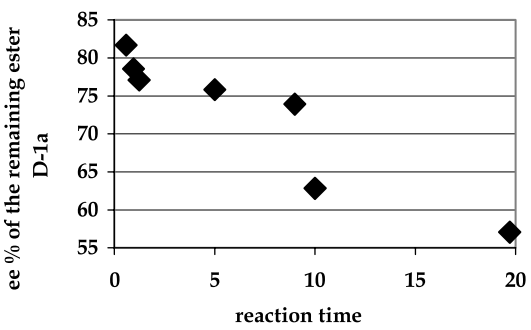


Figure 1. The enantiomeric excess of the retained methyl ester **1a** from different biotransformations (Table 1) in dependence of the reaction time.

ester, the transformation should be transferred to a preparative scale.

2.3. Enzyme inhibition at higher substrate concentrations

On a more preparative scale at higher substrate concentrations the enzymatic hydrolysis unexpectedly stopped after a few percent conversion with several proteases and ester substrates (rac. **1a**; **1b**, **1c**, **2b**; **2d**). The enzyme inhibition seemed to be a robust feature in this biocatalytic conversion of asparagine derivatives and could be observed already at a substrate concentration of 2% (Table 3).

When we tried to overcome the poor substrate solubility by adding an organic co-solvent we found—again unexpectedly—that this inactivation was overcome in the presence of the co-solvent. The addition of an organic co-solvent like THF in a concentration of ~16% before addition of the enzyme eliminated inhibition previously encountered with the different substrates and proteases. The effect was observed with water- miscible and immiscible solvents. Interestingly, the inhibition seems to be irreversible. In control experiments with addition of an organic co-solvent after the inhibition had set in the enzyme activity was not recovered. Therefore, the prevention of the inhibition can not be explained with substrate solubilization as mode of action of the solvent (Table 3).

The different organic co-solvents affected the activity of the

Table 2. Preparation of enantiopure remaining methyl ester **1a** with different proteases at approximately 50% conversion

Protease[a,b]	Microbial origin	Reaction time (h)	ee of **1a** (%)
ALC	*Bacillus licheniformis*	0.4[b]	>99
SAV	*Bacillus*	0.8[b]	>99
EA	not defined	2.0[b]	>99
FGF	*Bacillus subtilis*	1.4[b]	>99
BPN	*Bacillus subtilis*	0.5[a]	>99
NUE	not defined	2.3[a]	>99
K	*Tritirachium album*	2.1[a]	94
PZ	*Aspergillus melleus*	1.5[a]	98

[a] The abbreviations are given in Section 4.2. Reaction conditions were similar to General procedure (Section 4.4), except for (i) a lower substrate concentration of 30 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

[b] The abbreviations are given in Section 4.2. Reaction conditions were similar to General Procedure (Section 4.4), except for (i) a lower substrate concentration of 15 mg rac. **1a**, (ii) a protease aliquot of only 3 mg lyophilisate or 30 μl solution and (iii) a pH of 7.0 was used.

Table 3. Enzyme inhibition and the influence of the addition of an organic co-solvent

#	Protease	Organic co-solvent	Compound	Amount enzyme	Substrate concentration (%)	Conversion[a] (%)	Reaction time (h)	ee ester (%)
a	ALC	—	**1a**	100 μL	5	13	Reaction stops, no work up	
b	ALC	Ethanol	**1a**	100 μL	5	48	22	98
c	ALC	Ethyl acetate	**1a**	100 μL	5	54	3.5	>99
d	ALC	TBME	**1a**	100 μL	5	48	5.5	>99
e	ALC	Acetone	**1a**	100 μL	5	49	5.0	>99
f	SAV	—	**1a**	100 μL	5	<1	Reaction stops, no work up	
g	SAV	THF	**1a**	100 μL	5	50	18	>99
h	K	—	**1b**	4.0 mg	2	7	Reaction stops, no work up	
I	K	THF	**1b**	4.2 mg	2	48	3.2	97
j	PSG	—	**1b**	4.0 mg	2	5	Reaction stops, no work up	
k	PSG	THF	**1b**	4.0 mg	2	48	12	92
l	ALC	—	**2b**	50 μL	2	10	Reaction stops, no work up	
m	ALC	THF	**2b**	50 μL	2	53	17	97
n	SAV	—	**2b**	50 μL	2	11	Reaction stops, no work up	
o	SAV	THF	**2b**	50 μL	2	45	46	93
p	ALC	—	**1c**	50 μL	2	3	Reaction stops, no work up	
q	ALC	Dioxane	**1c**	50 μL	2	51	48	98
r	ALC	—	**2d**	50 μL	2	14	Reaction stops, no work up	
s	ALC	Acetone	**2d**	50 μL	2	47	10	>99

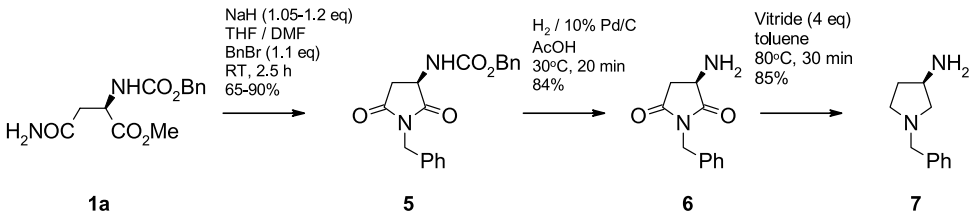
[a] The conversion was calculated from the base consumption in the pH-static experiments. Reaction conditions, see General procedure (Section 4.4). The abbreviations are explained in Section 4.2.

proteases with the methyl ester **1a**, but the selectivity remained nearly absolute. Ethanol as co-solvent reduced the activity roughly four times stronger as compared to the best water-miscible solvent acetone. The water-immiscible solvent TBME provided nearly the same activity as acetone. Homogeneity of the transformation mixture did not seem to play a role: the bi-phasic co-solvent ethyl acetate afforded the highest activity (Table 3). As a further result of the experiments shown in Table 3, the methyl ester **1a** turned out to be the best substrate with respect to enzymatic activity.

Nevertheless, different esters (**1a**; **1b**; **1c**; **2b** and **2d**) were synthesized in gram-amounts with high enantiomeric purities (>95% ee) and in a good chemical yields (>40%). Besides, also the formed acids **3** and **4** were isolated in high optical purities (>99% ee) and good chemical yields (>40%). The separation and isolation of the enantiomerically pure reaction products, D-esters and L-acids, was achieved by conventional extraction.

On the preparative scale substrate concentrations above 7% (overall) became problematic due to the viscosity of the suspension formed. Therefore, an even larger scale the stirring conditions are expected to be a critical issue.

With these esters in hand, it was possible to elaborate them into key precursors, for example ester **1a** was submitted to sequential cyclization, selective deprotection and reduction yielding efficiently the benzyl protected (*R*)-3-amino-pyrrolidine,[7] a homo-chiral building block utilized in the preparation of numerous drug candidates (Scheme 2).[8] In particular, we have exploited the use of this pyrrolidine in the assembly of a broad spectrum cephalosporin.[7a] Although each step had some literature precedent, some modifications were necessary; thus although a one-pot cyclization and benzylation of ester **1a** to benzylmide **5** has been described for its enantiomer (aq. NaOH/BnBr/cat. NBu_4I),[9] it proved not possible to perform this procedure without incurring significant racemization (47% yield, 55.4% ee). However replacing the base with NaH gave only minor racemization which could be rectified by one re-crystallization (65% yield, >99.9% ee). Additionally deprotection of the Cbz-protected imide **5** had been described for its enantiomer using $Pd(OH)_2$[9] or Pd/C[10] in MeOH. We found no conversion using these systems; however employing AcOH as the solvent, allowed the hydrogenation to proceed smoothly in just 20 min at room temperature, facilitating the isolation of the unstable amine **6** as the corresponding acetate salt in 84% yield, which could be stored without problems. Carefully controlled liberation of the free amine at pH 8 (quantitative) and immediate reduction with Vitride in toluene (borane led to decomposition and $LiAlH_4$ gave isolation problems) resulted in the recovery of the desired amine **7** in 87% yield with no racemization.



Scheme 2. Synthesis of *N*-benzyl (*R*)-3-aminopyrrolidine from enantiopure ester **1a** via cyclyzation, selective deprotection and reduction.

3. Conclusion

An attractive chemo-enzymatic route for the preparation of N-protected (*R*)-3-aminopyrrolidines has been established based on an efficient racemic resolution of N-protected D,L-asparagine esters with proteases as the key step. Beside the retained D-esters also the respective L-acids were obtained in high enantiomeric purity and chemical yield by this enzymatic step. The enzyme inhibition encountered at higher substrate concentrations could be overcome by application of an organic co-solvent. The reaction has been successfully carried out on the multi-gram scale.

4. Experimental

4.1. General

NMR-spectra: Bruker DPX 400 MHz. IR-spectra: Nicolet, FT-IR 20 SXB. EI-MS-spectra: SSQ7000 (Finnigan MAT). EI-, ISN- and ISP-MS-spectra. Optical rotations: Perkin–Elmer Polarimeter 241.

4.2. Materials

The proteases used for the preparative experiments were Savinase 6.0T (*Bacillus*, *SAV*); *SP539* (*Bacillus*); Alcalase 2.4L (*Bacillus sp.*, *ALC*); *NUE* 6.0 S, Typ 1; Esperase 6.0 T (*EA*) from Novo Nordisk, subitilsin *BP N*(Nagarse) from Sigma, Protase *K* (*Tritichium album*); Pronase E (*Streptomyces griseus*, *PSG*) from Fluka, Prozyme 6 (*Aspergillus melleus*, *PZ*); Proleather *FG-F* from Amano. All other reagents were purchased from Fluka or Merck and used directly.

4.2.1. *N*-Benzyloxycarbonyl-D,L-asparagine methyl ester 1a. A suspension of 40.0 g (150.2 mmol) *N*-benzyloxycarbonyl-D,L-asparagine **3** [Bachem C-1310] in 500 mL methanol was cooled to 0 °C. Then 34.0 mL (462.6 mol, 3.08eq.) of thionyl chloride was added dropwise so that the temperature remained below 10 °C. The mixture was stirred at 0 °C for another 15 min. The reaction mixture was evaporated together with 1 L toluene (after 500 mL of volume was reached, 200 mL methanol was added and evaporation continued; 40 °C bath temperature). The resulting white, crystalline residue was triturated/digested overnight in 600 mL TBME. The residue was filtered off and dried at HV to give 38.45 g of the rac. methyl ester **1a** as a white crystalline powder (yield: 91%). Analytics: SFC: 99.5% area. EI-MS: 280.1, 221.2 ($M-CO_2Me$), 173.1 ($M-COPh$). IR (Nujol) (NH) 3318, (CO_2Me) 1742, (NCO_2) 1682, (NCO) 1665 and 1558, 1219, 757, 698 cm^{-1}. ^1H NMR ($CDCl_3$): 2.76 (dd, J=4, 17 Hz, 1H, CH), 2.98 (dd, J=4, 17 Hz, 1H, CH), 3.76 (s, 3H, OCH_3), 4.60 (m, 1H, CHCOO), 5.13 (s, 2H, $-CH_2O$), 5.45 and 5.55 (2×bs, 2H, $CONH_2$), 6.00 (bd, $J\sim8$ Hz, <1H, OCONH), 7.26–7.37 (m, 5H, Ph).

4.2.2. *N*-Benzyloxycarbonyl-D,L-asparagine ethyl ester 1b. Similar to the above (Section 4.2.1) the racemic crystalline ethyl ester **1b** was prepared with a yield of 79%. Analytics: HPLC: >99% area. ISP-MS: 317.2 ($M+Na^+$), 295.3 ($M+H^+$). ^1H NMR (DMSO): 1.15 (t, J=6 Hz, 3H, CH_3), 2.42–2.58 (m, ~2H, CH_2), 4.07 (q, J=6 Hz, 2H, CH_2O), 4.39 (m, 1H, CH), 5.03 (s, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.31–7.37 (m, 6H, Ph and $CONH_2$), 7.60 (d, $J\sim8$ Hz, 1H, CONH).

4.2.3. *N*-Benzyloxycarbonyl-D,L-asparagine butyl ester 1c. Similar to the above (Section 4.2.1) the racemic solid butyl ester **1c** was prepared with a yield of 67%. Analytics: HPLC: >99% area. ISP-MS: 345.3 ($M+Na^+$), 323.3 ($M+H^+$). ^1H NMR (DMSO): 0.87 (t, J=6 Hz, 3H, CH_3), 1.31 (m, 2H, CH_2), 1.51 (m, 2H, CH_2), 2.42–2.59 (m, ~2H, CH_2), 4.03 (m, 2H, CH_2O), 4.40 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.29—7.39 (m, 6H, Ph and $CONH_2$), 7.60 (d, $J\sim4$ Hz, 1H, CONH).

4.2.4. *N*-Benzoyl-D,L-asparagine 4. A solution of 20.0 g (0.15 mol) D,L-asparagine, 120 mL deionized H_2O, 20 g sodium carbonate and 20 mL THF was heated to 55 °C. A solution of 20 mL THF and 21 mL of benzoyl chloride (0.18 mol) was added dropwise during 2 h at 55 °C. The reaction mixture was stirred for another 1 h and then cooled to 15 °C. The pH was adjusted to 2.5 and the reaction mixture was stirred for 0.5 h at 15 °C. The crystalline asparagine **4** was filtered, washed thrice with 75 mL H_2O and dried at HV. The yield was 32.4 g (87%). Analytics: HPLC: 95.9% area. ISN-MS: 235.2 ($M-H^-$), 217.2 ($M-H_2O$). ^1H NMR (DMSO): 2.50–2.72 (m, 2H, CH_2), 4.72 (m, 1H, CH), 6.94 (bs, 1H, $CONH_2$), 7.39 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.64 (d, J=8 Hz, 1H, CONH), 12.6 (s, 1H, COOH).

4.2.5. *N*-Benzoyl-D,L-asparagine ethyl ester 2b. Similar to the above (Section 4.2.1) the racemic crystalline ethyl ester **2b** was prepared with a yield of 65%. Analytics: HPLC: >99% area. EI-MS: 264.2 (M), 191.2 ($M-COOEt$). ^1H NMR (DMSO): 1.16 (t, J=8 Hz, 3H, CH_3), 2.57–2.72 (m, 2H, CH_2), 4.09 (q, J=8 Hz, 2H, CH_2O), 4.75 (m, 1H, CH), 6.95 (bs, 1H, $CONH_2$), 7.40 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.75 (d, J=8 Hz, 1H, CONH).

4.2.6. *N*-Benzoyl-D,L-asparagine benzyl ester 2d. Similar to the above (Section 4.2.1) the racemic crystalline benzyl ester **2d** was prepared with a yield of 33%. Analytics: HPLC: >99% area. ISP-MS: 349.4 ($M+Na^+$), 327.3 ($M+H^+$). ^1H NMR (DMSO): 2.61–2.78 (m, 2H, CH_2), 4.84 (m, 1H, CH), 5.14 (s, 2H, OCH_2), 6.98 (bs, 1H, $CONH_2$), 7.30–7.85 (m, 11H, 2xPh and $CONH_2$), 8.84 (d, J=8 Hz, 1H, CONH).

4.3. Activity assay

Into 96 well plates pre-loaded with 0.5 mg enzyme/well a buffered pH-indicator solution (190 μl, 7.5 mM Tris/HCl, pH 8.0, 0.02% NaN_3, 50 mg/l cresol red) and the substrate solution (1 mg **1a** in a mixture of 2.5 μl DMF and 7.5 μl EtOH) were added with a liquid handler (Lissy; Zinsser Analytics). The color change of the indicator from red to yellow was monitored for up to 1 day at 410 nm with a well plate reader (Tecan Sunrise) and a well plate autosampler (Twister).

4.4. General procedure for the preparation of different N-protected D-asparagine esters (cf. Table 3)

A suspension of 0.5 g (1.76–1.53 mmol) [2 g (7.0 mmol)] *N*-protected asparagine ester in 23 mL [28 mL] 0.1 M sodium chloride solution, 2 mL [4 mL] 0.1 M sodium phosphate buffer pH 7.0 and in the presence or absence (Tables 1 and 2) of 4 mL [8 mL] organic co-solvent was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of the protease (Table 3). The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. The experiments without organic co-solvent showing incomplete conversion (Table 3) were not worked up, and the experiments with organic co-solvent were treated as follows: After approximately 50% conversion the reaction mixture was extracted with 2×25 mL [3×30 mL] dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate and evaporated at 35 °C. The residue containing the retained ester was subjected to ee-determination (methods see the corresponding experiments on a larger scale).

4.4.1. *N*-Benzyloxycarbonyl-D-asparagine methyl ester 1a. A suspension of 18.5 g (65.7 mmol) methyl ester rac. **1a** (99.5%) in 190 mL 0.1 M sodium chloride solution, 40 mL 0.1 M sodium phosphate buffer pH 6.5 and 25 mL THF was vigorously stirred. 1.0 mL Alcalase 2.4 L was added and the pH maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. The hydrolysis was terminated in 8 h with a consumption of 32.0 mL (49% conversion) of titrating agent. After 16 h the reaction mixture was extracted trice with 350 mL dichloromethane. The combined organic phases were washed with 500 mL 20 mM sodium phosphate buffer pH 7.0, dried on anhydrous sodium sulfate and evaporated (at 25 °C bath temperature). The residue was triturated overnight in 200 mL TBME. The suspension was filtered and the filter cake dried at HV to give 8.2 g the methyl ester **1a** as a white crystalline powder (yield: 46%). Enantiomeric excess: >99.5% (Chiracel ODH, 15 cm×300 μm, 85% *n*-hexane+15% isopropanol, 6 μl/min, 30 °C, 210 nm). $[\alpha]_D$=+0.97 (*c*=1.6; AcOH). SFC: 98.8% area. EI-MS: 280.2 (M), 221.1 (M−CO_2Me). IR (Nujol): (NH) 3349, (CO_2Me) 1741, (NCO_2) 1665, (NCO) 1550, 733, 698 cm^{-1}. ^{1}H NMR (CDCl3): 2.76 (dd, *J*=4, 16 Hz, 1H, CH), 2.98 (dd, *J*=4, 16 Hz, 1H, −CH), 3.76 (s, 3H, OCH_3), 4.60 (m, 1H, $CHCO_2$), 5.13 (s, 2H, CH_2O), 5.42 and 5.53 (2×bs, 2H, $CONH_2$), 6.00 (bd, *J*~8 Hz, <1H, OCONH), 7.29–7.37 (m, 5H, Ph).

4.4.2. *N*-Benzyloxycarbonyl-L-asparagine 3. In analogy to Section 4.4.1 10.0 g (35.1 mmol) of the methyl ester rac. **1a** (98%) was suspended in 140 mL 0.1 M sodium chloride solution, 20 mL 0.1 M sodium phosphate buffer pH 6.5 and 40 mL THF under vigorous stirring. 0.5 mL Alcalase 2.4 L was added and the pH maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 16.42 mL of 1.0 N sodium hydroxide solution after 2.1 h (corresponds to 47% conversion) the reaction mixture was extracted with 3×200 mL dichloromethane to remove the uncleaved methyl ester. The aqueous phase was concentrated to approximately 50 mL volume using 200 mL of toluene. The pH was adjusted to 3.5 with 25% hydrochloric acid. The formed precipitate was filtered off and triturated overnight in 300 mL of deionized water. The suspension was filtered and the filter cake dried on HV to give 4.07 g (44%) of **3** as white crystals. Analytics: enantiomeric excess: >99% (Chiracel ODH, 25 cm×4.6 mm, 85% *n*-heptane+15% isopropanol, 0.8 mL/min, room temperature, 220 nm). $[\alpha]_D$=+5.4 (*c*=2.0; AcOH)[†] ($[\alpha]_D$=−6.7 (*c*=1.1; DMSO) determined in another experiment). HPLC: >99% area. ISN-MS: 265.3 ($M-H^-$). IR (Nujol): (NH) 3337, (CO_2H) 1697, (NCO_2) 1643, 1536, 1268, 737, 695 cm^{-1}. ^{1}H NMR (DMSO): 2.41–2.58 (m, 2H, CH_2), 4.34 (m, 1H, CH), 5.03 (s, 2H, CH_2O), 6.92 (bs, 1H, $CONH_2$), 7.26–7.40 (m, 6H, Ph and $CONH_2$), 7.44 (bd, *J*~8 Hz, 1H, OCONH), 12.67 (bs, 1H, −COOH).

4.4.3. *N*-Benzyloxycarbonyl-D-asparagine ethyl ester 1b. A suspension of 10.0 g (34.0 mmol) ethyl ester rac. **1b** in 460 mL 0.1 M sodium chloride solution, 40 mL 0.1 M sodium phosphate buffer pH 7.0 and 80 mL THF was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 1.0 mL Savinase 16 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 16.45 mL 1.0 N sodium hydroxide solution (48.2% conversion; after 17 h) the reaction mixture was extracted with 2×500 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue dried on HV to give 5.02 g of the ethyl ester **1b** as white crystals (yield: 50%). Enantiomeric excess: 98.9% (Chiracel ODH, 15 cm×2.1 mm, 87% *n*-heptane+13% isopropanol+0.1% TFA, 100 μl/min, room temperature, 220 nm). $[\alpha]_D$=+12.2 (*c*=1.0; EtOH). HPLC: >99% area. ISP-MS: 317.2 ($M+Na^+$), 295.3 ($M+H^+$). ^{1}H NMR (DMSO): 1.16 (t, *J*=8 Hz, 3H, CH_3), 2.42–2.58 (m, ~2H, CH_2), 4.07 (q, *J*=8 Hz, 2H, CH_2O), 4.39 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.31–7.37 (m, 6H, Ph and $CONH_2$), 7.60 (d, *J*~8 Hz, 1H, CONH).

4.4.4. *N*-Benzyloxycarbonyl-D-asparagine *n*-butyl ester 1c. A suspension of 4.0 g (12.41 mmol) *n*-butyl ester rac. **1c** in 180 mL 0.1 M sodium chloride solution, 16 mL 0.1 M sodium phosphate buffer pH 7.0 and 30 mL dioxan was vigorously stirring. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 400 μl Alcalase 2.4 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After 66 h and 87 h an additional 200 μl of Alcalase-solution was added to the reaction. After a total consumption of 5.73 mL 1.0 N sodium hydroxide solution (46% conversion; after totally 91 h) the reaction mixture was extracted with 2×200 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue triturated overnight in 50 mL TBME. The solid was filtered off, the filter cake washed with TBME and dried on HV to give 1.61 g of the butyl ester **1c** as white solid (yield: 40%). Enantiomeric excess: 95% (Chiracel ODH, 15 cm×0.3 mm, 90% *n*-heptane+10% isopropanol, 5 μl/min, 30 °C, 210 nm). $[\alpha]_D$=+19.9 (*c*=1.0; DMSO). HPLC:

98.9% area. ISP-MS: 345.3 ($M+Na^+$), 323.3 ($M+H^+$). ^1H NMR (DMSO): 0.87 (t, J=8 Hz, 3H, CH_3), 1.31 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 2.43–2.59 (m, ~2H, CH_2), 4.03 (m, 2H, CH_2O), 4.40 (m, 1H, CH), 5.03 (m, 2H, CH_2O), 6.93 (bs, 1H, $CONH_2$), 7.29–7.39 (m, 6H, Ph and $CONH_2$), 7.60 (d, J~12 Hz, 1H, CONH).

4.4.5. *N*-Benzoyl-D-asparagine ethyl ester 2b. A suspension of 3.5 g (13.2 mmol) ethyl ester rac. **2b** in 55 mL 0.1 M sodium chloride solution, 5 mL 0.1 M sodium phosphate buffer pH 7.0 and 8 mL THF was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 350 μl Alcalase 2.4 L The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-stat) of 1.0 N sodium hydroxide solution. After a consumption of 6.44 mL 1.0 N sodium hydroxide solution (49% conversion; after 23 h) the reaction mixture was extracted with 3×50 mL dichloromethane and 3×50 mL ethyl acetate. The combined organic phases were dried on anhydrous sodium sulfate, evaporated at 35 °C and the residue dried on HV to give 1.63 g of the ethyl ester **2b** as a white solid (yield: 47%). Enantiomeric excess: 98.2% (Chiralpak-AD, 25 cm×4.6 mm, 75% *n*-heptane+25% EtOH+0.2% TFA, 1 mL/min, room temperature, 220 nm). $[\alpha]_D$=+12.0 (c=1.1; DMSO). HPLC: >99% area. ISP-MS: 287.1 ($M+Na^+$), 265.3 ($M+H^+$). ^1H NMR (DMSO): 1.17 (t, J=8 Hz, 3H, CH_3), 2.57–2.72 (m, 2H, CH_2), 4.19 (q, J=8 Hz, 2H, CH_2O), 4.75 (m, 1H, CH), 6.95 (bs, 1H, $CONH_2$), 7.40 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.75 (d, J=8 Hz, 1H, CONH).

4.4.6. *N*-benzoyl-D-asparagine benzyl ester 2d and *N*-benzoyl-L-asparagine 4. A suspension of 2.50 g (7.66 mmol) benzyl ester rac. **2d** in 115 mL 0.1 M sodium chloride solution, 10 mL 0.1 M sodium phosphate buffer pH 7.0 and 20 mL acetone was vigorously stirred. The pH was adjusted to 6.5 with 1.0 N hydrochloric acid and the reaction started by addition of 250 μl Alcalase 2.4 L. The pH was maintained at 6.5 under vigorous stirring by the controlled addition (pH-static) of 1.0 N sodium hydroxide solution. After a consumption of 3.479 mL 1.0 N sodium hydroxide solution (46% conversion; after 17.9 h) the reaction mixture was extracted with 2×125 mL dichloromethane. The combined organic phases were dried on anhydrous sodium sulfate, evaporated (at 35 °C bath temperature) and the residue triturated in 20 mL TBME overnight. The solid was filtered off and dried on HV to give 1.10 g of the benzyl ester **2d** as a white solid (yield: 44%). Enantiomeric excess >99% (Chiracel-ODH, 15 cm×2.1 mm, 87% *n*-heptane+13% iPrOH+0.1% TFA, 0.1 mL/min, room temperature, 220 nm). $[\alpha]_D$=+13.2 (c=1.2; DMSO). HPLC: >99% area. ISP-MS: 349.5 ($M+Na^+$), 327.3 ($M+H^+$). ^1H NMR (DMSO): 2.61–2.78 (m, 2H, CH_2), 4.84 (m, 1H, CH), 5.14 (s, 2H, OCH_2), 6.97 (bs, 1H, $CONH_2$), 7.32–7.84 (m, 11H, 2×Ph and $CONH_2$), 8.83 (d, J=8 Hz, 1H, CONH).

The aqueous phase was acidified to pH 2 with 25% hydrochloric acid. The formed precipitate was stirred at 1 °C overnight and filtered off. The filter cake was washed with 10 mL 10 mM hydrochloric acid and dried on HV to give 0.77 g of **4** as a white powder (yield: 43%). Enantiomeric excess: >99% (Chiralpak-AD, 25 cm×4.6 mm, 85% *n*-heptane+15% EtOH+0.12% TFA, 0.7 mL/min, room temperature, 220 nm). $[\alpha]_D$=−16.5 (c=1.0; DMSO). HPLC: 99% area. ISN-MS: 235.2 ($M-H^-$). ^1H NMR (DMSO): 2.57–2.72 (m, 2H, $-CH_2-$), 4.72 (m, 1H, −CH−), 6.94 (bs, 1H, $CONH_2$), 7.39 (bs, 1H, $CONH_2$), 7.46–7.85 (m, 5H, Ph), 8.64 (d, J=8 Hz, 1H, −CONH−), 12.6 (s, 1H, COOH).

4.4.7. (*R*)-(1-Benzyl-2,5-dioxo-pyrrolidin-3-yl-carbamic acid benzyl ester 5. A suspension of 1.12 g of 60% NaH in 75 mL of THF is treated with 7.50 g of methyl ester **1a** (99.9% (*R*)-isomer) over 5 min at room temperature. After 20 min, 3.57 mL of benzyl bromide was added followed by 120 mL of DMF. After 3 h, HPLC indicated conversion was complete. The reaction was quenched with 150 mL H_2O and extracted three times with 120 mL of toluene. The organic layer was washed with H_2O, dried over $MgSO_4$, filtered and the filtrate evaporated to dryness. The residue was triturated in 100 mL of TBME, the resultant suspension filtered and dried (35 °C/10 mbar) to give 8.13 g (90%) of the pure benzyl imide **5** as white crystals. Analytics: enantiomeric excess: 93% (Chiracel ODH, 15 cm×300 μm, 85% *n*-hexane+15% isopropanol, 6 μl/min, 30 °C, 210 nm). SFC: 100% area. Mp 143.3–144.5 °C (lit. 140.5 °C).[11] SFC: 100% area. ISP-MS: 339.2 ($M+H^+$). IR (Nujol): (NH) 3321, (NCO) 1773, 1706, (CO_2Bu) 1685 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): 2.77 (dd, J=18, 4 Hz, 1H, $COCH_2$), 3.05 (dd, J=18, 9 Hz, 1H, $COCH_2$), 4.27 (m, 1H, NCH), 4.70 and 4.65 (2× d, J=12 Hz, 2H, NCH_2), 5.08 (bs, 2H, OCH_2), 5.23 (bd, 1H, NH), 7.37–7.25 (m, 10H, Ph). Optically pure material could be obtained from crystallization from CH_2Cl_2/*n*-hexane, 72% recovery. Analytics: enantiomeric excess 99.9%. Mp 145.9–146.7 °C. Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.36; N, 8.28. Found: C, 67.25; H, 5.27; N, 8.29.

4.4.8. (*R*)-3-Amino-1-benzyl-pyrrolidine-2,5-dione diacetic acid salt 6. A solution of 7.80 g of the benzyl imide **5** (93% (*R*)-isomer) in 160 mL of acetic acid was treated with 0.78 g of 10% Pd/C (Degussa 1835) and hydrogenated at 30 °C for 20 min whereupon TLC and HPLC indicated the reaction was complete. The reaction mixture was filtered, evaporated and the residue crystallized from EtOAc and *n*-hexane to give 5.80 g (78%) of the pure amine acetate salt **6** as white crystals. Analytics: enantiomeric excess: 91% as trifluoroacetamide (GC (BGB-177): 15 m×0.25 mm, carrier gas: He; program: 150 °C-200 °C at 1 °C /min; injector temp. 210 °C; FID: 220 °C). HPLC 100% area. GC 99.8% area (as free amine). ISP-M.S: 205.2 ($M+H^+$). ^1H NMR ($CDCl_3$, 1.6 equiv. AcOH) 2.08 (s, 2×CH_3CO_2, 6H), 2.50 (dd, J=18, 5.4 Hz, $COCH_2$, 1H), 3.05 (dd, J=7.8, 18 Hz, $COCH_2$, 1H), 3.92 (dd, J=5.4, 7.8 Hz, 1H, NCH), 5.64 (bs, 4H, NH), 4.65 (s, 2H, $PhCH_2$, 7.32 (m, 5H, Ph). MA (1.6 equiv. AcOH): Calcd for $C_{11}H_{12}N_2O_2$. 1.6 equiv. $C_2H_4O_2$: C, 56.79; H, 6.18; N, 9.33. Found: C, 56.66; H, 6.33; N, 9.23.

4.4.9. (*R*)-1-Benzyl-pyrrolidin-3-ylamine 7. A solution of 10.87 g of the amine salt **6** in 100 mL of H_2O was treated with 100 mL of CH_2Cl_2 followed by 67.60 mL of 1 N NaOH at room temperature to pH 8.0. After saturation with NaCl, the mixture was extracted seven times with 100 mL of CH_2Cl_2, dried over $MgSO_4$ and evaporated at 35 °C/10 mbar to give 6.32 g (97%) of the free base as a pale

yellow solid. NMR ($CDCl_3$, 250 MHz): 2.43 (dd, J=5, 17.5 Hz, 1H, $COCH_2$), 3.04 (dd, J=7.5, 17.5 Hz, 1H, $COCH_2$), 3.88 (dd, J=5, 7.5 Hz, 1H, NCH), 4.64 (s, 2H, $PhCH_2$), 7.30 (m, 5H, Ph).

5.90 g of this yellow oil was treated at 0 °C over 20 min with 33 mL of a 3.5 M solution of Vitride in toluene and the resultant yellow–orange solution was warmed to 80 °C for 30 min (MS indicated the reaction was complete), cooled to 0 °C and treated with 80 mL of 1 N NaOH solution. The phases were separated and the aqueous phase extracted with two further portions of 15 mL toluene. The combined organic phases were washed with 76 mL 1 N NaOH, 70 mL brine, dried and evaporated to give 4.42 g (87%) of the NMR pure amine **7**, as a light brown oil. Analytics: enantiomeric excess 93% as trifluoroacetamide (GC (BGB-177): 15 m×0.25 mm, carrier gas: He; program: 150–200 °C at 1 °C/min; injector temp. 210 °C; FID: 220 °C). GC 97% area. ISP-MS: 177.1 ($M+H^+$). IR (Film): (NH) 3357, (NCH) 2789. ^1H NMR ($CDCl_3$, 250 MHz): 1.45 (bm, 3H, NH_2 and CH_2), 2.15 (m, 1H, CH_2), 2.24 (dd, J=4.5, 9.5 Hz, 1H, NCH_2), 2.44 (m, 1H, NCH_2), 2.69 (m, 2H, 2×NCH_2), 3.48 (m, 1H, $CHNH_2$), 3.60 (dd (AB), 2H, $PhCH_2$), 7.28 (m, 5H, Ph).

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An electrokinetic bioreactor: using direct electric current for enhanced lactic acid fermentation and product recovery

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Abstract—The microbiological production of organic acids by fermentation processes is growing in commercial importance. However, the removal of product and pH control are two main issues that limit the technical and commercial viability of such processes. A laboratory scale bioreactor combining conventional electrodialysis and bipolar membrane electrodialysis has been developed for in situ product removal and pH control in lactic acid fermentation. The electrokinetic process enabled removal of the biocatalytic product (lactic acid) directly from the bioreactor system, in a concentrated form, as well as enabling good pH control without generation of troublesome salts. Moreover, end-product inhibition of glucose catabolism was reduced, resulting in a greater generation of the end-product lactic acid. An automatic pH sensor and current application system was developed and successfully implemented for lactic acid fermentation in the electrokinetic bioreactor.

1. Introduction

Lactic acid has been produced commercially by fermentation since 1883.[1] The major application of lactic acid is in the food industry as an additive and preservative. Other applications include use as a pharmaceutical intermediate, lactate ester, which is an alternative solvent to glycol ether. Lactic acid-derived polymers are becoming increasingly important because of their application within drug delivery systems and their biodegradable and thermoplastic nature means that they can be produced as high volume biodegradable plastics for packaging and other applications. However, this potential can only be realised if the cost of production is competitive on a global scale.[2–5]

Lactic acid is produced microbially from a variety of feedstocks by fermentation using lactic acid bacteria (*Lactobacilli*). In the conversion of glucose to lactic acid by homofermentative *Lactobacilli*, two molecules of lactic acid are produced for every molecule of glucose consumed. pH control of this organic acid fermentation is crucial; in the absence of such pH control, the final lactic acid concentration may be less than half of that obtained with pH control.[6] Conventionally, calcium carbonate has been used to neutralize the pH during lactic acid fermentation, producing calcium lactate. Ammonium hydroxide and sodium hydroxide have also been used for neutralization, however the cost of sodium hydroxide for the neutralization of the process is dominant in the operational cost in the fermentation step.[7] As a result of pH neutralization, the product is an organic acid salt. After fermentation, the process of separating the product from the medium and converting the salt to an organic acid is complicated, involving precipitation and acidification using a mineral acid (sulfuric acid). These steps contain the major economic hurdles for organic acid production. Moreover, the processing produces large quantities of an effluent containing high concentrations of salts. For instance, in the case of calcium carbonate neutralization, one ton of gypsum by-product will be produced for every ton of lactic acid produced.[2]

A low cost, environmentally sustainable process for organic acid downstream processing is desirable. Many investigations have been carried out into the separation, concentration and purification of organic acids from fermentation broth.[1,2] Among these, conventional electrodialysis has been developed for the separation, purification and concentration of organic acid salts from a fermentation broth, leading to economic product formation and low environmental impact of the downstream processing. Conventional electrodialysis is a membrane separation technology that uses ion-exchange membranes under the influence of direct current for separating and concentrating ions in solution. It has been widely used for separating and concentrating organic acid salts from fermentation broths.[2,8] Bipolar

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electrodialysis involves water splitting within a bipolar membrane, which is a laminate of cation and anion exchange membranes, with the efficient generation of protons and hydroxyl ions, thus producing acid and base from the salt solution with high energy efficiency.[9–11] Bipolar electrodialysis, integrating conventional electrodialysis with bipolar membrane water splitting, provides a potentially attractive complement to organic acid fermentation, enabling the separation, purification and concentration of salts and converting these salts into acid and base without producing effluents containing high concentrations of salts or gypsum, thereby avoiding discharge to the environment. Another advantage of bipolar membrane electrodialysis is that the base produced can be recycled and used for neutralization of the fermentation process.[2,10–12]

There is the potential that separation and product recovery can be applied in situ, during the fermentation process. As lactic acid fermentation is product inhibited, the removal of lactic acid produced in the fermentation medium will relieve this inhibition.[1] Studies of product removal following fermentation have been carried out in a number of systems by means of extraction,[13] dialysis[14] or conventional electrodialysis[15] as well as bipolar membrane electrodialysis.[16] The in situ extraction of lactic acid is possible, but it has yet to be demonstrated economically.[1] Conventional electrodialysis during fermentation with the cathode located in the fermentation compartment damages the bacterial cells if they come into direct contact with the electrodes. Therefore, an ultrafiltration step must be used before electrodialysis to prevent the cells from contacting the electrode.[17] A second issue for the microbiology is that of pH control in the fermentation chamber during electrodialysis. This can be partially achieved by electrodialytic removal of the acid, however the pH remained lower than optimal because some acid remained in the medium.[15,18] In order to maintain precise pH control, an additional pH control system with addition of NaOH solution was incorporated into the electrodialysis fermentor.[18]

Bipolar membrane electrodialysis has been used as a single unit operation for industrial organic acid production,[19] however, when coupled to fermentation, it is currently used as a separate second downstream stage for acid formation and base recycling.[9,10] In this format it follows conventional electrodialysis and enables recycling of base generated in the fermentor, leading to the production of the organic acid salt in the medium, which will also need to be converted into acid in a downstream process.

The aim of the current research is to combine the advantages of both conventional electrodialysis and bipolar membrane electrodialysis within the bioreactor configuration for lactic acid fermentation. This electrokinetic membrane bioreactor couples fermentation directly with both in situ lactic acid separation and product concentration. The use of a positively charged buffering system (bis-Tris) in the fermentation and concentration chambers leads to the migration of the negatively charged lactate ion alone into the concentration chamber. There is in situ pH control through removal of lactate and the direct use of hydroxyl ions produced by bipolar electrodialysis. The potential benefits of the bioreactor include relief of product inhibition, and hence an increase in yield, plus separation

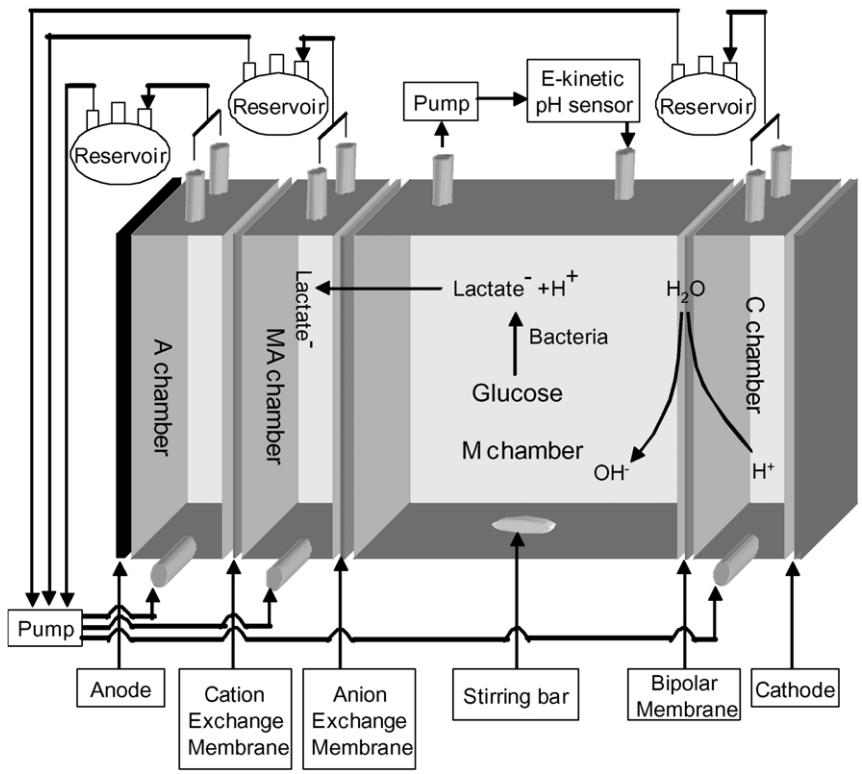

Figure 1. Electrokinetic bioreactor Each compartment has a circulation loop and reservoir for pH control, sampling and product recovery. Chambers A, MA, C and M were recirculated at 120 ml/min using a peristaltic pump with Norprene tubing (Cole-Parmer Instrument Co. Ltd, London, UK). The anode and cathode were connected to a computer-controlled DC power supply, with a pH sensor in chamber M connected to a computer interface.

and concentration of lactic acid and direct use of hydroxyl ions produced by water splitting to neutralize pH without the requirement for additional acid or base. pH control is coupled to the power supply to enable lactic acid to be removed as it is produced.

2. Results

2.1. Bioreactor design and electromigration of lactic acid in the electrokinetic bioreactor utilising bipolar electrodialysis

The electrokinetic bioreactor consisted of four chambers, labelled A, MA, M and C (Fig. 1). These chambers were separated by membranes, and the complete system was sealed by means of double O-rings between chambers and the system was clamped together. Chambers A and C were adjacent to the anode and cathode, respectively. Chambers A, MA, and C had a total capacity of 100 ml, however they were each connected to separate reservoirs, which enabled a total volume of 500 ml solution to be recirculated through each chamber. The reservoir for chamber MA contained a pH control system for monitoring and control of pH. Chamber M had a total capacity of 1400 ml. A cation exchange membrane (Nafion 450, DuPont Fluoroproducts, Fayetteville, USA) separated chambers A and MA, an anion exchange membrane (AMH, Tokuyama Co. Ltd., Tokyo, Japan) separated chambers MA and M, and a bipolar membrane (BP-1, Tokuyama Co. Ltd., Tokyo, Japan) separated chambers M and C. Chamber M was stirred at 250rpm using a magnetic stirring bar. The anode was fabricated from titanium-plated steel, and the cathode was a stainless steel plate. They were both square and had a working surface area of 11×8.5 cm^2. Chamber A and its reservoir contained 500 ml 0.1 M phosphate buffer, initially at pH 6.5. Chambers MA and C (including their reservoirs) each contained 500 ml 0.1 M bis-Tris buffer, starting pH 8.5 and 6.5, respectively. Chamber M contained 1400 ml 0.1 M bis-Tris buffer, pH 6.5, 20% (w/v) glucose. Harvested bacterial cells were resuspended in this medium to a density as indicated in the text for each bioreactor run. The anode and cathode were connected to a DC programmable power supply (PL-P, Thurlby Thandar Instruments Ltd. Cambridgeshire, UK). Within bipolar electrodialysis, pH control was achieved through water splitting and migration of hydroxyl ions into chamber M and through electromigration of lactate across the anion exchange membrane into chamber MA.

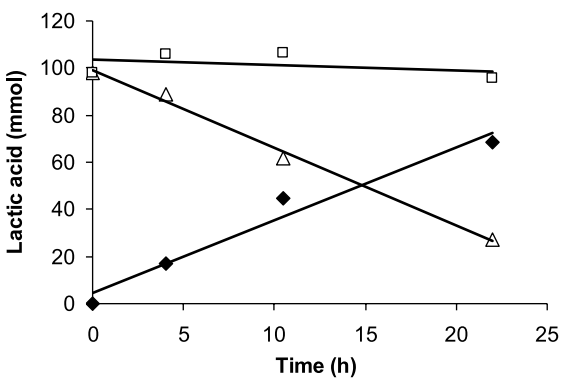

Figure 2. Lactic acid migration in the electrokinetic bioreactor with bipolar electrodialysis Bioreactor configuration and buffer content were as described in Section 4.1. Lactate (79.3 mM) migrated from chamber M (△) into chamber MA (◆) giving total lactic acid in the bioreactor (M+MA) (□) as determined by ion chromatography. The current density was fixed at 0.86 mA cm^{-2}. pH in chamber MA was controlled at pH 8.5 by addition of 4 M NaOH.

An initial experiment was conducted using buffer and lactic acid alone (i.e. no bacteria or glucose) to ensure that lactic acid migrated across the anion exchange membrane and could be recovered in compartment MA. The initial concentration of lactic acid in the buffer solution was 79.3 mM (10 g lactic acid in 1.4 l). Electromigration was observed, and a mass balance of 95% recovery of lactic acid was maintained as it migrated from chamber M across the membrane into chamber MA (Fig. 2). The current efficiency under these conditions was approximately 100%.

2.2. Lactic acid fermentation in the electrokinetic bioreactor with the application of current part way through the fermentation process

The bioreactor configuration and buffers in chambers A, MA, C and M were the same as described in Section 2.1. When glucose (20 g/l) was added to harvested cells and the fermentation was allowed to proceed in the absence of an applied current, lactic acid accumulated in chamber M giving a total of 81 mmol after 86 h, with some diffusion into compartment MA to a total of 93 mmol in the reactor (Fig. 3). After 86 h, the current was applied and lactic acid began to migrate into chamber MA. The amount of lactic acid in M remained constant to 106 hours and thereafter decreased significantly to approximately 10 mmol after a further 20 hours, whilst the amount of lactic acid in MA increased to a final level of 130 mmol after 48 h of applied current. In the control bioreactor, lactic acid production was very similar to that in the electrokinetic bioreactor up to 86 h (Fig. 4). However, after 86 h, there was very little further increase in lactic acid production, such that total lactic acid production in the electrokinetic bioreactor was

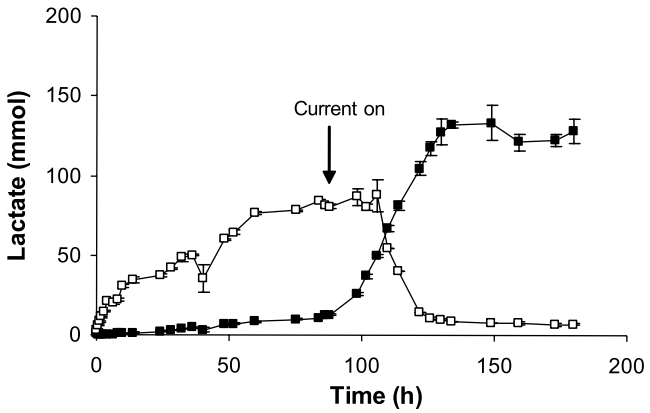

Figure 3. Lactic acid production in the electrokinetic bioreactor, with application of current after 86 h and no pH control. The configuration of the electrokinetic bioreactor and operating conditions are described in Sections 2.1 and 2.3, respectively. Starting glucose concentration was 111 mM (20 g/l) and applied current density was 1.8 mA/cm^{-2}. The pH in chamber M was not externally controlled. Lactate in chambers M (□) and MA (◆) was determined by ion chromatography. Harvested cells were used at a concentration of 0.87 g dry cell weight/l in both bioreactors. Values are the means of three determinations±SEM.

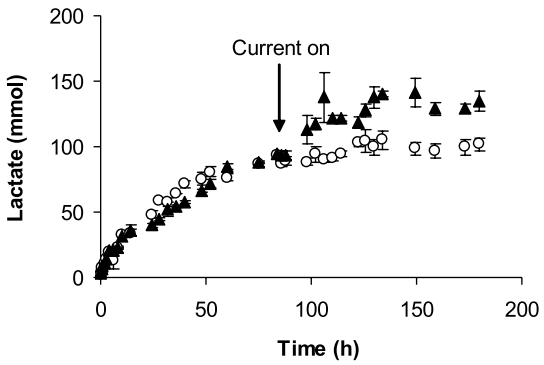

Figure 4. Total lactic acid production in the electrokinetic and control bioreactors without pH control. Lactic acid, measured as lactate, in the electrokinetic (▲) and control (○) bioreactors was determined by ion chromatography. Bioreactor configurations, operating conditions and harvested cells used were the same as in Figure 3. Values are the means of three determinations±SEM.

approximately 34% higher in comparison with the production in the control bioreactor (Fig. 4). The average lactic acid production rate over 180 h was 0.86 mmol/h/g dry cell weight for the electrokinetic bioreactor and 0.64 mmol/h/g dry cell weight for the control bioreactor. The increased lactic acid production also correlated to differences in glucose consumption in the electrokinetic and control bioreactors (Fig. 5). The total mass balance of glucose to lactic acid conversion was 57% for the electrokinetic bioreactor and 46% for the control bioreactor at the end of the fermentation.

As a result of lactic acid production, the pH in both bioreactors had decreased to approximately pH 4 after 86 h. After the current was switched on, the pH in chamber M in the electrokinetic bioreactor increased dramatically and finally stabilized at approximately pH 12 (Fig. 6).

2.3. pH controlled lactic acid fermentation in an electrokinetic bioreactor

Having show that the application of current could recover the pH in the fermentation chamber, leading to increased lactic acid production, it was necessary to control the applied current such that the pH could be maintained at a

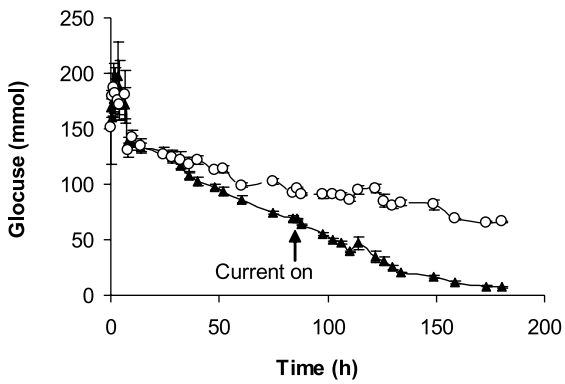

Figure 5. Glucose content in the electrokinetic and control bioreactors without pH control. Glucose content in the electrokinetic (▲) and control (○) bioreactors was determined by a glucose oxidase-kit (Section 4.5). Bioreactor configurations, running conditions and harvested cells used were the same as in Figure 3. Values are the means of three determinations± SEM.

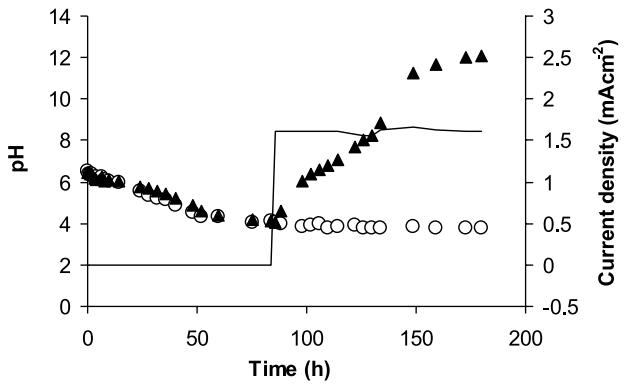

Figure 6. pH levels in the electrokinetic and control bioreactors during fermentation and applied current density for the electrokinetic bioreactor without pH control. pH in the EK bioreactor (▲) and control bioreactor (○) was determined using a microelectrode. Bioreactor configurations, operating conditions and harvesting of cells used were the same as in Figure 3.

suitable value. Manual pH control was achieved by increasing the applied current when the pH fell below pH 6.5, and decreasing or switching off the current when the pH increased above pH 6.5. The pH in the control bioreactor was controlled at pH 6.5 using a pH controller and addition of 4 M NaOH. The lactic acid production rate was 0.78 mmol/h/g dry cell weight in the electrokinetic bioreactor and 0.51 mmol/h/g dry cell weight for the control bioreactor (averages calculated over a period of 197 h). The production of lactic acid in the electrokinetic bioreactor, proceeded at a constant rate to 100 h and continued to increase to 197 h, giving a maximum production of 168 mmol, whereas in the control reactor lactate production stopped after 100 hours, with a maximum production of 112 mmol (Fig. 7). pH was maintained at pH 6.5 in both bioreactors. The increased production of lactic acid in the electrokinetic bioreactor may be due to the removal of lactic acid from chamber M into chamber MA and due to the different conditions of the pH control systems (Fig. 8). Moreover, a higher concentration of lactic acid was achieved in chamber MA (approximately double) at the end of the fermentation compared to that in chamber M

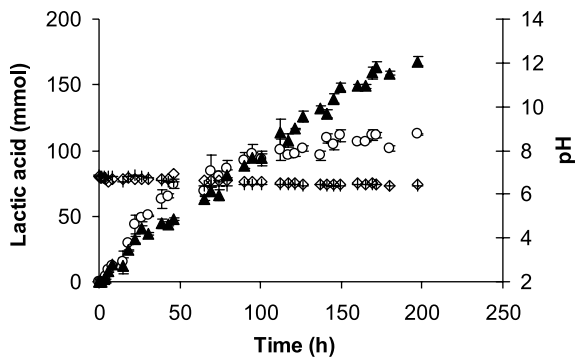

Figure 7. Lactic acid production within an electrokinetic bioreactor with manual pH control. Lactic acid levels in the electrokinetic bioreactor (▲) and control bioreactor (○) were determined by ion chromatography. The pH in the electrokinetic bioreactor (+) was controlled by manually adjusting the current according to changes in pH in chamber M. The pH in the control bioreactor (◇) was controlled using a conventional pH controller and addition of 4 M NaOH. Both bioreactors were controlled at pH 6.5. Harvested cells were used at 0.79 g dry cell weight /l in both bioreactors. Values are the means of three determinations ±SEM.

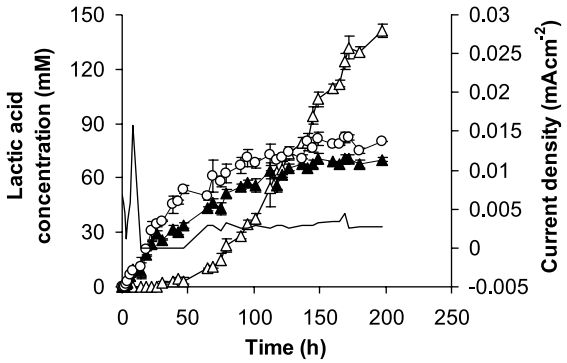

Figure 8. Lactic acid concentrations in chambers M and MA of the electrokinetic bioreactor and in the control bioreactor together with current density applied to the electrokinetic bioreactor with manual pH control. Lactic acid concentrations (mM) in chambers M (▲) and MA (△) in the electrokinetic bioreactor and in the control bioreactor (○) were determined by ion chromatography. Bioreactor configurations and other conditions, including current (—) were the same as in Figure 7. Values are the means of three determinations±SEM.

(Fig. 8). The total mass balance of glucose to lactic acid conversion was 61% for the electrokinetic bioreactor and 46% for the control bioreactor.

2.4. Lactic acid fermentation using the electrokinetic bioreactor and bipolar electrodialysis with automatic pH control

To increase the effectiveness of the bipolar electrodialysis system, manual pH control was replaced with an automatic pH controller with which applied current was linked to measured pH. In this experiment, the bioreactor configuration and operating conditions were the same as in Section 2.3, except that the pH in the electrokinetic bioreactor was controlled by an automatic pH controller set to pH 6.5 (Section 4.1). In the control bioreactor, the pH was maintained at the same value by addition of sodium hydroxide.

Lactic acid migrated electrokinetically into chamber MA from chamber M and the concentration of lactic acid in chamber MA at the end of the fermentation was approximately 20 times higher than that in chamber M (Fig. 9). The

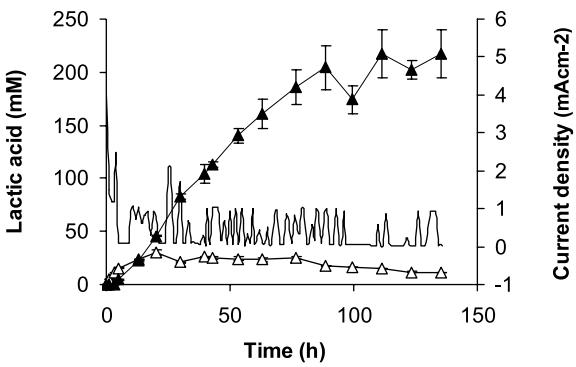

Figure 9. Lactic acid concentration during fermentation in the electrokinetic bioreactor with automatic pH control. Lactic acid concentration (mM) in chambers MA (▲) and M (△) was determined by ion chromatography. Current density (—)was supplied by a computerized automatic pH control system. Harvested cells were 1.3 g dry cell weight/l. Values are the means of three determinations±SEM.

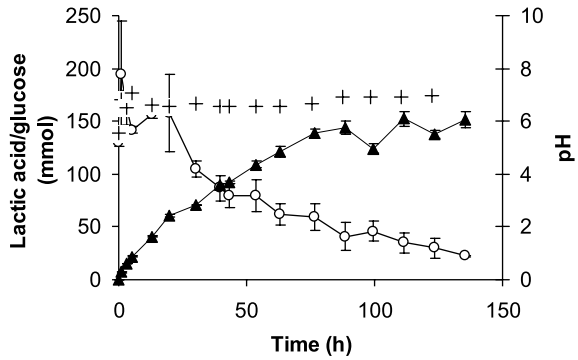

Figure 10. Total lactic acid, glucose and pH in the electrokinetic bioreactor during fermentation under automatic pH control. The total lactic acid (▲) combined from chambers MA and M, was determined by ion chromatography. Current was supplied by a computerized automatic pH controller system. The results are means of three determinations±SEM.

average rate of lactic acid production in the electrokinetic bioreactor was 0.61 mmol/h/g dry cell weight over 135.5 h. The yield was 62% of glucose consumed. The pH was maintained constant throughout the experiment (Fig. 10).

3. Discussion and conclusion

Under the application of DC current, it has been demonstrated that the negatively charged lactate ion generated by fermentation in chamber M of the electrokinetic bioreactor migrated across the anion exchange membrane into the concentrating chamber (MA). This electromigration and concentration of lactic acid was coupled with maintenance of the pH at near neutral conditions in contrast to the otherwise acidic condition in the fermentation medium in the absence of electrokinetics. In the electrokinetic bioreactor, higher lactic acid production was also achieved. This was coupled to higher yield when pH was maintained at the same level as in the control bioreactor (to which no DC current had been applied). This is most likely due to overcoming product inhibition in the electrokinetic bioreactor as the lactate generated was removed to a separate chamber. The health, as measured by culturability, of the bacteria during the electrokinetic process was not affected by the application of current at different levels up to 1.6 mA/cm^{-2} even after 150 h (data not shown).

Since pH control did not rely only on the removal of the acid produced in the electrokinetic bioreactor, the hydroxyl ion produced by water splitting at the bipolar membrane, which migrated into chamber M, also played an important role. This approach of combining the advantages of conventional electrodialysis and bipolar water splitting technology could be used to precisely control the pH while the acid migrated into MA. The pH could even be controlled at a value higher than neutral without addition of base. This is not possible by conventional electrodialysis and removal of the lactic acid alone.[18] This feature is important because conductivity of the organic acid was strongly dependent on the pH during electrodialysis.[20] Control of the pH at an optimal level is desirable both for the bacterial fermentation and for a maximum rate of electrodialysis to occur. Moreover, the direct use of the hydroxyl ions produced from bipolar membrane water splitting to control pH means that, in

contrast to other production processes, the lactic acid salt is not produced in the fermenting media.[15,18] There is also the potential to recover the accumulated lactic acid in chamber MA in its protonated form as it combines with protons migrating from chamber A into MA.

This bioreactor changes the conventional method of pH control through addition of a base to that of applying a current to control the pH. It is much simpler to control the system by this approach, which can also be automated very effectively. The overall benefit from the electrokinetic bioreactor was extremely positive in that it was a one stage integrated process, with in situ pH control, integral product (lactic acid) removal in a concentrated form, and overall enhancement of yield. The approach taken in this study is potentially applicable in a wide range of biocatalytic and fermentation processes.

4. Experimental

4.1. Bioreactor configuration and its running conditions

The electrokinetic bioreactor configuration is described in Section 2.1. The pH in chamber MA was controlled at pH 8.5 by means of a pH controller (Electrolab Ltd, Gloucestershire, UK) with addition of 4 M NaOH. In all experiments, the pH in chambers A and C was not controlled. The pH in chamber M was controlled through a combination of water splitting and migration of hydroxyl ions into chamber M, and electromigration of lactic acid across the anion exchange membrane into chamber MA.

As a further development of the process, automatic pH control (Fig. 1. electrokinetic pH controller) was later developed. A pH probe was fitted into a recirculating loop attached to chamber M in the bioreactor. This pH probe was connected to a computer programmed with software that controlled a programmable power supply unit. When the pH fell below a set value, due to production of lactic acid, the power supplied was increased, and when the pH rose to above another set value, the power supplied was decreased.

Control experiments were conducted in a 2 liter flask or in a bioreactor in which chamber M was isolated such that no current was applied. Control experiments always used the same batch of cells as used in the bioreactor and were carried out simultaneously and under the same conditions of volume, temperature and stirring speed.

4.2. Microorganisms and culture conditions

Lactobacillus rhamnosus NCIMB 6375 was obtained as a freeze-dried culture from NCIMB Ltd. (Aberdeen UK). The culture was resuscitated in MRS broth (Merck Ltd, Poole, UK) and stored on MRS agar plates and as glycerol stocks at −70 °C.

A single colony from an MRS agar plate was innoculated into 50 ml MRS broth in a 250 ml conical flask. The flask was incubated in a shaking incubator for 48 h at 30 °C, 100 rpm. An aliquot of 20 ml of the culture was then used to innoculate 500 ml of the same medium in a 2 l flask which was incubated until the cells had grown to late exponential phase (approximately 18 h). Cells were harvested by centrifuging at 3840*g* (Beckman JH-2 centrifuge with JA-14 rotor, Beckman Instruments, Bucks, UK) at 4 °C for 25 min and washed twice with 0.1 M bis-Tris buffer (pH 6.5) and re-suspended in the same buffer to a total volume of approximately 1 l. The bacterial suspension was divided into two equal parts, one part for the experiment in the electrokinetic bioreactor and one for the control bioreactor.

4.3. Fermentation conditions

A final volume of 1.4 l harvested cell suspension (in 0.1 M bis-Tris buffer, pH 6.5) containing glucose at a concentration of 20 g/l was added to chamber M and the control chamber. Fermentation commenced with the addition of glucose. Samples were withdrawn at each time point for analysis.

4.4. Determination of bacterial cell density and culturability

Bacterial cell density was determined by measuring optical density (OD) at 600 nm using a spectrophotometer (Thermo UNICAM, Cambridge, UK) and correlated to dry cell weight using a reference curve. For dry cell weight determination, cell suspensions were dried at 105 °C overnight in pre-weighed glass weighing vessels. Bacterial colony forming units (cfu's) were determined by serial dilution in sterile water and plating onto MRS agar. Colonies were counted following incubation for 24 h at 30 °C.

4.5. Sample analysis

The individual pH values for samples were determined using a semi-micro electrode (Merck Ltd., Poole, UK) attached to a pH meter (Jenway 3305, Essex, UK). Samples were centrifuged for 3 min at 10,000 rpm using a micro-centrifuge (Labnet, Berkshire, UK). Supernatants were stored at −20 °C for future analysis.

Glucose was measured using either a glucose oxidase-based system (GOD-PERID, Roche Diagnostics Corp., Indiana, USA) or a hexokinase-based system (Gluco-Quant Glucose/HK Assay, Roche Diagnostics Corp, Indiana, USA).

Lactic acid was analysed by ion chromatography using an Anion Dual 2 column (Metrohm, Buckingham, UK). The mobile phase was 2.4 mM $NaHCO_3$ and 2 mM Na_2CO_3 at a flow rate of 0.8 ml/min. Lactic acid was determined by conductivity detection and was quantified according to a standard curve.

Acknowledgements

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Ketoreductases in the synthesis of valuable chiral intermediates: application in the synthesis of α-hydroxy β-amino and β-hydroxy γ-amino acids

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Abstract—A general method for the synthesis of β-alkyl α-hydroxy β-amino and α- and γ-alkyl substituted β-hydroxy-γ-amino acids is described. The synthesis of all three classes of amino acids proceeds through a common chiral alcohol intermediate that is generated from a pro-chiral ketone diester via the action of a nicotinamide-dependent ketoreductase. Regioselective chemical or enzymatic hydrolysis followed by rearrangement under Hofmann or Curtius conditions gives the final amino acid products. High yields of single diastereomers of the final amino acids are obtained. Amino acids with both natural and unnatural alkyl substituents can be accessed using this methodology.

1. Introduction

Amino alcohols, and in particular α-hydroxy β-amino acids (**1**, Fig. 1) and β-hydroxy γ-amino acids (**2**, **3**, Fig. 1), also known as statines, are valuable chiral compounds that are present in a variety of natural and synthetic compounds possessing a spectrum of biological activities. These key structural units are found in molecules showing several types of pharmacological activity including protease inhibitors, anti-neoplastic agents, antibacterials and anti-cancer drugs.[1] The significance of these molecules is also evident given the number of research publications and books dedicated to their synthesis.[1–6]

Some methods for the synthesis of such compounds are based on resolutions of ester derivatives using lipases and give low yields.[5] A number of elegant chemical synthetic routes have been reported, however, most require multi-step reaction sequences, chiral catalysts or starting materials, air-sensitive reaction conditions, or unstable intermediates.[1–3] In addition, few if any of these methodologies are generally useful for the synthesis of each of the individual diastereomers of a range of statine analogs, and most are not useful for preparing compounds with alkyl substitutions derived from non-naturally occurring amino acids.[4–6] Gaining control over the stereochemistry of the chiral carbons bearing both the amino and the alcohol groups at reasonable cost and high enantiomeric purity is the key to the successful production of these important chemical intermediates.

Herein we describe a chemo-enzymatic method for the synthesis of individual diastereomers of α-hydroxy β-amino and β-hydroxy γ-amino acids and analogs that are shown in Figure 1. Using non-chiral, readily available starting materials, a range of vicinal amino alcohols can be produced. The key step is a diastereoselective enzymatic reduction that generates two chiral centers in a single step, setting the absolute stereochemistry in the molecule. The remaining chemical steps are straightforward and can be

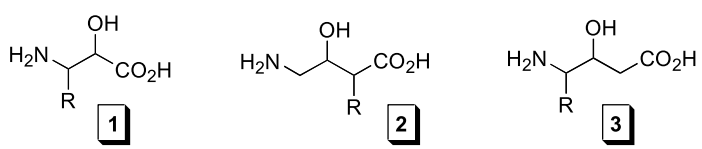

Figure 1. Targeted amino alcohols and amino acids.

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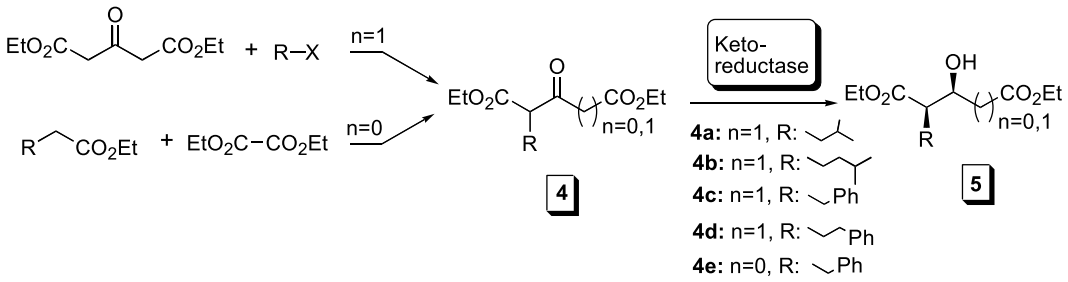


Figure 2. Synthesis of ketones and enzymatic reduction.

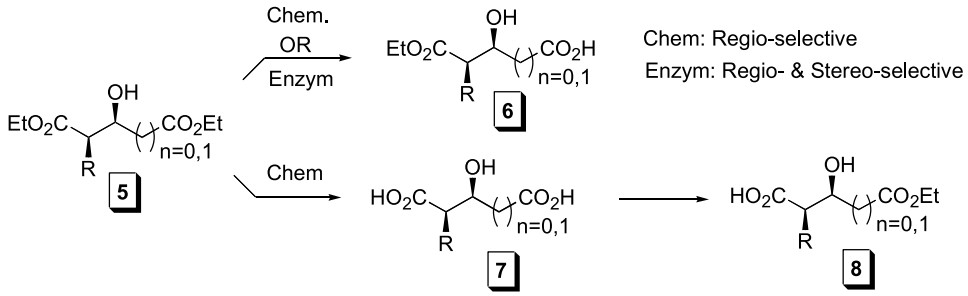


Figure 3. Synthesis of each mono ester can be achieved by either chemical or enzymatic techniques.

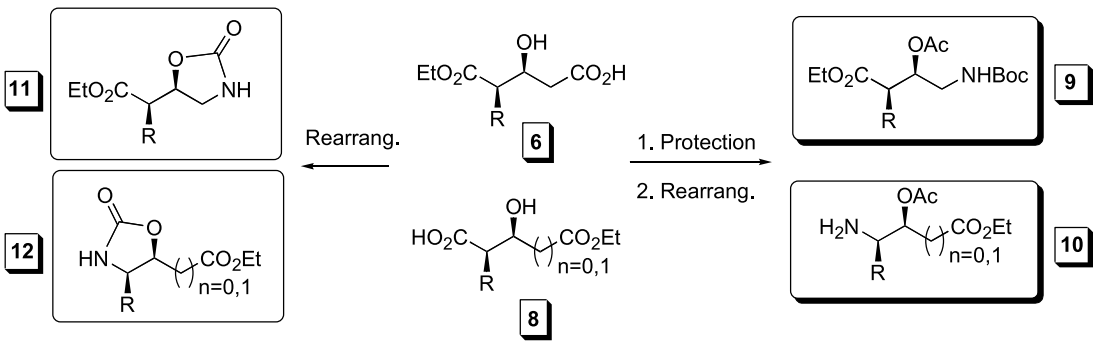


Figure 4. Both the cyclic carbamates or the free amines can be obtained from the rearrangement of the mono-acids.

carried out with readily available reagents. In the basic synthetic method, a ketodiester **4** (Fig. 2) is reduced diastereoselectively to form a key hydroxydiester intermediate **5** in high yield by the action of a ketoreductase enzyme. Both chiral centers present in the final statines and their analogs are generated in this enzymatic step. Regioselective chemical or enzymatic hydrolysis of either ester group of the key intermediate diester, followed by rearrangement under Hofmann or Curtius conditions, gives the final amino alcohol products (Figs. 3 and 4). In a recent report, we described the synthesis of statine and phenylstatine (R=isobutyl, **10a** and R=benzyl, **10c**, Fig. 4) using this methodology.[7] We now expand the method to include the synthesis of a range of statine analogs including α-hydroxy β-amino acids bearing natural and unnatural alkyl substitutions (Fig. 1).

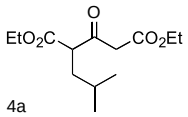
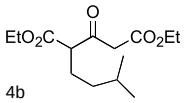
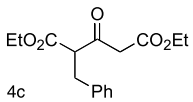
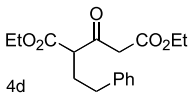
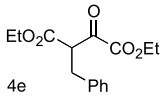
2. Results and discussion

2.1. Synthesis of substituted ketone diesters 4 (Fig. 2) and enzymatic reduction

Synthesis of 2-substituted diethyl ketoglutarates (**4a**–**d**, Fig. 2) is accomplished in a straightforward fashion via the mono-alkylation of diethyl 1,3 acetonedicarboxylate with the appropriate alkyl halide. The homo-statine **4b** and homo-phenylstatine **4d** (Table 1) precursors were synthesized by the alkylation of diethyl 1,3 acetonedicarboxylate with 1-iodo-3-methyl butane and 1-bromo-2-phenyl ethane, respectively, using methods based on previously reported methodology for the synthesis of **4a** and **4c** (Table 1).[7] The analogous compound 2-benzyl diethyl oxaloacetate (**4e**, Fig. 2) was synthesized by a Claisen condensation of ethyl hydrocinnamate and diethyl oxaloacetate using an adaptation of a reported method.[8] As an illustration of the generality of the method, we describe herein two compounds with natural side chains (**4a** and **4c**) and two compounds bearing non-natural alkyl substitutions (**4b**, **4d**, Table 1).

To identify the appropriate ketoreductase for a desired reaction, all ketodiester substrates were screened for reduction in the presence of each of 10 different commercially-available ketoreductases (KRED-10,000 Ketoreductase Screening Set, BioCatalytics Inc, Pasadena, CA, USA) using the protocol provided by the manufacturer. At least one enzyme was shown to be active with every ketone

Table 1. Reductions of ketone substrates using KRED enzymes

Substrate	Yield (A/B/C/D)[a]	
	KRED101	KRED108
 4a	>95% (0/5/0/95)[b]	
 4b	85% (0/13/0/87)	
 4c		95% (0/99/0/0)[b]
 4d	~5%	35% (95/5)[c]
 4e		>90% (0/99/0/0)[d]

[a] A/B/C/D represents the ratio of diastereomers. For statine (**4a**) and phenylstatine (**4c**): A: (3*S*,4*S*); B: (3*R*,4*R*); C: (3*R*,4*S*); D: (3*S*,4*R*).[7]
[b] Reactions described in Ref. 7.
[c] The ratio of diastereomers *SS*+*RR*/*SR*+*RS* is reported. No base-line separation of all diastereomers was achieved by either chiral GC or HPLC.
[d] Major diastereomer: (2*R*,3*S*).

substrate; the results from the best enzyme for each ketodiester are shown in Table 1.

As described earlier, the KRED-catalyzed diastereoselective reduction generates two chiral carbons during this reaction, and as a result, the absolute stereochemistry of the final statines and their analogs is determined at this point. For most reactions, the diastereomeric purity of the products is high, >90%. In cases where the optical purity is lower than desired, it can be further improved by stereoselective enzymatic hydrolysis using a lipase enzyme (vide infra).

For statine and phenylstatine precursors (**4a** and **4c** respectively, Table 1) the absolute stereochemistry of the enzymatic reduction was identified earlier after rearranging to the final amine (**10a** and **10c**) and comparing it with authentic material that was synthesized chemically.[7] For the homo-statine **4b** (Table 1) no standards and no stereo-selective chemical syntheses to either **5b** or **10b** were available. However, based on the chiral GC retention time of the alcohol **5b** (Table 1) and the known diastereoselectivity of the reaction catalyzed by KRED101 on ketodiester **5a** (Table 1), we assume that the major diastereomer of the reduction of **5b** and KRED101 is (3*S*, 4*R*). For the homo-phenylstatine precursor **5d** we were unable to obtain baseline separation of all diastereomers using either chiral GC or HPLC chromatography, and as a result, only the diastereomeric ratio is reported (**4d**, Table 1). Finally, the absolute stereochemistry for the reduction of **4e** by KRED 108 was assigned using chiral HPLC by comparison with authentic material that was synthesized from *R*- and *S*-diethyl malate and benzyl bromide according to reported methodology.[9]

Cell-free enzymatic reaction conditions were developed to carry out the enzymatic reductions. Nicotinamide co-factor was required by all ketoreductases, but with efficient recycling methods, the amount of added co-factor is normally less than 1 mol% relative to starting ketone. All the ketoreductases used in this study require NADPH as the co-factor for hydride transfer. We therefore used a recycling system based on reducing NADP+ to NADPH with glucose and the commercially available enzyme glucose dehydrogenase (GDH-101, BioCatalytics Inc, Pasadena, CA, USA). Glucose is an inexpensive, water-soluble reductant, and the immediate reaction product of the oxidation of glucose, gluconolactone, spontaneously hydrolyses to gluconic acid under the reaction conditions employed, making the overall process essentially irreversible. The pH of the reaction mixture was maintained at 6.7–6.8 using a pH-stat.

2.2. Regioselective hydrolysis and rearrangement

We have previously shown that the two ester groups of **5a** and **5c** (Fig. 3) were hydrolyzed chemically with different reaction rates.[7] As a result, mild basic chemical hydrolysis gave preferably the mono acids **6a** and **6c** (Fig. 3) while complete hydrolysis to the mono acid followed by esterification in ethanol gave mono-acids **8a** and **8c** (Fig. 3).[7] This methodology has been successfully applied to all diethyl 2-alkyl 3-hydroxy glutarates **5a**–**d** as well as the diethyl 2-benzyl 3-hydroxy succinate **5e** (Fig. 3).

Both regio- and stereo-selectivity can also be obtained when a lipase is utilized for the hydrolysis of **5** (Fig. 3). Twenty-four lipases (ICR Screening Set, BioCatalytics, Inc. Pasadena, CA, USA) were screened and enzymes were identified that were active against every diester **5**, giving compounds **6** as the only or the preferred product. In some cases, enzymatic hydrolysis of the second ester yielding the diacid was observed when the reactions were allowed to continue for very long periods or when large excesses of enzyme were used. When a diastereomeric mixture of alcohols was utilized as a starting material for enzymatic hydrolysis, in addition to regioselectivity, stereoselectivity can also be obtained. Such an effect was previously shown in the hydrolysis of a diastereomeric mixture of 2-methyl 3-hydroxy glutarate and ICR 112.[7] Similarly, lipases ICR 113 and ICR 114 selectively hydrolyzed the major component [(2*R*,3*S*)] of a diastereomeric mixture [(2*R*,3*S*)/(2*S*,3*S*), 9:1] of diethyl 2-benzyl-3-hydroxy succinate **5e**, giving mono-acid **6e** (n=0, Fig. 3) in 50–60% yield.

As shown earlier for statine (**5a**) and phenylstatine (**5c**) precursors and now confirmed for the rest of compounds in Table 1, rearrangement of the free alcoholic acids **8** (Fig. 4) under either Curtius or Hofmann conditions gave the cyclic carbamates **12**.[7] Formation of these products was shown to be independent of both the rearrangement reagents and the reaction conditions. The simplest and most straightforward one step synthesis of carbamates **12** was shown to be the heating of the free acid **8** with diphenylphosphoryl azide (DPPA) in toluene.[7] We now show that the opposite cyclic carbamates **11** can be obtained in good yields (50–70%)

when instead of **8**, the less hindered mono-acids **6a–d** were utilized for rearrangement with DPPA. We therefore conclude that this is a general method for the preparation of cyclic carbamates **11** and **12** from the corresponding mono-acids **6** and **8** (Fig. 4).

Formation of the amino acids **9** and **10** was accomplished after the alcohol was protected as an acetate prior to the rearrangement. Under these reaction conditions, the corresponding amides of **8a–d** were prepared and rearranged to the free amine **10a–d** using bis[(trifluoroacetoxy)iodo] benzene [$(CF_3CO_2)_2IPh$]. This methodology was utilized earlier for the synthesis of statine (**10a**) and phenylstatine (**10c**).[7] Besides the β-hydroxy γ-amino acids **10a–d** (n=1, Fig. 4), the 2-benzyl α-hydroxy β-amino acid **10e** (n=0, R=Bz, Fig. 4) was prepared using this methodology in similar overall yield. Therefore, this reaction sequence of acetyl protection of the alcohol followed by rearrangement is a general method for the preparation of statine and analogues **10** (Fig. 4).

The previous rearrangement conditions using bis[(trifluoroacetoxy)iodo]benzene were shown to be inadequate for the preparation of the primary amines **9** (Fig. 4) from the corresponding amides of **6**. This rearrangement was achieved using lead tetracetate [$Pb(OAc)_4$] according to a previously described procedure.[10]

3. Conclusions

This report describes a general method for the synthesis of statines and a number of analogs. An advantage of the methodology reported here is that in most cases each of the four diastereomers can be produced in high stereochemical purity. All compounds are produced from non-chiral, readily available starting materials, and the reactions are performed at room temperature and pressure.

From the cloned commercially-available ketoreductases that we have screened to date against compounds **4a–e**, two commercially available ketoreductases (KRED-101, KRED-108, BioCatalytics, Inc. Pasadena, CA, USA) gave products with good to excellent diastereoselectivity (Table 1). We plan to further broaden the applications for this general method by the development of new ketoreductase enzymes. These will be reported in due course.

4. Experimental

4.1. Synthesis of ketones 4 (Fig. 2)

The methodology that was published earlier for the synthesis of **4a** and **4c** (Table 1) was utilized for the synthesis of homo-statine **4b** and homo-phenylstatine **4d** precursors.[7] These products can be enzymatically reduced to the alcohol without any further purification, or they can be purified using silica gel chromatography (Hex/EtOAc, v/v, 8:2) giving 88% yield (3.6 g) for 1-iodo-3-methylbutane and 65% for 1-bromo-2-phenylethane. NMR verification of the products was performed after enzymatic or chemical reduction (vide infra).

Synthesis of diethyl 2-benzyl diethyl oxaloacetate **4e** (Table 1, Fig. 2) was achieved by a modification of standard methodology.[8] Ethyl hydrocinnamate (1 mL, 5.6 mmol) was dissolved in THF (10 mL) and the solution was cooled at −18 °C before butyl lithium (2.9 mL, 2.5 M in hexanes, 7.3 mmol) was slowly added. After stirring at this temperature for 10 min, diethyl oxalate (0.8 mL, 8.4 mmol) was added through a syringe and the reaction was slowly left to reach room temperature overnight. The reaction mixture was then added to an ice-cold water (50 mL, 0.15 M HCl) solution and extracted twice with diethyl ether (30 mL×2). The combined organic layers were back-extracted with brine, dried with Na_2SO_4 and evaporated to dryness. After silica gel purification a clear oil of 2-benzyl ethyl hydroxy succinate (0.9 g) (**4e**, Table 1) was isolated (yield, 58%). NMR verification of the products was performed after enzymatic or chemical reduction (vide infra).

4.2. Enzymatic reduction to alcohols 5 (Fig. 2)

The Ketoreductase Screening Set (KRED-10,000; Bio Catalytics, Inc., Pasadena, CA, USA) containing 10 different ketoreductases was screened to determine the best enzyme for the diastereoselective reduction of every 2-alkyl-3-ketoglutarate diethyl ester. In addition to the 10 ketoreductases both NADPH co-factor and glucose dehydrogenase (GLDH) are products available from BioCatalytics. Individual reactions containing each ketoreductase (2 mg/mL), NADPH (5 mM), NaCl (100 mM), DMSO (2.5 or 5% v/v) each substrate (25 mM), glucose (100 mM) and glucose dehydrogenase (GLDH, 2 mg/mL) for co-factor recycling were prepared in a phosphate-buffered (1 mL, 300 mM, pH 6.5) solution. The reactions were incubated at 37 °C overnight before they were extracted with ethyl acetate and analyzed by GC or HPLC chromatography.

Larger-scale enzymatic reductions (0.5–2 g of ketone) were prepared according to previously described conditions.[7] The optical purity of each product was determined by chiral GC chromatography using a ChiralDex column (Chiral Technologies, 130 °C, 2 min and then to 180 °C, 0.5 or 1 °C/min) after each enzymatically-produced alcohol was derivatized as the triflate ester. The enantiomeric purity was also examined using chiral HPLC analysis (column: CHIRALCEL OD-RH from Chiral technologies, H_2O/CH_3CN, v/v 60:40, 0.6 mL/min). All products were oily compounds and were analyzed by ^1H NMR.

4.2.1. Compound 5b. From KRED-101 reduction ^1H NMR (400 MHz, $CDCl_3$): δ=0.87+0.88 (d+d, J=3.20 Hz, 6H, $CH_2CH_2CH(CH_3)_2$), 1.17 (m, 2H, $CH_2CH_2CH(CH_3)_2$), 1.27+1.29 (t+t, J=3.60 Hz+J=3.99 Hz, 6H, $CO_2CH_2CH_3$), 1.55 (m, 2H, $CH_2CH_2CH(CH_3)_2$), 1.75 (m, 1H, $CH_2CH_2CH(CH_3)_2$) 2.47+2.51 and 2.55+2.58 (d+d J=9.19 Hz and d+d J=3.99 Hz first d+d overlaps with multiplet, 3H, $CH_2CO_2CH_2CH_3$ and $CHCO_2CH_2CH_3$), 4.2 (pent, J=7.19 Hz, 5H, $CO_2CH_2CH_3$, CHOH).

4.2.2. Compound 5d. From $NaBH_4$ reduction ^1H NMR (400 MHz, $CDCl_3$): δ=1.25 (two overlapping triplets ~2:1 ratio, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.31 (two overlapping

triplets ~2:1 ratio, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.9+2.05 (m+m, 2H, CH_2CH_2Ph), 2.55 (m, 3H, $CH_2CO_2CH_2CH_3$ and $CHCO_2CH_2CH_3$), 2.7 (m, 2H, CH_2CH_2Ph), 4.15+4.2 (q+q J=7.19 Hz, and a multiplet, 5H, $CO_2CH_2CH_3$, CHOH), 7.18+7.25 (m+m, 5H, CH_2CH_2Ph).

4.2.3. Compound 5e. From KRED-108 reduction ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.20 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.27 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 3.00 (m, 1H, $CHCO_2CH_2CH_3$), 2.95+3.23 (m+asymmetric triplet, 2H, CH_2Ph), 4.15+4.22 (m+m, 5H, $CO_2CH_2CH_3$, CHOH), 7.3 (m, 5H, CH_2Ph).

The spectra of **5a** and **5c** have been reported earlier.[7]

4.3. Hydrolysis to the mono acid 6 (Fig. 3)

Hydrolysis to the mono acid **6** (Fig. 3) using mild chemical or enzymatic conditions has been described earlier for the compounds **5a** and **5c**.[7] The same conditions were applied to the rest of the compounds of Table 1 including 2-benzyl-3-hydroxy succinate **5e** which was hydrolyzed to the mono-acid **6e** (R:benzyl, n=0, Fig. 3) by either chemical or enzymatic methods. Under chemical conditions, 2-benzyl-3-hydroxy succinate **5e** (0.15 g, 0.53 mmol) was added to an aqueous/ethanol (2 mL, 8:2, v/v) solution containing NaOH (0.042 g, 1.1 mmol) and the mixture was stirred for 45 min at room temperature until complete reaction to the mono acid was detected by HPLC analysis. Acidification with HCl (pH ~2) and extraction with EtOAc (10 mL, ×2) gave after solvent evaporation the mono-acid **6e** (0.11 g, 0.44 mmol, 83% isolated yield). The same mono-acid was obtained by enzymatic hydrolysis using ICR125, since ^{1}H NMR, GC and HPLC analysis of the chemical and enzymatic hydrolysis products were identical. Under the enzymatic reaction conditions, 2-benzyl-3-hydroxy succinate **5e** (0.15 g, 0.53 mmol) was dissolved in 0.3 mL of DMSO and was mixed with a potassium phosphate solution (200 mM, pH 7, 10 mL) also containing lyophilized ICR125 lipase (10 mg). After stirring at 37 °C for 2–3 h, the mixture was acidified with HCl (2 M) to pH ~2.5 and extracted with EtOAc (5 mL, ×2). Solvent drying with Na_2SO_4 and evaporation to dryness gave pure mono-acid **6e** as an oil in 90% isolated yield.

4.3.1. Compound 6e. ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.20 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 3.00+3.05 and 3.21 (d+d, J=9.99 Hz and m, 3H, CH_2Ph and $CHCO_2CH_2CH_3$), 4.15 (m, 3H, $CO_2CH_2CH_3$, CHOH), 7.25 (m, 5H, CH_2Ph).

The hydrolysis of **5b** under mild chemical or enzymatic conditions follows the same regio-selectivity, giving the less hindered acid **6b**. This selectivity was confirmed by ^{1}H NMR analysis after making **8b** by the standard hydrolysis/esterification sequence and rearranging it to **12b** (vide infra). The hydrolysis selectivity and product characterization of **5a** and **5c** has been reported earlier.[7]

4.4. Rearrangement of mono acids 6 to the amines 9 and 11 (Fig. 4)

When rearrangement reactions were performed without prior protection of the alcohol, cyclic carbamates **11** (Fig. 4) were obtained. Following the same rearrangement conditions described earlier,[7] **6a** (reduced by KRED-101, hydrolyzed by ICR116) was mixed in toluene with diphenylphosphoryl azide (1.1 equiv. DPPA) and triethylamine (1.1 equiv.) and the solution was heated at 85 °C for 1 h before the temperature was lowered to 60 °C where it was stirred overnight. Product isolation and purification gave an oily compound **11a** in 55–60% yield.

4.4.1. Compound 11a. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.90+0.92 (d+d, J=4.0 Hz, 6H, $CH_2CH(CH_3)_2$), 1.12 (m, 1H, $CH_2CH(CH_3)_2$), 1.27 (t, J=6.8 Hz, 3H, $CHCO_2CH_2CH_3$), 1.60 (m, 2H, $CH_2CH(CH_3)_2$), 2.78 (m, 1H, $CHCO_2CH_2CH_3$), 3.32+3.34 and 3.61 (d+d and t, J=7.20, 8.79 Hz, 2H, $CH_2NH-CO-OCH$), 4.2 (m, 2H, $CO_2CH_2CH_3$), 4.75 (q, J=7.80 Hz, 1H, $CH_2NH-CO-OCH$). ^{13}C NMR ($CDCl_3$, 100 MHz) δ=14.16 (s, $CHCO_2CH_2CH_3$), 21.55 and 23.36 (s and s, $CH_2CH(CH_3)_2$), 26.06 (s, $CH_2CH(CH_3)_2$), 36.06 (s, $CH_2CH(CH_3)_2$), 43.78 (s, $CHCO_2CH_2CH_3$), 48.02 (s, $CHCO_2CH_2CH_3$), 61.06 (s, $CH_2NH-CO-OCH$), 77.18 (s, $CH_2NH-CO-OCH$), 159.74 (s, $CH_2NH-CO-OCH$), 172.42 (s, $CHCO_2CH_2CH_3$).

Treatment of **6c** with ICR125 and rearrangement with DPPA gave **11c** in 60% yield.

4.4.2. Compound 11c. ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.24 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 2.9+3.00 (m+m, 3H, $CHCO_2CH_2CH_3$, CH_2Ph), 3.45+3.62 (t+t, J=7.59+8.79 Hz, 2H, $CH-O-CO-NH-CH_2$), 4.10 (q, J=7.19 Hz, 2H, $CO_2CH_2CH_3$), 4.81 (m, 1H, $CH-O-CO-NH-CH_2$), 6.02 (s, 1H, $CH-O-CO-NH-CH_2$), 7.25 (m, 5H, CH_2Ph). ^{13}C NMR (100 MHz, $CDCl_3$): δ=13.99 (s, $CO_2CH_2CH_3$), 33.62 (s, $CHCO_2CH_2CH_3$), 43.68 (s, CH_2Ph), 51.56 (s, $CO_2CH_2CH_3$), 61.13 (s, $CH-O-CO-NH-CH_2$), 75.95 (s, $CH-O-CO-NH-CH_2$), 128.93+128.67+128.96+137.39 (s, CH_2Ph), 159.33 (s, $CH-O-CO-NH-CH_2$), 171.36 (s, $CO_2CH_2CH_3$).

Protection of the free alcohol prior to the rearrangement was necessary to synthesize the free amines **9**. In an illustration of the methodology **9a** and **9c** were prepared. Protection of the alcohol as an acetate ester was accomplished by reacting it with acetic anhydride (Ac_2O, 1.1 equiv.) in the presence of catalytic amounts (1–2% mol/mol) of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as described previously.[7] Under these conditions, the alcohols of **6a** and **6c** was acetylated in 85–90% isolated yield. In a typical rearrangement reaction, *O*-acetylated carboxylic acid of **6c** (0.3 g, 0.97 mmol) was dissolved in CH_2Cl_2 (4 mL, containing one drop of DMF), oxalyl chloride (0.1 mL, 1.2 mmol) was added and the reaction was stirred at room temperature for 30 min. The solvent was then evaporated; the oily residue was redissolved in THF and cooled in an ice bath for 5 min before ammonia gas was bubbled for 5 min through the solution under vigorous stirring. The reaction was left stirring at 4 °C for 3 more hours in a tightly closed vessel, before it was diluted with 10 mL EtOAc and extracted once with aqueous acid (1 N HCl) until neutral solution was obtained. After washing with brine, the organic layer dried with Na_2SO_4 and evaporated to dryness. The oily amide residue redissolved in 10 mL *tert*-butanol containing

one drop of $SnCl_4$, lead tetraacetate [$Pb(OAc)_4$, 0.53 g, 1.2 mmol] was added and the reaction was stirred at 80 °C for 4 h. At the end of the reaction *tert*-butanol was evaporated, product was redissolved in Et_2O (15 mL) and the solution was filtered through celite. After evaporation of Et_2O and silica gel purification the *O*-acetylated N-Boc protected compound **9c** was obtained (0.22 g, 60% yield from the *O*-acetylated carboxylic acid) as a white solid. The same reaction sequence gave **9a** as a white solid in 55% overall yield.

4.4.3. Compound 9c. ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.12 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.46 (s, 9H, $C(CH_3)_3$), 2.09 (s, 3H, $CHOCOCH_3$), 2.9 (m broad, 3H, $CHCO_2C_2H_5$ and CH_2Ph), 3.45+3.5 (m broad+m broad, 2H, CH_2-NHBoc), 4.02 (q, J=7.19 Hz, 2H, $CO_2CH_2CH_3$), 4.75 (m, broad, 1H, $CHOCOCH_3$), 5.17 (m, broad, 1H, CH_2NHBoc), 7.2 (m, 5H, CH_2Ph). ^{13}C NMR (100 MHz, $CDCl_3$) δ=14.08 (s, $CO_2CH_2CH_3$), 20.90 (s, $CHOCOCH_3$), 28.35 (s, $CO_2C(CH_3)_3$), 34.41 (s, $CHCO_2C_2H_5$), 41.73 (s, $CO_2C(CH_3)_3$), 49.54 (s, CH_2Ph), 60.68 (s, $CO_2CH_2CH_3$), 73.44 (s, $CHOCOCH_3$), 79.75 (s, CH_2NHBoc), 126.60+128.46+128.89+138.26 (all s, CH_2Ph), 155.89 (s, $CO_2C(CH_3)_3$), 170.11 (s, $CO_2CH_2CH_3$), 171.90 (s, $CHOCOCH_3$).

4.4.4. Compound 9a. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.88+0.90 (d+d, J=4.00 Hz, 6H, $CH_2CH(CH_3)_3$), 1.25 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.45 (s, 9H, $C(CH_3)_3$), 1.52+1.65 (m broad+m broad, 2H, $CH_2CH(CH_3)_3$), 2.05 (s, 3H, $CHOCOCH_3$), 2.75 (m, 1H, $CHCO_2C_2H_5$), 3.35+3.45 (m broad+m broad, 2H, CH_2NHBoc), 4.1 (q, J=7.19 Hz, 2H, $CO_2CH_2CH_3$), 4.7 (s, broad, 1H, $CHOCOCH_3$), 5.1 (m, broad, 1H CH_2NHBoc).

4.5. Hydrolysis to the mono acids 8 and rearrangement to 10 and 12 (Fig. 4)

Synthesis of statine **10a**, phenylstatine **10c** as well as the cyclic carbamates **12a** and **12c** from the corresponding mono-acids **8** has been described earlier.[7] For the formation of **12b** and **12e** the same reaction sequence was applied. Using the same reaction sequence **12e** was synthesized from **5e** (n=0, Fig. 4) in 67% isolated yield. The only difference is that esterification of the di-acid **7e** was performed at room temperature, since a small amount (5–10%) of diester is forming under the esterification conditions.

4.5.1. Compound 12b. ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.89+0.91 (d+d, J=1.6 Hz, 6H, $CH_2CH_2CH(CH_3)_2$), 1.22 (m, 2H, $CH_2CH_2CH(CH_3)_2$), 1.28 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 1.60 (m, 3H, $CH_2CH_2CH(CH_3)_2$), 2.65+2.7 and 2.78+2.82 (d+d and d+d, J=6.80, 6.40 Hz, 2H, $CH_2CO_2C_2H_5$), 3.51 (q, J=5.19, 7.19 Hz, 1H, CH–NHCO–O–CH), 4.20 (q, J=7.19 Hz, 2H, $CO_2CH_2CH_3$), 4.58+4.60+4.62 (d+d+d, J=4.80 Hz, 1H, CH–NHCO–O–CH), 5.8 (s, broad, 1H, CH–NHCO–O–CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ=14.14 (s, $CO_2CH_2CH_3$), 22.34 and 22.54 (s and s, $CH_2CH_2CH(CH_3)_2$), 27.92 (s, $CH_2CH_2CH(CH_3)_2$), 33.15 (s, $CH_2CH_2CH(CH_3)_2$), 34.06 (s, $CH_2CH_2CH(CH_3)_2$), 39.42 (s, $CH_2CO_2C_2H_5$), 57.84 (s, $CO_2CH_2CH_3$), 61.13 (s, CH–NHCO–O–CH), 78.02 (s, CH–NHCO–O–CH), 158.58 (s, CH–NHCO–O–CH), 169.32 (s, $CO_2CH_2CH_3$).

4.5.2. Compound 12e. ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.28 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 2.89+2.92 and 3.03+3.06 (d+d and d+d, J=8.0, 5.20 Hz, 2H, CH_2Ph), 4.12 (m, 1H, CH–NHCO–O–CH), 4.25 (q, J=7.19 Hz, 2H, $CO_2CH_2CH_3$), 4.68 (d, J=4.80 Hz, 1H, CH–NHCO–O–CH), 5.67 (s, 1H, CH–NHCO–O–CH), 7.2+7.35 (m+m, 5H, CH_2Ph). ^{13}C NMR (100 MHz, $CDCl_3$): δ=14.04 ($CO_2CH_2CH_3$), 41.70 (CH_2Ph), 57.02 ($CO_2CH_2CH_3$), 62.27 (CH–NHCO–O–CH), 77.23 in between the three $CDCl_3$ peaks (CH–NHCO–O–CH), 127.52+129.08+129.21+135.21 (CH_2Ph), 157.33 (CH–NHCO–O–CH), 168.52 ($CO_2CH_2CH_3$).

Protection of the free alcohol of **8e** as an acetate using TMSOTf catalyst and rearrangement to the free amine using [bis(trifluoroacetoxy)iodo]benzene [$(CF_3CO_2)_2PhI$] was performed as described earlier.[7] Under these conditions **8e** (0.3 g, 1.2 mmol) was protected and rearranged giving **10e** (0.15 g, 47% yield from **8e**) as a white solid.

4.5.3. Compound 10e. ^{1}H NMR (400 MHz, D_2O): δ=1.27 (t, J=7.19 Hz, 3H, $CO_2CH_2CH_3$), 2.32 (s, 3H, $CHOCOCH_3$), 3.14 (m, 2H, CH_2Ph), 4.25 (q, overlaps with m, 3H, $CO_2CH_2CH_3$ and $CHNH_2$), 5.17 (d, J=3.20 Hz, 1H, $CHOCOCH_3$), 7.4 (m, 5H, CH_2Ph). ^{13}C NMR (100 MHz, D_2O): δ=15.95 (s, $CO_2CH_2CH_3$), 22.60 (s, $CHOCOCH_3$), 37.96 (s, CH_2Ph), 55.23 (s, $CO_2CH_2CH_3$), 66.98 (s, $CHOCOCH_3$), 73.10 (s, H_2NCH), 130.83+132.13+132.23+136.97 (s, CH_2Ph), 171.25 (s, $CHOCOCH_3$), 174.95 (s, $CO_2CH_2CH_3$).

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Aldehyde-based racemization in the dynamic kinetic resolution of *N*-heterocyclic α-amino esters using *Candida antarctica* lipase A

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Abstract—The present research introduces approaches for the dynamic kinetic resolution of the methyl esters of proline and pipecolic acid. As the result, a method was developed which is based on the acylation of the secondary amino group of the amino esters with vinyl butanoate by *Candida antarctica* lipase A. In the optimized method, acetaldehyde as a racemizing agent is released in situ from vinyl butanoate in the presence of triethylamine, allowing ca. 90% of the racemic proline and 70% of the pipecolic acid methyl esters to be acylated in the forms of highly enantiopure (ee=97%) butanamides with the *S*-absolute configurations.

1. Introduction

N-Heterocyclic amino acids, such as proline and pipecolic acid, are valuable building blocks of many pharmaceuticals.[1] In our previous paper the enzymatic kinetic resolution of methyl pipecolinate (*rac*-**2**; Scheme 1) as a non-proteinogenic α-amino ester was studied.[2] As the most important result, *Candida antarctica* lipase A (CAL-A) was reported to catalyze the acylation of the secondary ring nitrogen with 2,2,2-trifluoroethyl esters in a highly enantioselective manner in organic solvents (enantioselectivity ratio $E>100$).

Dynamic kinetic resolution enables the transformation of a racemate to a new product as one enantiomer. This becomes possible through the racemization of one of the enantiomers whereas the other reacts to a stable product. Most racemizing methods of α-amino acids are based on the acidic α-hydrogen at an amino acid or its derivative. This allows enol formation under acidic and enolate formation under basic conditions. However, free amino acids are generally difficult to racemize. Derivatization of an amino acid as a Schiff base, hydantoin or oxazolone results in a more acidic α-proton, and accordingly promotes racemization.[3–5] Acidic and basic conditions are known to aid aldehyde-promoted racemization through a Schiff base.[6–8] The previously published racemizations of amino acids or esters including the racemization of (*S*)-proline were effectively performed in acetic acid or in the mixture of acetone–acetic acid using various aldehydes or ketones at elevated temperatures.[6,9,10] Traditional racemization by aldehydes is assisted by metal catalysts.[11] Metal catalysts also alone are able to provoke racemization particularly in alkaline solutions.[12]

Several biocatalytic dynamic kinetic resolutions of amino acids have been published including both enzymatic and chemical racemization steps. Especially oxazolones, thiazolones and hydantoins have been used as a starting material.[13] Among amino acid racemases, hydantoin and *N*-acylamino acid racemases have previously gained significance in industrial dynamic kinetic resolution processes.[14] Regarding aldehyde-based methods, salicylaldehyde and pyridoxal have been utilized for the racemization of phenylglycine methyl ester during the lipase-catalyzed ammoniolysis in organic solvents.[15,16] The pyridoxal 5-phosphate-catalyzed racemization of amino esters together with alcalase-catalyzed resolution is also known.[7] Moreover, Schiff bases of α-amino esters have served as starting materials for enzyme-catalyzed ester hydrolysis.[8,17]

The above results prompted us to start work in order to develop an aldehyde-based dynamic kinetic resolution method for *N*-heterocyclic amino esters *rac*-**1** and *rac*-**2** in the presence of CAL-A and an acyl donor (Scheme 1). In order to study the general usability of CAL-A, the normal kinetic resolution of *rac*-**1** and *rac*-**2** as secondary amines and that of some common α-amino esters as primary amines with 2,2,2-trifluoroethyl butanoate have been investigated. Racemization of (*S*)-**1** and (*S*)-**2** in the presence of additives (acids or bases) and aldehydes has been studied. Finally, the dynamic kinetic resolution method was optimized and the preparative scale experiment performed using racemic proline methyl ester as a substrate.

Keywords: Dynamic kinetic resolution; Proline methyl ester; Pipecolic acid methyl ester; *Candida antarctica* lipase A; Aldehyde-based racemization.
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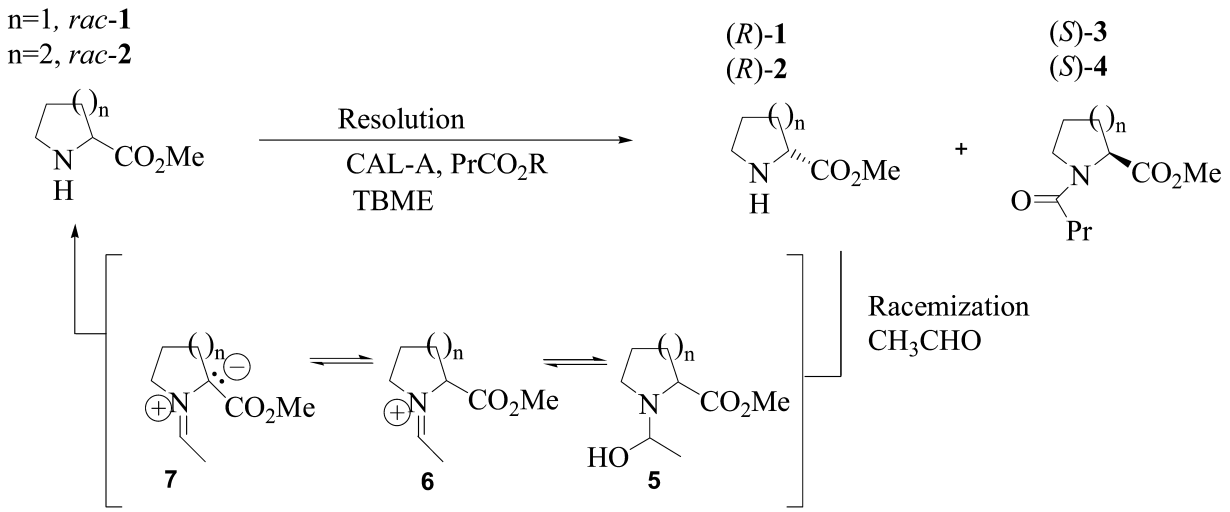

Scheme 1.

2. Results and discussion

In order to develop a satisfactory method for dynamic kinetic resolution where the product enantiomer is formed with high yield and enantiomeric excess, the resolution reaction should be highly enantioselective and the less reactive enantiomer rapidly racemized under the conditions where the product of an enzyme-catalyzed reaction remains stable and the enzyme catalytically active. Under such conditions, the starting material is always practically racemic, allowing the maximal enantiopurity (as determined by the *E* value of the corresponding kinetic resolution) to be reached at the theoretical zero conversion for the more reactive enantiomer throughout the reaction. Thus, it can be calculated that in order to achieve the product with ee>95%, the *E* value for kinetic resolution should be 40 or higher.[18]

2.1. Kinetic resolution

We have shown earlier that CAL-A catalyzes highly enantioselective *N*-acylations between β-amino esters and achiral esters with absolute chemoselectivity while some other common lipases tend to lead interesterification products.[19–22] We have also shown that CAL-A is exceptional among lipases by catalyzing acylations of the secondary amino group of (*S*)-**2**.[2] In order to find the general usability of CAL-A for the resolution of α-amino esters, various methyl esters (Table 1) were subjected to enzymatic resolution under the best conditions of the previous works. According to the results in Table 1, enantioselectivity requirements for a successful dynamic kinetic resolution are properly fulfilled only for the acylation of sterically hindered *N*-heterocyclic amino esters **1** and **2** with 2,2,2-trifluoroethyl esters (entries 16–21). Aspartic acid dimethyl ester as a primary amine is also acceptable with *E*=33 and with a calculated theoretical ee=94% at zero conversion for the (*S*)-amide product (entry 1). However, the observed high stability against racemization did not persuade us to continue studies for its dynamic kinetic resolution. Thus, there was only a small drop in ee from 99 to 73% when the (*S*)-amino ester (0.1 M) was incubated in TBME (*tert*-butyl methyl ether) for 24 h while the enzymatic acylation of the racemic compound with 2,2,2-trifluoroethyl butanoate in TBME smoothly proceeded to 53% conversion in 3 h. On the above basis and because 2,2,2-trifluoroethyl butanoate as an acyl donor (entries 16 and 19) was more effective than the corresponding acetate (entries 18 and 21) the studies were continued using butanoates as achiral acyl donors and *rac*-**1** and *rac*-**2** as substrates.

2.2. Racemization experiments

Racemization of amino esters with aldehydes is a process where an aldehyde reacts with the starting material in the formation of carbinolamine **5** which in the presence of an acid forms a Schiff base **6** (Scheme 1). The subsequent base-catalyzed release of a proton is responsible for the deterioration of enantiopurity in the formation of the carbanion intermediate **7**.[3,7,8] When racemization is planned to proceed in situ at the same time with the enzymatic acylation mild reaction conditions are necessary for the enzyme to stay active. In the present work, methyl ester (*S*)-**1** (0,1 M, ee=99%) and acetaldehyde (0.1 M) were chosen for racemization in TBME with and without additives (Table 2). Carboxylic acids and ammonium acetate (entries 2–4) clearly favour racemization while bases such as triethylamine (entry 6) do not have an impact over the effect of acetaldehyde alone (entry 1). As another observation, the amount of **1** clearly decreases in the presence of carboxylic acids (entries 2 and 3). Salt formation between **1** and the acid is one explanation for the decrease. Indeed, some precipitation was observed in the mixture of **1** or **2** (0.1 M) and acetic or butanoic acid (0.1 M) in TBME at room temperature while the precipitate disappeared at elevated temperature (48 °C). Another possible explanation for the loss of an amino ester is the stabilization of a species such as **6** through salt formation. The loss of **1** when no additives were added shows that part of the starting material is in the form of a Schiff base (entry 1). The formation of various condensation products (although not detected by the present GC method) cannot be totally ruled out either.

Different aldehydes were next tested for the acetic acid-catalyzed racemization of (*S*)-**1** in TBME (Table 3).

Table 1. CAL-A-catalyzed acylation at room temperature of the methyl esters of α-amino acids (0.1 M) with trifluoroethyl butanoate (0.2 M) in TBME (A) and in CH_3CN (B) and with butyl butanoate as a solvent and an acyl donor (C)

Entry	Methyl ester of	Conditions	*E*	*N*-Acylated product formed (%)	Time (h)
1	aspartic acid	A	33±2	16[a]	0.5
2	aspartic acid	B	20±1	19[a]	0.5
3	aspartic acid	C	17±1	9[a]	0.5
4	glutamic acid	A	12±1	37[b]	0.5
5	glutamic acid	B	12±1	33[b]	0.5
6	glutamic acid	C	13	53[c]	0.5
7	methionine	A	10±1	49[c]	0.5
8	methionine	B	7±1	100[c]	0.5
9	methionine	C	8±0	90[c]	0.5
10	phenylglycine	A	7±0	77[c]	0.5
11	phenylglycine	B	1.5±0.1	54[c]	0.5
12	phenylglycine	C	6±0	35[c]	0.5
13	valine	A	15±1	38[c]	0.5
14	valine	B	9±0	44[c]	0.5
15	valine	C	18±0	24[c]	0.5
16	proline (**1**)	A	>100	50[c]	0.5
17	proline (**1**)	C	>100	44[c]	0.5
18	proline[d] (**1**)	A	>100	2	0.5
19	pipecolic acid[e] (**2**)	A	>100	49[c]	9
20	pipecolic acid[e] (**2**)	B	>100	39[c]	22
21	pipecolic acid[d,e] (**2**)	A	>100	29[c]	24

[a] 5 mg/mL of the enzyme preparation.
[b] 10 mg/mL of the enzyme preparation.
[c] 75 mg/mL of the enzyme preparation.
[d] $CH_3CO_2CH_2CF_3$ as an acyl donor.
[e] Ref. 2.

Acetaldehyde (entry 2) is clearly most favourable for the purpose. Increasing acetic acid (entries 1–4, Table 4) and acetaldehyde (entries 5–9) concentrations have a positive effect on racemization although acetic acid (0.1 M, entry 5) alone is not effective. Increasing acetaldehyde concentrations also decrease the amount of **1** in the presence of acetic acid (0.1 M; entries 5–9). This indicates improved possibilities for the adducts like **6** and/or improved possibility for side reactions. As the best compromise, 1 equiv. of acetaldehyde and acetic acid with respect to the starting material were chosen for the subsequent dynamic kinetic resolution experiments.

Contrary to the above results for proline methyl ester, the racemization of (*S*)-**2** proceeds slowly in the presence of acetaldehyde (0.1 M) and various acetic acid concentrations in TBME (Table 4, entries 10–12). Elevated temperature (48 °C) favours racemization and also leads to the enhanced disappearance of the starting material (entries 13–15).

2.3. Dynamic kinetic resolution

As shown in Table 1, the traditional kinetic resolution of *rac*-**1** and *rac*-**2** with 2,2,2-trifluoroethyl butanoate and CAL-A smoothly proceeds in a highly enantioselective manner with *E*>100 in TBME (entries 16 and 19). High enantiopurity is also observed for the formed (*S*)-**3** when vinyl butanoate is used in the place of 2,2,2-trifluoroethyl butanoate although the acylation of *rac*-**1** then is approaching toward the conditions of dynamic kinetic resolution due to the acetaldehyde produced from vinyl alcohol (Fig. 1; Table 5, entry 2). Thus, after an hour 75% of the racemate (●) was transformed to the product (■) with $ee^{(S)\text{-}\mathbf{3}}$=98% and at this point the reaction mixture contained only 4% of the unreacted starting material. The enantiopurity of (*S*)-**4** is also excellent (entry 8) for the acylation of *rac*-**2** with vinyl butanoate, but the yield of the product only slightly increases after 40% is reached (Fig. 2). It is clear according to Figures 1 and 2 that a considerable amount of the starting material has disappeared somewhere.

Three different strategies were now chosen for improving the yield of the dynamic kinetic resolution of *rac*-**1** and *rac*-**2**. In method I, acetic acid and acetaldehyde (both 0.1 M) were added to the reaction mixture where 2,2,2-trifluoroethyl butanoate served as an acyl donor. In methods II and III vinyl butanoate served as an acyl donor and as the in situ

Table 2. Effect of additives on the racemization of (*S*)-**1**[a] (0.1 M) with acetaldehyde (0.1 M) in TBME after 24 h at room temperature

Entry	Additive (0.1 M)	ee (%)	**1** left (%)
1	No additive	72	92
2	Acetic acid	0	86
3	Butanoic acid	0	85
4	$CH_3CO_2^-NH_4^+$	3	96
5	DMAP[b]	72	98
6	Triethylamine	76	93

[a] Original $ee^{(S)\text{-}\mathbf{1}}$=99%.
[b] *N*,*N*-Dimethylaminopyridine.

Table 3. Effect of different aldehydes on the racemization of (*S*)-**1**[a] (0.1 M) in the presence of acetic acid (0.1 M) in TBME after 2 h at room temperature

Entry	Aldehyde (0.1 M)	ee (%)	**1** left (%)
1	Pivalaldehyde	96	100
2	Acetaldehyde	0	95
3	Pyridoxal	97	29
4	Benzaldehyde	10	93
5	4-Methylbenzaldehyde	4	89

[a] Original $ee^{(S)\text{-}\mathbf{1}}$=99%.

Table 4. Effects of acetaldehyde and acetic acid concentrations on the racemization of (*S*)-**1**[a] (0.1 M) and (*S*)-**2**[b] (0.1 M) in TBME after 2 h at room temperature

Entry	Acetic acid (M)	Acetaldehyde (M)	$ee^{(S)\text{-}\mathbf{1}}$ or $^{(S)\text{-}\mathbf{2}}$ (%)	**1** or **2** left (%)
1	0	0.1	**1**; 86	95
2	0.01	0.1	**1**; 79	96
3	0.05	0.1	**1**; 27	96
4	0.1	0.1	**1**; 0	95
5	0.1	0	**1**; 99	97
6	0.1	0.05	**1**; 66	100
7	0.1	0.1	**1**; 0	95
8	0.1	0.25	**1**; 1	86
9	0.1	0.5	**1**; 2	46
10	0	0.1	**2**; 95	100
11	0.1	0.1	**2**; 93	98
12	0.5	0.1	**2**; 83	93
13	0.1[c]	0.1	**2**; 90	100
14	0.25[c]	0.1	**2**; 82	97
15	0.5[c]	0.1	**2**; 76	74

[a] Original $ee^{(S)\text{-}\mathbf{1}}$=99%.
[b] Original $^{(S)\text{-}\mathbf{2}}$=95%.
[c] Temperature 48 °C.

source of acetaldehyde. In method II, the reaction proceeded in the presence of acetic acid (0.1 M) with the purpose to enhance racemization. In method III, triethylamine (or ammonium acetate) was added to the mixture of a substrate (0.1 M) and vinyl butanoate (0.2 or 0.4 M) in TBME in order to enhance racemization and to bind the liberated butanoic acid. It is worth mentioning that 2,2,2-trifluoroethyl and vinyl butanoates as activated esters are partly hydrolyzed by water in the seemingly dry enzyme preparation, always giving some butanoic acid in the reaction mixture independent of which method is considered.

In accordance with the fast racemization in the case of (*S*)-**1** with acetic acid (Table 4, entry 4), *rac*-**1** (0.1 M) was effectively transformed to (*S*)-**3** in TBME using either method I or II (Table 5, entries 1, 3, and 4). The same methods were useless for the dynamic kinetic resolution of *rac*-**2** (entries 7 and 9). Accordingly, the reaction by method I almost stopped at ca. 30% yield for (*S*)-**4** after 16 h and thereafter was slowly approaching to 43% yield at 75% total conversion in 3–4 days. The reaction closely followed the progression curves shown in Figure 2 where no acid was added. There was no increase in the amount of (*S*)-**4** either when normal kinetic resolution was allowed to proceed first to 48% conversion ($ee^{(R)\text{-}\mathbf{2}}$=91% and $ee^{(S)\text{-}\mathbf{4}}$=99%) before acetic acid (0.1 M) and acetaldehyde (0.1 M) were added. Instead and as expected, the less reactive enantiomer was subject to slow racemization ($ee^{(R)\text{-}\mathbf{2}}$=73%, $ee^{(S)\text{-}\mathbf{4}}$=99% after 70 h). Under the conditions of method II, the enzymatic acylation of *rac*-**2** stopped when only 25% of (*S*)-**4** was obtained ($ee^{(S)\text{-}\mathbf{4}}$=96%) at the point where 33% ($ee^{(R)\text{-}\mathbf{2}}$=3) of the starting material was left unreacted (entry 9). Replacing acetaldehyde with benzaldehyde did not alleviate the problem and the acylation of *rac*-**2** in the presence of acetic acid (0.1 M) stopped at 28% yield for the product. The above-suggested improved existence of stable adducts like **6** with an added acid (or that formed from an acyl donor) and the precipitation of amino esters as acetate (or butanoate) salts can explain where at least part of the original substrate disappeared.

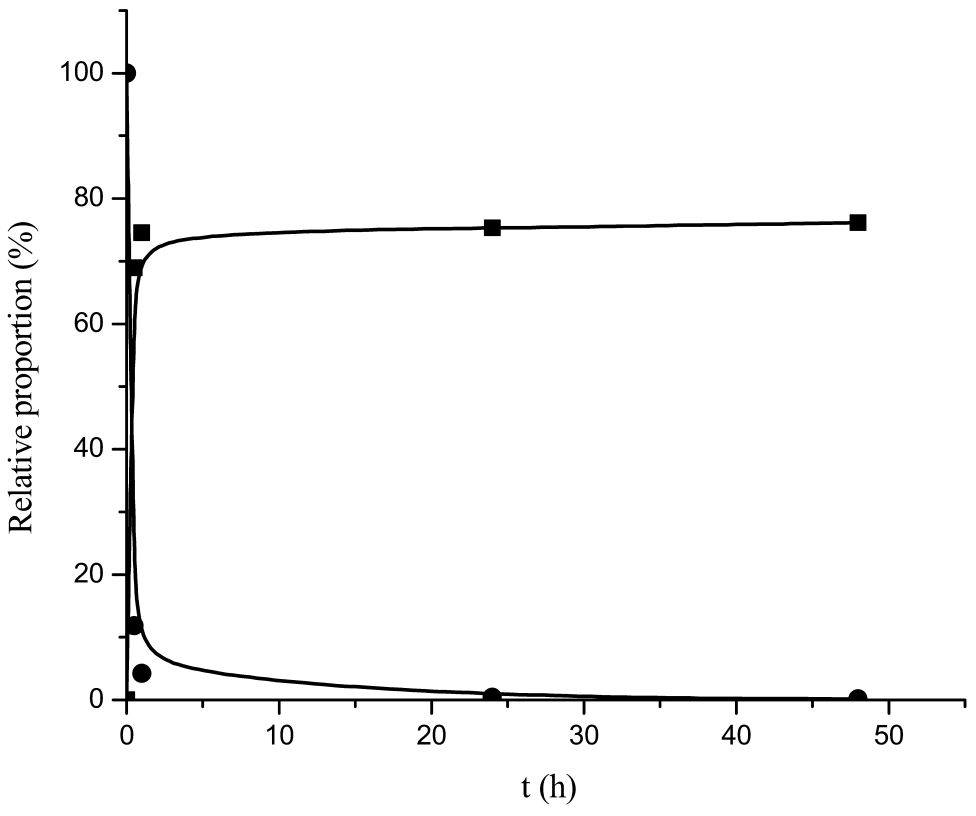

Figure 1. Progression curves for the formation of (*S*)-**3** (■) and disappearance of *rac*-**1** (●) when *rac*-**1** (0.1 M) reacts with vinyl butanoate (0.2 M) in TBME in the presence of CAL-A preparation (75 mg/mL).

Table 5. Dynamic kinetic resolution of *rac*-**1** and *rac*-**2** (both 0.1 M) with $PrCO_2R$ in TBME in the presence of CAL-A-preparation (75 mg/mL) and additives (0.1 M)

Entry	Substrate	R	ee$^{(S)\text{-}\mathbf{1}\text{ or }(S)\text{-}\mathbf{2}}$	(*S*)-**3** or (*S*)-**4** formed[a] (%)	Time (h)	Temperature (°C)	Additive
1	*rac*-**1**	CH_2CF_3[b]	99	97	25	25	CH_3CO_2H
2	*rac*-**1**	$CH{=}CH_2$[b]	98	75	1	25	None
3	*rac*-**1**	$CH{=}CH_2$[b]	96	82	3	25	CH_3CO_2H
4	*rac*-**1**	$CH{=}CH_2$[b]	97	88	3	48	CH_3CO_2H
5	*rac*-**1**	$CH{=}CH_2$[c]	97	86	1	25	$CH_3CO_2^-NH_4^+$
6	*rac*-**1**	$CH{=}CH_2$[c]	97	86	1	25	TEA
7	*rac*-**2**	CH_2CF_3[b]	97	43	187	48	CH_3CO_2H
8	*rac*-**2**	$CH{=}CH_2$[b]	97	39	24	25	None
9	*rac*-**2**	$CH{=}CH_2$[b]	96	25	45	48	CH_3CO_2H
10	*rac*-**2**	$CH{=}CH_2$[c]	97	50	24	25	TEA
11	*rac*-**2**	$CH{=}CH_2$[c]	97	61	24	48	TEA
12	*rac*-**2**	$CH{=}CH_2$[c]	97	69	24	56	TEA

[a] Values indicate maximum yields obtained in the reaction.
[b] 0.2 M.
[c] 0.4 M.

Finally in method III we decided to use base-induced racemization of the Schiff base (although the racemization tests of Table 2 were not promising) and to neutralize the formed butanoic acid by adding triethyl amine (0.1 M) in the enzymatic reaction mixture of *rac*-**1** and *rac*-**2** with vinyl butanoate in TBME. At this stage the concentration of vinyl butanoate was also doubled to 0.4 M. Surprisingly, the addition of triethyl amine caused ca. 10% enhancements in the product yield of (*S*)-**3** (Table 5, entry 6) compared to the case where no additives were present (entry 2). The same result was obtained when 0.05 and 0.1 M triethylamine was used. The presence of ammonium acetate affected in the same way (entry 5). For the acylation of *rac*-**2**, the positive effect of triethyl amine was even more pronounced and still increased at elevated temperature. Finally, ca. 70% of *rac*-**2** was transformed to (*S*)-**4** by refluxing the mixture of *rac*-**2** (0.1 M), vinyl butanoate (0.4 M) and CAL-A preparation (75 mg/mL) in TBME in the presence of triethyl amine (0.1 M) (entry 12).

From an economical point of view, vinyl acetate should be preferred over vinyl butanoate as an achiral acyl donor. When vinyl butanoate was replaced with vinyl acetate in the presence of triethyl amine 84% of initially *rac*-**1** was transformed to (*S*)-**3** with only 92% ee in 24 h at 48 °C. The reaction at room temperature stopped when 77% product was yielded. Due to higher enantiopurity, the dynamic kinetic resolution of *rac*-**1** in gram-scale was finally performed with vinyl butanoate as described in Section 4 using method III.

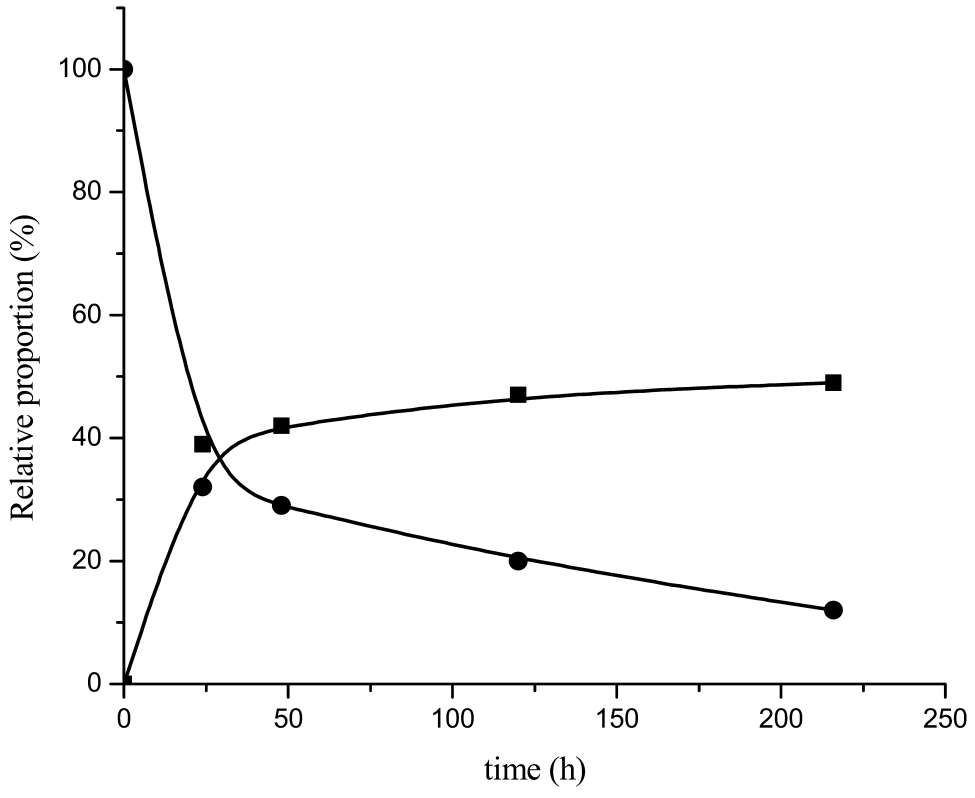

Figure 2. Progression curves for the formation of (*S*)-**4** (■) and disappearance of *rac*-**2** (●) when *rac*-**2** (0.1 M) reacts with vinyl butanoate (0.2 M) in TBME in the presence of CAL-A preparation (75 mg/mL).

3. Conclusions

The previously reported[2] exceptional property of lipase A from *Candida antarctica* to catalyze highly enantioselective *N*-acylations of *N*-heterocyclic amino esters as secondary amines has been confirmed in the present work. This property was used for dynamic kinetic resolution studies where the methyl esters of proline (*rac*-**1**) and pipecolic acid (*rac*-**2**) have served as racemic substrates and vinyl butanoate and 2,2,2-trifluoroethyl butanoate as achiral acyl donors in TBME (Tables 1 and 5). Negligible to moderate enantioselectivity with *E* values from 1 to 33 did not give reasons to use CAL-A for the dynamic kinetic resolution of various natural α-amino esters through the acylation of primary amino groups (Table 1).

For the enzymatic acylation of *rac*-**1** with vinyl esters the reaction was clearly approaching the conditions of dynamic kinetic resolution with 75% yield for (*S*)-**3**, showing the aldehyde-based racemization ability of the less reactive (*R*)-**1** under the acylation conditions (Scheme 1; Fig. 1; Table 5, entry 2). For the acylation of *rac*-**2** the reaction tended to stop when only 40% of the starting material was transformed to (*S*)-**4**. Three different strategies were chosen for improving the yields. In methods I and II, acetic acid served to catalyze aldehyde-based racemization. The methods failed badly with *rac*-**2** as the substrate. Salt formation between the acid and amino esters and/or intermediates such as **6** was suggested to explain the observed loss of the substrate and accordingly the low yield for the produced amide (*S*)-**4**. In method III, triethyl amine was added to the acylation mixture of *rac*-**1** and *rac*-**2** with vinyl butanoate in order to bind the butanoic acid which is liberated when the acyl donor is enzymatically hydrolyzed by the water in the enzyme preparation. In methods II and III the use of vinyl butanoate allows the releasing acetaldehyde to racemize the amino ester in situ. Under optimized conditions, ca. 90% of the racemic proline and 70% of the pipecolic acid methyl esters were acylated to highly enantiopure (ee=97%) butanamides with the *S*-absolute configurations.

4. Experimental

4.1. Materials and methods

Racemic proline, butyl butanoate and (*S*)-glutamic acid were obtained from Acros, methyl pipecolinate hydrochloride, valine, (*S*)-valine methyl ester hydrochloride, racemic and (*R*)-phenylglycine, racemic and (*R*)-methionine, *tert*-butyl methyl ether and dihexyl ether from Aldrich, methanol and petroleum ether from J. T. Baker and thionyl chloride from Riedel De-Haën. Vinyl butanoate, hexadecane, ethyl acetate, glutamic acid and (*S*)-proline were the products of Fluka. Dimethyl aspartate was a product of Sigma. All solvents were of the best analytical grade. (*S*)-methyl pipecolinate (ee=95%) was the product of the enzymatic kinetic resolution.[2] Amino acid methyl esters were synthesized by esterifying the acid with thionyl chloride in methanol followed by bubbling with ammonia in chloroform. Trifluoroethyl esters were synthesized from trifluoroethanol and an acid chloride. Absolute configurations were determined by synthesizing an enantiopure amino ester from a commercially available amino acid and by comparing the peak of the enantiomer with the peaks of a racemic compound in a chromatogram.

CAL-A was the product of Roche (Chirazyme L5, lyo.). Before use, CAL-A (20% (w/w)) was adsorbed on Celite by dissolving the enzyme (5 g) and sucrose (3 g) in Tris–HCl buffer (250 mL, 20 mM, pH 7.8) followed by the addition of celite (17 g). The mixture was dried by letting water evaporate. The enzyme preparation gave the initial velocity of 0.028 mmol/min/g for the acylation of racemic valine methyl ester (0.1 M) with trifluoroethyl butanoate (0.2 M) in TBME ($E=15.5\pm0.2$).

^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ on a Jeol Lambda 400 Spectrometer tetramethylsilane being as an internal standard. MS-spectrum was recorded on a VG Analytical 7070E instrument equipped with a VAXstation 3100 M76 computer. Optical rotation was measured using a Jasco DIP-360 polarimeter. The determination of *E* was based on equation $E=\ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$.[18] Using linear regression *E* was achieved as the slope of a line.

Typical reaction volume for enzymatic reactions was 1–3 mL. Substrate (0.1 M) and an acyl donor (0.2 M) were dissolved in TBME and an acid or base and acetaldehyde were added. CAL-A preparation (5–75 mg/mL) started the reaction. The progress of the reactions was followed by GC on Chrompack CP-Chirasil-DEX CB or Chrompack CP-Chirasil-L-Valine column by taking samples (0.1 mL) at intervals and derivatizing them with acetic anhydride (butanoate as an acyl donor) or butanoic anhydride (acetate as an acyl donor) and *N*,*N*-dimethylaminopyridine in pyridine (1% solution). *N*,*N*-dimethylaminopyridine solution was not used for those samples where aldehyde-induced racemization was present. Quantitative analysis of the reactions was performed by using dihexyl ether or hexadecane as internal standards (0.1 or 0.2 M).

4.1.1. Enzymatic gram-scale resolution. Before performing a gram scale reaction the amount of the enzyme preparation was optimized and as the best compromise 25 mg/mL was chosen. Vinyl butanoate (5.9 mL, 46.5 mmol) and *rac*-**1** (1.50 g, 11.6 mmol) were dissolved in TBME (116 mL). Addition of triethylamine (1.6 mL, 11.6 mmol) and CAL-A on Celite (2.904 g, 25 mg/mL) started the reaction. The reaction was stopped when 90% of *rac*-**1** was transformed to (*S*)-**3** after 5 h by filtering off the enzyme. The crude product was purified by column chromatography on Silicagel. After first purification (EtOAc–petroleum ether (3/7)) the product still contained butanoic acid. It was removed by dissolving the crude product in methanol and by adding thionyl chloride (200 μL) in an ice bath. After evaporation the product was repeatedly purified by column chromatography yielding (*S*)-**3** (viscous liquid, 1.79 g, 78%, 9.0 mmol, ee=97%, $[\alpha]_D^{20}$ −96.4 (*c*=1.04, MeOH)).

(*S*)-**3**. ^{1}H NMR: δ (ppm) 0.99 (t, *J*=7.4 Hz, 3H, CH_3CH_2), 1.69 (m, 7.6 Hz, 2H, CH_3CH_2), 1.90 (m, 2H, CH_2CON), 2.0–2.4 (m, 4H, CH_2CH_2CHN), 3.53 (m, 2H, CH_2N), 3.72

(s, 3H, CO_2CH_3), 4.49 (m, J=4.0, 8.5 Hz, 1H, C*H*), ^{13}C NMR: δ (ppm) 13.7 (CH_3CH_2), 17.9 (CH_3CH_2), 24.7 (CH_2CH_2CH), 29.1 (CH_2CH), 36.2 (CH_2CO), 46.9 (CH_2N), 52.0 (CO_2CH_3), 58.4 (*C*H), 171.8 (*C*O), 172.9 (*C*O). HRMS calculated for $C_{10}H_{17}NO_3$ M^+=199.1208. Found M^+=199.1216.

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Novel reaction systems for the synthesis of *O*-glucosides by enzymatic reverse hydrolysis

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Abstract—Our studies are presented to replace alcohols as solvents in reverse hydrolytic reactions catalyzed by immobilized β-glucosidase to synthesize *O*-substituted β-D-glucopyranosides in preparative-scale. We found that 1,2-diacetoxyethane is a suitable solvent and *O*-alkyl or aryl β-D-glucosides were synthesized in moderate yields (after isolation 12–19%). In these reactions proportion of glucose and glucosyl acceptor hydroxy compounds was 1:20. We suggest that 1,2-diacetoxyethane can be useful not only for alcohols but for other glucosyl donor compounds unsuitable for the role of solvent (e.g., phenols) in the synthesis of *O*-β-D-glucosides by reverse hydrolysis.

1. Introduction

It is known that enzymes are important catalysts in non-conventional media.[1] In chemical, pharmaceutical and food industry different hydrolases—proteases, esterases and glycosidases are used to catalyze the synthesis of biologically active peptides, esters and glycosides. In aqueous solutions, enzymatic hydrolytic reactions catalyzed by hydrolases are practically irreversible, but hydrolysis can be converted to synthesis by reducing the water content of the medium via using organic solvents—this is reverse hydrolysis.[2] Stereoselectivity and regioselectivity in enzymatic reactions are generally higher than in chemical syntheses.[3] In the presence of organic solvents, the solubility of substrate and/or product may be improved but the solubility, stability and activity of some enzymes can be diminished,[4] therefore immobilized enzymes are often used.[5] In most cases, some water is required to maintain the native conformation of the enzyme.[6]

Environmentally friendly *O*-alkyl glucosides are non-ionic and non-toxic surfactants[7] with antimicrobial ability and biodegradability.[8,9] Some of them are used in the detergent, food and pharmaceutical industry.[10,11] The synthetic activity of β-glucosidase in reverse hydrolytic processes was tested by Vulfson[12] then later by Laroute and Willemot.[13]

Earlier we found that native glucosidases have significant *O*-glucosylation activity using glucose and different alcohols, but upscaling of the reactions reduced yields sharply because of heterogenity of reaction mixtures and enzyme deactivation in organic media. Yields were enhanced when immobilized forms of glucosidases on a modified polyacrylamide-type bead support (Acrylex C-100) were used.[14] It was a general supposition in the literature that alcohols have to be both glucosyl acceptors and solvents in these glucosylation reactions.[13]

Recent results of our study are presented to replace alcohols in solvent function in preparative-scale reverse hydrolytic synthesis of *O*-substituted β-D-glucopyranosides.

2. Results and discussion

The study of synthetic reactions was connected with the examination of enzyme deactivation in these reactions. The water content of the mixtures was chosen on the basis of our earlier results.[14] Deactivation of immobilized β-glucosidase was characterized in two different ways. The effect of reverse hydrolytic reaction mixtures on enzyme activity was characterized by immediate deactivation when the enzyme activities in different reaction mixtures were compared to the original enzyme activity measured in aqueous mixture (it was taken as hundred percent). The effect of reverse hydrolytic reaction mixtures on enzyme stability was characterized by deactivation kinetics when the loss in enzyme activity during reverse hydrolysis was compared to the enzyme activity at the start of the reaction (immediate deactivation value was taken as hundred percent).

Keywords: β-Glucosidase; Immobilization; Enzyme reaction in non-conventional media; Reverse hydrolysis; *O*-Glucosylation; 1,2-Diacetoxyethane.
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2.1. Stability of immobilized β-glucosidase in alcohol–organic solvent mixtures of different water content

Different organic solvents as co-solvents were tested: hydrophilic aprotic solvents (e.g., dimethylformamide), ethers (e.g., dioxane), ketones (e.g., acetone), hydrocarbons (e.g., hexane), esters (e.g., ethyl acetate, 1,2-diacetoxyethane) and other solvents (e.g., acetonitrile). Immediate deactivation and deactivation kinetics of immobilized β-glucosidase were tested in reaction mixtures containing different alcohols of water content 5–10–15% and organic solvent content 0–5–50%. Generally the organic solvents tested decreased stability of immobilized β-glucosidase, moreover most of them caused an immediate and complete loss in enzyme activity. However, we found that in the presence of 1,2-diacetoxyethane in reaction mixtures the enzyme activity values of immobilized β-glucosidase (Table 1) were reduced only to one-half and its stability values (Table 2) were similar to data in pure alcohols.

Table 1. The immediate deactivation of immobilized β-glucosidase (expressed in residual activity %) in alcohol–1,2-diacetoxyethane mixtures

Alcohol (5–15% water content in the reaction mixture)	1,2-Diacetoxyethane content %		
	0	5	50
n-butanol	52±5	33±5	25±6
n-pentanol	42±4	27±5	22±5
n-hexanol	65±5	37±5	30±6
Cyclohexanol	71±7	38±5	28±7

The activity of the enzyme in aqueous reaction mixture was taken as hundred percent. For the mixtures of different water content mean values are given and differences (±) are presented.

Table 2. Residual activity (%) of immobilized β-glucosidase in alcohol–1,2-diacetoxyethane mixtures at the end of six-day reverse hydrolytic reaction at room temperature

Alcohol (5–15% water content in the reaction mixture)	1,2-Diacetoxyethane content %		
	0	5	50
n-butanol	42±4	40±4	32±6
n-pentanol	42±5	38±4	33±6
n-hexanol	43±6	41±5	34±8
Cyclohexanol	41±4	39±5	35±7

The activity of the enzyme at the start of the reaction was taken as hundred percent.For the mixtures of different water content mean values are given and differences (±) are presented.

Organic solvents may affect the properties of enzymes in several ways including interactions with the hydration layer essential for catalysis and proper folding as well as alteration of protein conformation by direct interaction with protein solvation sites either hydrophobic or hydrogen bonding.[4] It is known that glucosidases show higher affinity for hydrophobic substrates than hydrophilic ones. In transglucosylation reactions, hydrophobic acceptors like alcohols and diols produce glucosides but very hydrophilic alcohols, for example, glycerol cannot not be transglucosylated.[15] It was presumed that this kind of co-solvent can weaken the hydrophobic interaction between substrate alcohol and amino acid side chains of the enzyme binding sites.[16] It is attributed to the hydrophobic character of 1,2-diacetoxyethane (on the basis of log *P* values slightly stronger than that of the alcohols examined) that the decrease in hydrophobic enzyme–substrate interactions was small.

Rate constants (k) ($10^{-3}\,h^{-1}$) of deactivation kinetics of immobilized β-glucosidase during reverse hydrolysis were determined for hexanol-1,2-diacetoxyethane–water mixtures at room temperature. They were obtained from the slope of the regression in the plots of lg residual enzyme activity versus reaction time [$\lg(A/A_o)=-kt$] (detailed only for hexanol of 50% 1,2-diacetoxyethane content in Fig. 1). The results compiled in Table 3 and Figure 1 suggest first order kinetics for inactivation.

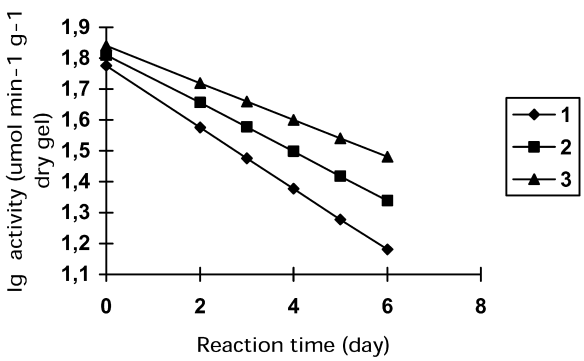

Figure 1. Plot of the logarithm of the residual enzyme activity as a function of reaction time in deactivation of immobilized β-glucosidase during reverse hydrolysis in hexanol with 50% 1,2-diacetoxyethane content at room temperature. Water content 5% (1), 10% (2), 15% (3).

Table 3. Rate constants (k) ($10^{-3}\,h^{-1}$) of deactivation of immobilized β-glucosidase during reverse hydrolysis in hexanol-1,2-diacetoxyethane–water mixtures at room temperature

Content of mixture	Water content		
	5	10	15
(1)	100	116.4	144.5
(2)	98	107	126.5
(3)	72	92.6	120.5

Content of mixture (1) hexanol, (2) hexanol with 5% 1,2-diacetoxyethane content, (3) hexanol with 50% 1,2-diacetoxyethane content.

2.2. Reverse hydrolysis catalyzed by immobilized β-glucosidase in alcohol–organic solvent mixtures of different water content

There are only a few published data in the literature for replacing glucosyl acceptor alcohols as solvents in reverse hydrolysis, for example, *O*-octyl-β-D-glucopyranoside was obtained in 8% yield using a 60:30:10 (v/v) *tert*-butanol–octanol–water mixture.[17] The yields of *O*-glucosylations with glucose as glucosyl donor and different alcohols both as glucosyl acceptors and solvents in reverse hydrolytic processes with different water content catalyzed by immobilized β-glucosidases in absence and presence of different organic solvents as co-solvents were studied. In most of the cases of the organic solvents examined as co-solvents, the synthetic reactions (e.g., dimethylformamide, dioxane, acetone, hexane, ethyl acetate and acetonitrile) practically failed. The yields in alcohol-1,2-diacetoxyethane

(1:1) mixtures were between 30–58% on the basis of HPTLC method. The best results were found for all alcohols studied at 10% water content after 6 days at room temperature (Table 4). We ascribe the efficiency of 1,2-diacetoxyethane as co-solvent in *O*-glucosylation reactions by reverse hydrolysis to its hydrophobic character (slightly stronger than that of the alcohols examined) and water solubility (143 mg ml^{-1}). We found no direct correlation between stability data of glucosidases or yield of reverse hydrolyses and different physical parameters (e.g., dielectric constant, log *P*, solubility in water) of both alcohols and organic solvents or 1,2-diacetoxyethane content of the reaction mixtures.

Table 4. Maximum yields (%) of *O*-glucosylation reactions catalyzed by immobilized β-glucosidase in different alcohol–1,2-diacetoxyethane mixtures (% water content) at room temperature (reaction time 6 days) on the basis of HPTLC method

Alcohol	1,2-Diacetoxyethane content (%)		
	0	5	50
n-butanol	45 (15)	43 (15)	43 (10)
n-pentanol	48 (15)	47 (15)	46 (10)
n-hexanol	57 (15)	58 (15)	60 (10)
Cyclohexanol	52 (15)	54 (10)	56 (10)

2.3. In preparative-scale reactions in 1,2-diacetoxyethane of 10% water content as solvent

We found that in reverse hydrolysis catalyzed by immobilized β-glucosidase to synthesize *O*-alkyl β-D-glucosides, alcohols as solvents can be replaced by 1,2-diacetoxyethane therefore the synthesis of *O*-alkyl-β-D-glucosides in presence of 1,2-diacetoxyethane in preparative-scale was attempted. The reaction mixtures were evaporated in vacuo on a larger scale (boiling point of 1,2-diacetoxyethane is only 79–81 °C at 1.45×10^{-3} Pa) and glucosides were isolated by thick layer chromatography of residues. We prepared not only *O*-alkyl β-D-glucosides which were synthesized earlier in alcohols as solvents[14] but some other *O*-alkyl and aryl β-D-glucosides were formed by reverse hydrolysis catalyzed by immobilized β-glucosidase in 1,2-diacetoxyethane in preparative-scale. *O*-Aryl β-D-glucosides have never been synthesized by reverse hydrolysis.

3. Conclusion

We found that 1,2-diacetoxyethane is a suitable solvent to replace alcohols as solvents in reverse hydrolytic reactions catalyzed by immobilized β-glucosidase to synthesize *O*-subsituted (alkyl or aryl) β-D-glucosides. In these reactions, proportion of glucose and glucosyl acceptor hydroxy compounds was 1:20. We suggest that 1,2-diacetoxyethane can be useful not only for alcohols but for other glucosyl donor compounds unsuitable for the role of solvent (e.g., phenols) in the synthesis of *O*-β-D-glucosides by reverse hydrolysis.

4. Experimental

4.1. Materials

β-Glucosidase (G 0395) from almonds (EC 3.2.1.21), *n*-butanol, *n*-pentanol, *n*-hexanol and cyclohexanol were SIGMA products. Other hydroxy compounds, D-glucose, D-cellobiose, 1,2-diacetoxyethane (ethylene glycol diacetate) and other chemical materials were purchased from FLUKA. β-Glucosidase was immobilized on a modified polyacrylamide-type bead support (Acrylex C-100) possessing carboxylic groups by the carbodiimide method described earlier.[14] Immobilized β-glucosidase showed an activity per gram dry gel of 153 μmol min^{-1} (V_{max} 233 μmol min^{-1}, K_M 0.1 M). Binding capacity of the support was 6.2±0.3 mequiv. per gram dry gel, particle size 100–320 μm. Activity of β-glucosidase was measured according to the literature[18] with substrate cellobiose (0.25 mol l^{-1}) in sodium acetate buffer (0.1 mol l^{-1}, pH 5.1).

4.2. General procedures for reverse hydrolysis

4.2.1. In different alcohol–organic solvent mixtures of different water content. Reaction mixtures (0.5 ml) containing immobilized β-glucosidase (3 mg) and glucose (9 mg, 0.05 mmol) in different alcohol–organic solvent mixtures of 5–10–15% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed twice with distillated water (1 ml). Combined filtrate was analysed for glucose and *O*-alkyl glucoside content and the activity of washed immobilized enzyme was determined.

Concentration of unreacted glucose was measured by enzymic analysis using hexokinase, glucose-6-phosphate dehydrogenase and NADP (Sigma) then mixtures were evaporated to dryness in vacuo. The *O*-alkyl glucoside content of residues was analysed by Chrompress LABOR-MIM (Hungary) HPTLC[19] and Ultrascan XL LKB laser densitometer. Yields were determined on the basis of HPTLC method.

4.2.2. In different alcohol–1,2-diacetoxyethane mixtures of different water content. Reaction mixtures (0.5 ml) containing immobilized β-glucosidase (3 mg) and glucose (9 mg, 0.05 mmol) in different alcohol–1,2-diacetoxyethane mixtures of 5–10–15% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed twice with distillated water (1 ml). Combined filtrate was analysed for glucose and *O*-alkyl glucoside content and the activity of washed immobilized enzyme was determined.

Concentration of unreacted glucose was measured by enzymic analysis using hexokinase, glucose-6-phosphate dehydrogenase and NADP (Sigma) then mixtures were evaporated to dryness in vacuo. The *O*-alkyl glucoside content of residues was analysed by Chrompress LABOR-MIM (Hungary) HPTLC[19] and Ultrascan XL LKB laser

densitometer. Yields are given on the basis of HPTLC method (Table 4).

4.2.3. In preparative-scale reactions in 1,2-diacetoxyethane of 10% water content as solvent. Reaction mixtures (5 ml) containing immobilized β-glucosidase (30 mg), glucose (0.18 g, 1 mmol) and hydroxy compound (20 mmol) in 1,2-diacetoxyethane of 10% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed three times with distillated water (5 ml). The isolation of glycosides by evaporation of filtrate in vacuo and preparative layer chromatography of residue on Kieselgel PF_{254} layer by chloroform–ethyl acetate–methanol mixtures as eluants gave pure *O*-alkyl and aryl β-D-glucosides.

4.3. Structure identification

All of *O*-alkyl and aryl β-D-glucosides synthesized in preparative-scale are known in the literature and they were identified by the authentic samples. Their optical purity was controlled by their ^{1}H-NMR spectra according to the literature[20] (δ 4.50–4.56 ppm 1 H, d, 1-H). ^{1}H-NMR spectra were taken on a Varian EM-390 90 MHz instrument using TMS as internal standard in DMSO-d_6. Yields after isolation are given in parentheses.

O-*n*-Butyl β-D-glucopyranoside[15] (15%);
O-*n*-Pentyl β-D-glucopyranoside[21] (17%);
O-*n*-Hexyl β-D-glucopyranoside[11] (19%);
O-Cyclohexyl β-D-glucopyranoside[21] (19%);
O-*n*-Octyl β-D-glucopyranoside[10] (18%);
O-*n*-Nonyl β-D-glucopyranoside[10] (16%);
O-*n*-Decyl β-D-glucopyranoside[10] (12%);
O-Phenyl β-D-glucopyranoside[22] (13%);
O-4-Nitrophenyl β-D-glucopyranoside[23] (11%).

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Enzymatic resolution of 4-*N*-phenylacetylamino-derivatives obtained from multicomponent reactions using PenG amidase and in silico studies

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Abstract—The three component coupling reaction of aldehydes, phenylacetamide and dienophiles gave the corresponding *N*-phenylacetamidocyclohexene derivatives in good yields (55–70%) and high regioselectivity. For the first time, enzymatic kinetic resolution of this class of compounds has been achieved. The enantioselective hydrolysis of 4-*N*-phenylacetylamino-*cis*-3a,4,7,7a-hexahydro*iso*indole-1,3-dione derivatives using Penicillin G amidase (PGA) from *Escherichia coli* gave the remaining enantiomer with ee-values from 30 to 70% at 50% conversion. On the basis of molecular modeling predictions, 1-*N*-phenylacetylamino-2-cyano-5-cyclohexene derivatives were synthesized. The kinetic resolution resulted in ees up to 99%. The differences of the observed selectivities confirmed the in silico predictions based on the simulation of enzyme–substrate interactions.

1. Introduction

The efficient synthesis of enantiomerically pure compounds is still a challenging task in organic synthesis.[1] In addition to diastereoselective syntheses and asymmetric catalysis, resolution procedures play an important role to obtain enantiomerically pure compounds. With regard to kinetic resolution methods, enzymes, especially hydrolases, are attractive catalysts for these reactions. In general, they are easy to handle, often there is no need to employ co-factors and they work under mild conditions. One of their major advantages of bio-catalysts is the fact, that their substrate tolerance is comparably broad.[2] Hence, they have been applied successfully for the resolution of various racemic compounds on laboratory and industrial scale.[3] Some of us have been involved in the synthesis of amino acid derivatives[4] and amines[5] applying domino or multicomponent coupling reactions (MCR). More recently, a new three-component coupling procedure for the preparation of highly substituted 1-amido-cyclohexenes and 1-amido-3,5-cyclohexadienes starting from simply available substrates like amides, aldehydes and dienophiles (AAD reaction) was discovered.[6] As shown in Scheme 1 these AAD reactions take advantage of the intermediacy of 1-(*N*-acylamino)-1,3-butadiene species[7] which are subject to in situ trapping with electron-deficient dienophiles. Up to four stereogenic centers arise from the formation of three carbon–carbon bonds and one carbon–nitrogen bond over the course of the reaction. Although these multicomponent coupling reactions exhibit very high *endo* selectivity in most cases, the products are obtained as racemic mixtures. Interestingly, a closer look at the structure of the MCR products reveal that they can be easily refined to pharmacologically attractive targets such as lycoricidene,[8] pancratistatine,[9] and narciclasine[10] analogues.

Having a variety of such AAD building blocks in hand, we became attracted by the potential enantioselective synthesis of the substrates.

Based on the selective cleavage of the *N*-acyl amino group by hydrolases a more general resolution of different substrates should be possible. Here, we report on the first successful kinetic resolution of this type of MCR products and the enantioselective synthesis of this class of compounds. Furthermore, the observed substrate specificity of the used Penicillin G amidase (PGA) is discussed on the basis of molecular modeling studies.

2. Results and discussion

At the beginning of our work we selected a small library of

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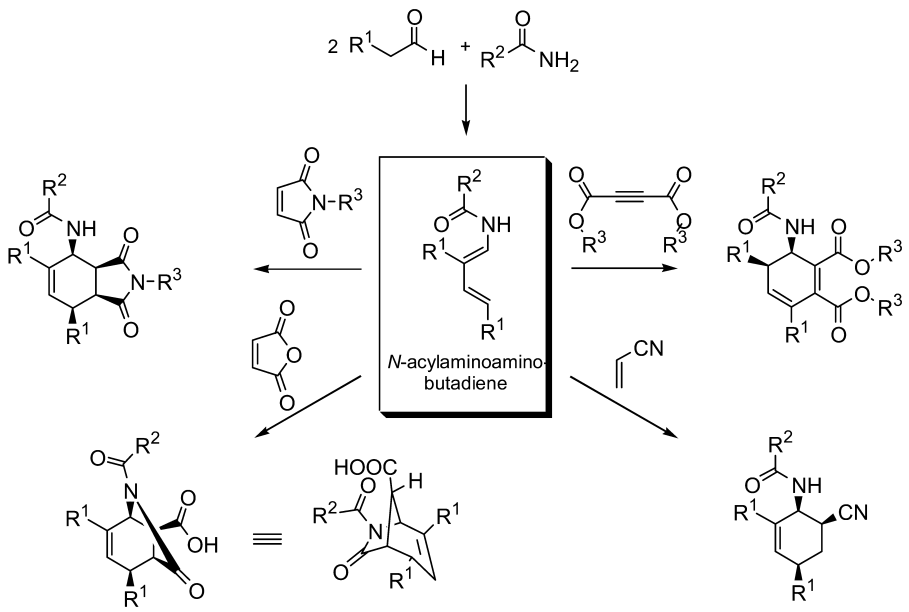
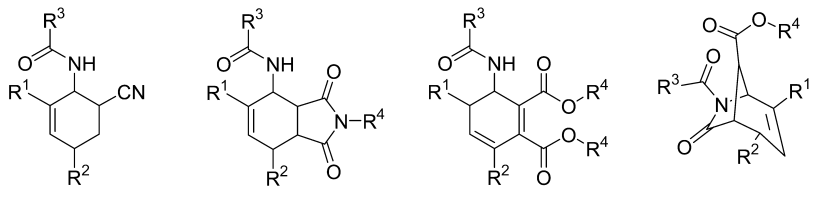


Scheme 1. MCR of aldehydes, amides and dienophiles (AAD reaction).

10 differently substituted AAD compounds **1**–**10** for kinetic resolution (Scheme 2). All substrates share, in common, the *N*-acyl amino group, which is caused by the use of an amide in the AAD reaction. The major difference in the structure of the starting materials is caused by the variation of the dienophile. More specifically we employed two 1-*N*-acylamino-2-cyano-5-cyclohexene derivatives (**1**–**2**), three 4-*N*-acylamino-*cis*-3a,4,7,7a-hexahydro*iso*indole-1,3-dione derivatives (**3**–**5**), two 1-*N*-acylamino-*trans*-3,4-dihydrophthalic acid ester derivatives (**6**–**7**) and three 6-acyl-7-oxo-azabicyclo[3.2.1]oct-3-ene-8-carbonic acid derivatives (**8**–**10**) for our investigations.

To find a suitable enzyme for kinetic resolution all products **1**–**10** were subjected to an enzyme-screening using a pH-indicator assay.[11] Despite the use of more than 150 different hydrolases (lipases, esterases, proteases, acylases, amidases and nitrilases), we did not observe any kinetic resolution reaction at all.

In order to realize an enantioselective hydrolysis of the amide bond, another approach was developed. Apparently, the selective hydrolysis of the acetamido or benzamido group is difficult. Hence, the question arose, whether other *N*-acyl amino groups can be more easily cleaved by enzymes. Various research groups demonstrated that PGA is able to cleave the phenylacetyl-moiety with high selectivity from a wide variety of unnatural substrates like



1, 2 **3, 4, 5** **6, 7** **8, 9, 10**

compound	R^1	R^2	R^3	R^4
1	Me	Me	Me	-
2	H	Me	Me	-
3	H	H	*o-Br*-Ph	Me
4	Et	Et	Ph	H
5	Me	Me	N(Me)2	H
6	i-Pr	i-Pr	Me	Et
7	Me	Me	Ph	Et
8	Bz	Bz	Me	H
9	i-Pr	i-Pr	Me	H
10	Bn	Bn	Ph	Me

Scheme 2. Products of the multicomponent reaction of aldehydes, amides and dienophiles (AAD reaction) used as substrates in the enzymatic resolution.

secondary amines and alcohols.[12] The natural role of this enzyme is the hydrolysis of the phenylacetyl-moiety of the antibiotic Penicillin G. In order to investigate a kinetic resolution of AAD-products by PGA, the phenylacetyl-moiety was introduced into the AAD-products by using phenylacetamide as amide source in the three-component reaction.

In Table 1 the synthesis of 'tailor-made' substrates for PGA hydrolysis is shown. By variation of aldehyde and dienophile we were able to synthesize a small library of new AAD-products in yields from 55 to 70%. Reactions with maleimide and acrylonitrile selectively led to the *endo* adducts, verifying that only the *s-cis*-1*E*,3*E*-amidodiene isomer cyclized in Diels–Alder fashion. However, we cannot exclude the existence of equilibrating isomeric amidodienes under the reaction conditions (20 h, 120 °C).

Next, we tested the different products in kinetic resolutions in the presence of PGA. In case of 4-*N*-phenylacetylamino-*cis*-3a,4,7,7a-hexahydro*iso*indole-1,3-dione derivatives (**11**–**16**), enantioselective hydrolysis with ee-values from 30% (**13**) to 70% (**11**) is observed (Table 2). The highest ee is obtained with the least sterically hindered substrate bearing only H-atoms. Clearly, in most cases the obtained ee-values are not sufficient for preparative scale use. Nevertheless, these are the first enantioselective resolution processes of this class of compounds.

Compounds **17**–**20** were rationally designed on the basis of in silico studies of their interactions with the active site of PGA. Applying 1-*N*-phenylacetylamino-2-cyano-5-cyclohexene derivatives the substitution pattern on the cyclohexene ring revealed a major influence on the enantioselectivity (Table 3). For example, hydrolysis of 1-*N*-phenylacetylamino-2-cyano-5-cyclohexene **17** led to 65% ee of the AAD product at 50% conversion. However, in case of the dimethyl- and diethyl-substituted substrates **18** and **19**, respectively, we did not observe any kinetic resolution reaction at all. As expected from the calculations as described below, resolution of the mono-methyl-substituted substrate **20** gave the best ee-value of >99%.

In order to understand the obtained experimental results in more detail, molecular mechanics calculations of the enzymatic resolution process were carried out. Recently, the GRID/tetrahedral intermediate approach was proposed as a powerful tool for the study of selectivity[13] and especially of the enantioselectivity of PGA[14] and proved to be effective for the enantioselectivity prediction and explanation. The predictions demonstrated to be in good agreement, although not quantitatively, with experimental data for eight different nucleophiles, mostly aminoesters. The same approach was used for substrates **17**–**20** in order to gain further information about the enantiospecific recognition of this class of substrates, which were synthesized in order to give better selectivities in the kinetic resolution. The model construction consisted in the calculation of GRID molecular interaction fields (MIFs) into the enzyme active site and the design of the tetrahedral intermediate of the hydrolysis reaction, using the MIFs as guide for the docking of the substrates into the enzyme.[13,14] Molecular dynamics simulations and energy minimisations of the chemical species allowed to calculate the difference of energy of the tetrahedral intermediates of the two enantiomers ($\Delta\Delta E$) that can be used as a measure of enantioselectivity. Scheme 3a–c shows the tetrahedral intermediates of the substrates 2*S*-**17**, 2*S*-**18** and 2*S*-**19** in their lowest energy conformation inside the active site of PGA. The double nature of the nucleophilic sub-site of PGA is the key for understanding the mechanism of substrate enantiorecognition, since this part of the active site is divided in two different portions, one clearly polar and one apolar. The polar zone is characterized mainly by Arg B:263, Ser B:386, Asn B:241 and Asn B:388, while many hydrophobic amino acids, namely Phe A:146, Phe B:71, Leu B:253, Phe B:256, lead to the apolar zone. As demonstrated in Scheme 3a the least substituted compound 2*S*-**17** fits well into the active center of PGA. The formation of a strong H-bond of the cyano group with Arg B:263 and the correct location of the hydrophobic portion of the molecule in the hydrophobic part of the site are probably important contributions to the good observed kinetic resolution. The low $\Delta\Delta E$ value (1.8 kcal/mol) shows that the same general binding geometry can be reached by both enantiomers, with only small differences, keeping the apolar part in the hydrophobic zone of the sub-site and cyano group near Arg B:263. To our opinion, this observation could explain the observed low enantioselectivity.

The steric hindrance of the methyl groups attached to the double bond of **18** causes a distortion of the minimum energy conformer bound to the enzyme, mainly due to the significant collision with Phe A:146 when the bind with enzyme is forced. The energy difference between the preferred enantiomer of **17** and **18** (2*S*-**17** and 2*S*-**18**) was calculated to be of more than 27 kcal/mol. This is probably the reason why substrate **18** cannot bind into the active site of the enzyme and no reaction was observed. The same reasons could also explain the failure of the kinetic resolution for the diethyl-substituted molecule **19**.

The situation for substrate **20** is different from that of all the other compounds (Scheme 3c). The 2*S*-1-*N*-phenylacetyl-amino-2-cyano-4-methyl-5-cyclohexene (2*S*-**20**) conformation is very similar to that of 2*S*-**17**, since it find a perfect arrangement into the active site, with the cyano group directed towards Arg B:263, and the apolar part of the molecule in the hydrophobic zone of the nucleophile sub-site. The steric hindrance created by the methyl in position 4—does not interfere with the positioning of the 2*S*—stereoisomer, while it is sufficient to force the 2*R*-**20** to a completely different conformation: the apolar portion of the compound has to be fitted into the hydrophobic zone, while the cyano group cannot be placed near Arg B:263. The lack of the interaction with Arg B:263 and the impossibility for the cyano group to find a good interaction with another protein polar residue destabilize the molecule ($\Delta\Delta E$=5.8 kcal/mol) and can give an explanation for the enantioselectivity of the enzyme.

3. Conclusions

In conclusion, new multicomponent coupling reactions of

Table 1. Three component coupling reaction of aldehydes, phenylacetamide and dienophiles[a]

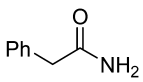
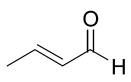
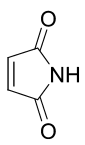
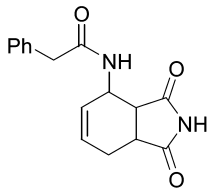
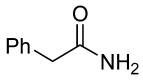
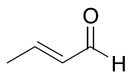
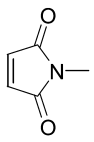
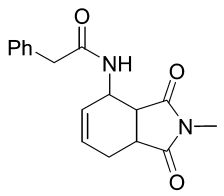
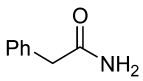
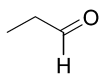
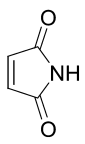
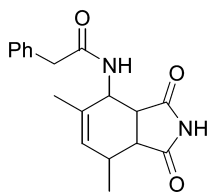
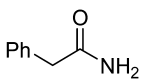
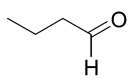
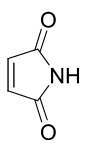
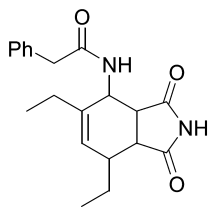
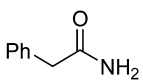
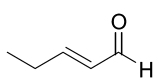
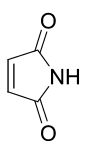
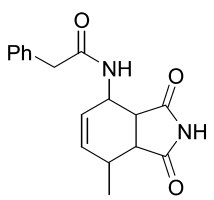
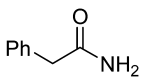
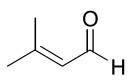
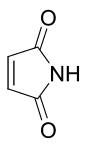
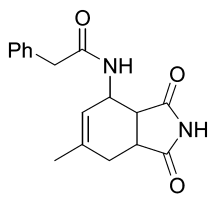
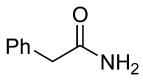
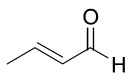
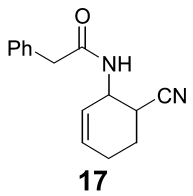
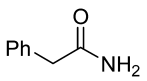
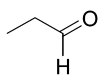
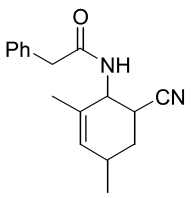
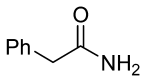
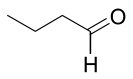
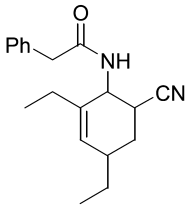
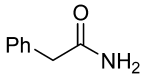
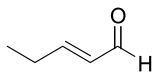
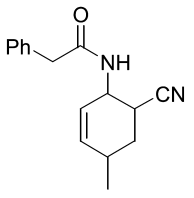
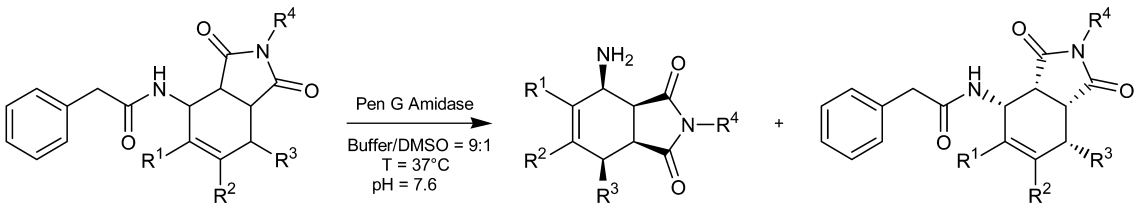
Entry	Amide	Aldehyde	Dienophile	Compound	Yield (%)[b]
1				 **11**	60
2				 **12**	58
3				 **13**	55
4				 **14**	70
5				 **15**	65
6				 **16**	62
7				 **17**	55

Table 1 (*continued*)

Entry	Amide	Aldehyde	Dienophile	Compound	Yield (%)[b]
8				 **18**	50
9				 **19**	70
10				 **20**	63

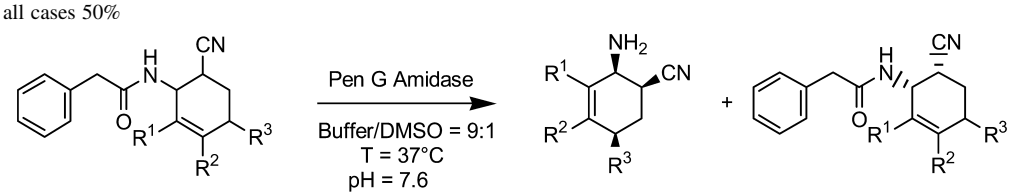
[a] Conditions: 15 mmol amide, 7.5 or 15 mmol aldehyde, 10 mmol maleimide or 80 mmol acrylonitrile, 7.5 mmol Ac_2O, 1.5 mol% *p*-TSA; 40 mL NMP; 120 °C, 24 h (120 h by the use of acrylonitrile).
[b] Isolated, non-optimized yields.

Table 2. PGA-catalyzed kinetic resolution of 4-*N*-phenylacetylamino-*cis*-3a,4,7,7a-hexahydro*iso*indole-1,3-dione derivatives; ee is given for the non-converted remaining enantiomer; conversion was in all cases 50%



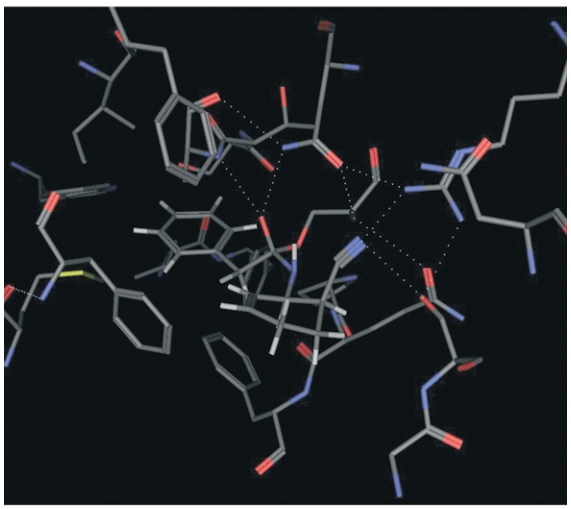
Compound	R^1	R^2	R^3	R^4	$ee_{Substrate}$ (%)	*E*
11	H	H	H	H	70	12
12	H	H	H	Me	35	3
13	Me	H	Me	H	30	2
14	Et	H	Et	H	45	4
15	H	H	Me	H	45	4
16	H	Me	H	H	35	3

Table 3. PGA-catalyzed kinetic resolution of 1-*N*-phenylacetylamino-2-cyano-5-cyclohexene derivatives. The ee is given for the remaining substrate, conversion was in all cases 50%

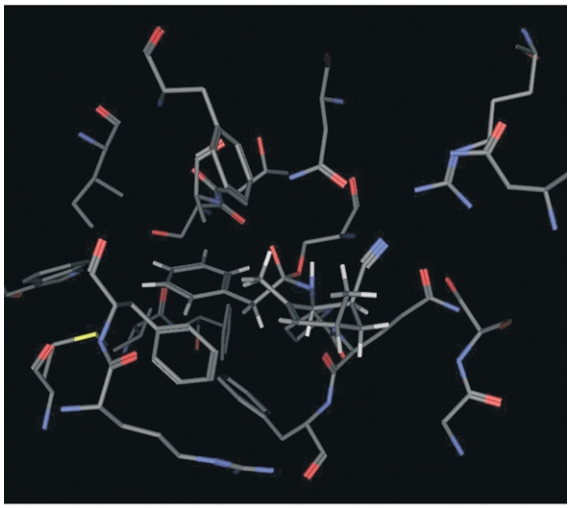


Compound	R^1	R^2	R^3	$ee_{Substrate}$ (%)	*E*-value
17	H	H	H	65	9
18	Me	H	Me	No conversion	—
19	Et	H	Et	No conversion	—
20	H	H	Me	>99	>200

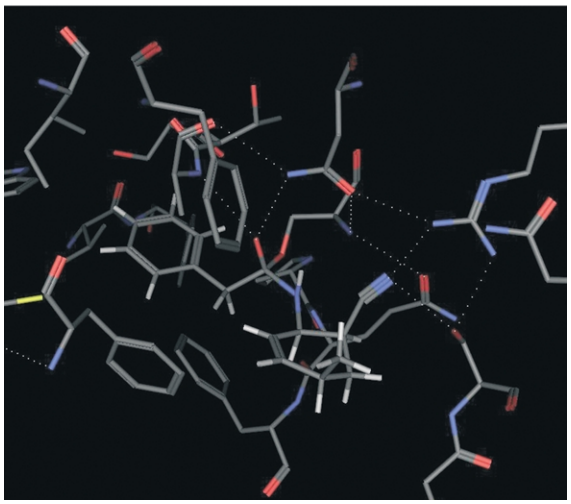
(a)



(b)



(c)



Scheme 3. (a–c) Molecular modeling of 1-*N*-phenylacetylamino-2-cyano-5-cyclohexene derivatives in the active center of PGA. (a) Representation of the tetrahedral intermediate of substrate 2*S*-**17**. (b) Representation of the tetrahedral intermediate of substrate 2*S*-**18**. (c) Representation of the tetrahedral intermediate of substrate 2*S*-**20**.

aldehydes, phenylacetamide and dienophiles have been described. By using PGA, kinetic resolution of this class of compounds is possible for the first time. Molecular mechanics calculations of the active center of the enzyme nicely explain the observed specificity of the resolution reactions. Further work is necessary to improve the scale up of the reaction due to the low solubility of substrate under the employed reaction conditions for the kinetic resolution as well as for the product isolation.

4. Experimental

4.1. General procedures

Reagents and solvents were used as received from commercial suppliers. PGA from *Escherichia coli* was obtained from Fluka. Threaded Aldrich ACE pressure tubes were used as reaction vessels. Exclusion of moisture and air was not necessary. Column chromatography was performed with 230–400 mesh ASTM silica gel from Merck. Uncorrected melting points were recorded on a Galen III Cambridge Instr. IR spectra were recorded as KBr pellets on a Nicolet Magna 550. ^{1}H and ^{13}C NMR data were recorded on a Bruker ARX 400 with QNP probe head and are reported relative to DMSO-d_6 or $CDCl_3$. MS data were obtained on AMD 402 (EI 70 eV). Combustion analyses were performed by the Microanalytical Laboratory, Department of Chemistry at the University of Rostock.

4.2. General procedure for multicomponent coupling reactions

Aldehyde (15 mmol), amide (15 mmol), maleimide (10 mmol) or acrylonitrile (60 mmol), acetic anhydride (15 mmol), and *p*-toluenesulfonic acid monohydrate (1.5 mol%) were combined in a threaded tube, and NMP (40 mL) was added. Then, the reaction was stirred at 120 °C. Over the course of the reaction, the solution turned slightly yellow or orange. After 24 h (in the case of acrylonitrile 120 h), the solvent and other volatile compounds were removed by oil pump vacuum to give an orange or red residue. Silica gel chromatography (ethyl acetate/heptane) afforded the corresponding AAD products.

4.3. General procedure for enzymatic screening

In a 96-well microtiter plate the following solutions were incubated at 37 °C: 100 μL of enzyme solution in 5 mM phosphate buffer, pH 7.3, 80 μL of 5 mM phosphate buffer, 20 μL of 20 mM substrate, containing 0.75 mM bromothymolblue as indicator, in DMSO. After 20 h the change of absorption at 600 nm was measured using a microplate reader. A color change from green to yellow indicates enzymatic activity towards a substrate. For control a blank without substrate was used.[11]

4.4. General procedure for PenG amidase catalysed kinetic resolution

Method (a). PGA (1.5 mg) is dissolved in 1350 μL 100 mM phosphate buffer (pH 7.3). After thermostating at 37 °C, 150 μL 20 mM substrate solution in DMSO is added. After

different time intervals 100 μL of sample are diluted with 500 μL ethylacetate. The ethylacetate is separated from the aqueous layer, dried with Na_2SO_4 and analysed by HPLC.

Method (b). 500 μL 20 mM substrate solution in DMSO are mixed in a thermostated vessel with 4.4 mL water at 37 °C. By using a pH-stat (Metrohm) the solution is adjusted to pH 7.6. The pH is maintained at this pH after addition of 2 mg enzyme dissolved in 100 μL water by addition of NaOH.

HPLC analysis. The enantiomeric excess is measured on a OD-H column on a Knauer HPLC-System (Knauer, Berlin) using as eluent 90% *n*-hexane and 10% EtOH at a temperature of 38 °C. UV-detection at 210 nm. This eluent is used for compounds **1**, **2** and **17**–**20**. Compounds **3**–**14** were analysed using a mixture of 70% *n*-hexane and 30% EtOH. Under these conditions baseline separation of the enantiomers is achieved in most cases.

4.5. Molecular modelling

All the calculations were performed on a SGI Octane workstation. GRID version 20 (distributed by Molecular Discovery Inc.) was used for the calculations of MIFs for chemical description of enzyme active site. SYBYL 6.7 (Tripos, USA) was used for molecular construction, visualisation and for molecular dynamics and energy minimisation calculations. AMBER force field in its SYBYL implementation was used for molecular dynamics and minimisations. The simulation protocols for the construction of the model were previously reported.[13,14]

4.5.1. 4-*N*-Phenylacetylamino-l-*cis*-3a,4,7,7a-hexahydro-*iso*indol-1,3-dione (11). R_f=0.26. Yield: 60% (white solid), mp 185–187 °C. Calcd for $C_{16}H_{16}O_3N_2$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.23; H, 5.58; N, 9.93. IR (KBr): 3410–2860 m, 2898 w, 1856 m, 1713 m, 1638 s. MS (EI): 284 ([**11**]$^+$, 11%); 165 ([**11**–Ac]$^+$, 43%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 11.27 (1H, s, CH$_2$–CH–CON*H*); 8.37 (1H, d, 7.5 CO–N*H*–CH); 7.42–7.23 (5H, m, *H*-Ar); 5.97 (1H, m, CH=C*H*–CH$_2$); 5.84 (1H, m, NHCH–*H*C=); 4.47 (1H, m, *H*CNH); 3.6 (1H, m, CHNH–C*H*–CONH); 3.38 (1H, m, CH$_2$–C*H*–CONH); 2.53 (2H, s, CH_2–CO–); 2.52 (2H, m, CH_2–CH–CONH). ^{13}C{^{1}H} 180.2 (CH$_2$–CH–*C*ONH); 178.8 (CHNH–CH–*C*ONH); 172.5 (CH$_2$–*C*O–NH); 136.9 (*i*-Ph); 131.5 (CH=*C*H–CHNH); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 128.2 (*C*H=CH–CHNH); 126.6 (*p*-Ph); 45.7 (NH*C*H); 45.6 (CH$_2$–*C*H–CONH); 43.4 (CH$_2$–*C*H); 42.6 (Ph–*C*H$_2$); 23.3 (*C*H$_2$–CH–CONH).

4.5.2. 4-*N*-Phenylacetylamino-5,7-dimethyl-*cis*-3a,4,7,7a-hexahydro*iso*indol-1,3-dione (12). R_f=0.32. Yield: 58% (white solid), mp 196 °C. Calcd for $C_{17}H_{18}O_3N_2$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.53; H, 6.12; N, 9.51. IR (KBr): 3210–2680 m, 2928 w, 1877 m, 1739 m, 1708 s, 1638 s. MS (EI): 298 ([**12**]$^+$, 19%); 179 ([**12**–Ac]$^+$, 32%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 8.39 (1H, d, 7.6, CO–N*H*–CH); 7.42–7.26 (5H, m, *H*-Ar); 6.01 (1H, m, CH=C*H*–CH$_2$); 5.89 (1H, m, NHCH–*H*C=); 4.43 (1H, m, *H*CNH); 3.63 (1H, m, CHNH–C*H*–CONH); 3.37 (1H, m, CH$_2$–C*H*–CONH); 2.55 (2H, s, CH_2–CO–); 2.49 (2H, m, CH_2–CH–CONH). ^{13}C{^{1}H} 179.2 (CH$_2$–CH–*C*ONMe); 177.8 (CHNH–CH–*C*ONMe); 172.6 (CH$_2$–*C*O–NH); 136.9 (*i*-Ph); 131.7 (CH=*C*H–CHNH); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 126.6 (*p*-Ph); 45.9 (CH$_2$–*C*H–CONH); 45.5 (NH*C*H); 43.5 (CH$_2$–*C*H); 42.7 (Ph–*C*H$_2$); 26.3 (CO–N*Me*); 23.2 (*C*H$_2$–CH–CONH).

4.5.3. 4-*N*-Phenylacetylamino-5,7-dimethyl-*cis*-3a,4,7,7a-hexahydro*iso*indol-1,3-dione (13). R_f=0.32. Yield: 55% (white solid), mp 125–127 °C. Calcd for $C_{18}H_{20}O_3N_2$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.44; H, 6.58; N, 6.62. IR (KBr): 3210–2680 m, 2928 w, 1877 m, 1739 m, 1708 s, 1638 s. MS (EI): 312 ([**13**]$^+$, 17%); 193 ([**13**–Ac]$^+$, 35%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 11.6 (1H, s, CH–CH–CON*H*); 8.4 (1H, d, 7.3 CO–N*H*–CH); 7.41–7.23 (5H, m, *H*-Ar); 5.35 (1H, s, C=C*H*–CH$_2$); 4.48 (1H, m, *H*CNH); 4.45 (1H, m, C*H*NH–CH–CONH); 3.16 (1H, m, CHNH–C*H*–CONH); 2.53 (2H, s, CH_2–CO–); 2.35 (1H, m, C*H*–CH–CONH); 1.52 (3H, s, CH_3–C=); 1.23 (3H, d, 7.4, CH_3–CH). ^{13}C{^{1}H} 179.2 (CHNH–CH–*C*ONH); 178.2 (CH–CH–*C*ONH); 170.2 (CH$_2$–*C*O–NH); 136.9 (*i*-Ph); 136.5 (CH=*C*(CH$_3$)–CH–NH); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 127.3 (*C*H=C(CH$_3$)–CHNH); 126.5 (*p*-Ph); 46.2 (NH*C*H); 44.1 and 44.2 (CO–*C*H–*C*H–CO); 42.6 (Ph–*C*H$_2$).

4.5.4. 4-*N*-Phenylacetylamino-5,7-diethyl-*cis*-3a,4,7,7a-hexahydro*iso*indol-1,3-dione (14). R_f=0.3. Yield: 70% (white solid), mp 167 °C. Calcd for $C_{20}H_{24}O_3N_2$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.86; H, 7.23; N, 8.46. IR (KBr): 3200–2960 m, 3108 w, 2985 w, 1788 s, 1698 s, 1564 s. MS (EI): 340 ([**14**]$^+$, 12%); 221 ([**14**–Ac]$^+$, 29%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 11.2 (1H, s, CH–CH–CON*H*); 8.1 (1H, d, 7.7, CO–N*H*–CH); 7.42–7.22 (5H, m, *H*-Ar); 5.28 (1H, s, C=C*H*–CH); 4.47 (1H, m, *H*CNH); 3.09 (1H, m, C*H*–CH–CONH); 3.06 (1H, m, CHNH–C*H*–CONH); 2.53 (2H, s, CH_2–CO–); 1.92 (2H, m, Me–CH_2–C=); 1.86 (2H, m, Me–CH_2–CH); 1.1 (3H, t, 7.8, CH_3–CH$_2$–C=); 1.02 (3H, t, 6.8, CH_3–CH$_2$–CH). ^{13}C{^{1}H} 180.1 (CH–CH–*C*ONH); 179.2 (CHNH–CH–*C*ONH); 171.5 (CH$_2$–*C*O–NH); 142.8 (CH=*C*–CHNH); 136.9 (*i*-Ph); 129.6 (*o*-Ph); 128.5 (*m*-Ph); 126.5 (*p*-Ph); 124.5 (*C*H=C–CHNH); 47.2 (NH*C*H); 44.9 (Et–CH–*C*H); 43.3 (CH–CH–CONH); 42.3 (Ph–*C*H$_2$); 24.2 (Me–*C*H$_2$–C=); 23.7 (Me–*C*H–CH); 23.6 (*C*H–CH–CONH); 12.3 (*C*H$_3$–CH$_2$–C=); 11.7 (*C*H$_3$–CH$_2$–CH).

4.5.5. 4-*N*-Phenylacetylamino-*cis*-3a,4,7,7a-hexahydro-*iso*indol-1,3-dione (15). R_f=0.18. Yield: 60% (white solid), mp 185–187 °C. Calcd for $C_{16}H_{16}O_3N_2$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.88; H, 5.73; N, 10.04. IR (KBr): 3300–2790 m, 3218 w, 3077 m, 2939 m, 1708 s, 1584 s. MS (EI): 284 ([**15**]$^+$, 22%); 165 ([**15**–Ac]$^+$, 34%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 11.27 (1H, s, CH$_2$–CH–CON*H*); 8.37 (1H, d, 7.2, CO–N*H*–CH); 7.43–7.27 (5H, m, *H*-Ar); 5.97 (1H, m, CH=C*H*–CH$_2$); 5.84 (1H, m, NHCH–*H*C=); 4.47 (1H, m, *H*CNH); 3.6 (1H, d, 7.1, CHNH–C*H*–CONH); 3.38 (1H, m, CH$_2$–C*H*–CONH) 2.53 (2H, s, CH_2–CO–); 2.52 (2H, m, CH_2–CH–CONH). ^{13}C{^{1}H} 180.2 (CH$_2$–CH–*C*ONH); 178.8 (CHNH–CH–*C*ONH); 172.5 (CH$_2$–*C*O–NH); 136.9 (*i*-Ph); 131.5 (CH=*C*H–CHNH); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 128.2 (*C*H=CH–CHNH); 126.6 (*p*-Ph); 45.7 (NH*C*H); 45.6 (CH$_2$–*C*H–CONH); 43.4 (CH$_2$–*C*H); 42.6 (Ph–*C*H$_2$); 23.3 (*C*H$_2$–CH–CONH).

4.5.6. 4-*N*-Phenylacetylamino-6-methyl-*cis*-3a,4,7,7a-hexahydro*iso*indol-1,3-dione (16). R_f=0.33. Yield: 62% (white solid), mp 183 °C. Calcd for $C_{17}H_{18}O_3N_2$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.58; H, 6.16; N, 9.56. IR (KBr): 3300–2760 m, 3058 w, 2765 w, 1748 s, 1658 s, 1534 s. MS (EI): 298 ([**16**]$^+$, 16%); 179 ([**16**–Ac]$^+$, 28%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 10.98 (1H, s, CH_2–CH–CON*H*); 8.41 (1H, d, 7.8, CO–N*H*–CH); 7.45–7.21 (5H, m, *H*-Ar); 5.81 (1H, m, NHCH–*H*C═); 4.42 (1H, m, *H*CNH); 3.63 (1H, m, CHNH–C*H*–CONH); 3.41 (1H, m, CH_2–C*H*–CONH); 2.85 (3H, s, CH_3–C═CH); 2.55 (2H, m, CH_2–CH–CONH); 2.54 (2H, s, CH_2–CO–). ^{13}C{^{1}H} 179.8 (CH_2–CH–*C*ONH); 178.6 (CHNH–CH–*C*ONH); 172.6 (CH_2–*C*O–NH); 136.8 (*i*-Ph); 131.7 (C═*C*H–CHNH); 129.5 (*o*-Ph); 128.4 (*m*-Ph); 128.2 (*C*Me═CH–CHNH); 126.5 (*p*-Ph); 45.8 (NH*C*H); 45.5 (CH_2–*C*H–CONH); 43.6 (CH_2–*C*H); 42.6 (Ph–*C*H_2); 31.4 (*C*H_3–C═CH); 23.5 (*C*H_2–CH–CONH).

4.5.7. 1-*N*-Phenylacetylamino-2-cyano-5-cyclohexene (17). R_f=0.22. Yield: 55% (white solid), mp 117–121 °C. Calcd for $C_{15}H_{16}ON_2$: C, 74.97; H, 6.71; N, 11.66. Found: C, 75.04; H, 6.94; N, 11.81. IR (KBr): 3045–2760 m, 2990 w, 2860 m, 2354 s, 1708 s, 1694 s. MS (EI): 240 ([**17**]$^+$, 15%); 121 ([**17**–Ac]$^+$, 26%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 8.77 (1H, d, 7.6, N*H*); 7.55–7.41 (5H, m, *H*-Ar); 6.12 (1H, d, 9.8, NH–CH–*H*C═); 5.70 (1H, m, CH═C*H*–CH_2); 4.74 (1H, m, *H*CNH); 3.12 (1H, m, *H*CCN); 2.73 (2H, s, CH_2–CO–); 2.29 (2H, m, ═CH–CH_2–CH_2); 2.11 (2H, m, CH_2–CH_2–CH–CN). ^{13}C{^{1}H} 172.5 (CH_2–*C*O–NH); 136.9 (*i*-Ph); 129.9 (*C*N); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 126.6 (*p*-Ph); 125.8 (CH_2–*C*H); 125.8 (CH═*C*H–CHNH); 44.9 (NH*C*H); 42.6 (Ph–*C*H_2); 30.8 (*C*HCN); 22.5 (CH_2–*C*H_2); 22.4 (CHCN–*C*H_2); 22.5 (CH_2–*C*H_2).

4.5.8. 1-*N*-Phenylacetylamino-2-cyano-4,6-dimethyl-5-cyclohexene (18). R_f=0.25. Yield: 50% (white solid), mp 87–92 °C. Calcd for $C_{17}H_{20}ON_2$: C, 76.09; H, 7.51; N, 10.44. Found: C, 76.23; H, 7.68; N, 10.64. IR (KBr): 346–2740 m, 3214 w, 3165 w, 2939 m, 1854 s, 1734 s. MS (EI): 268 ([**18**]$^+$, 15%); 149 ([**18**–Ac]$^+$, 26%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 8.33 (1H, d, 8.2, N*H*); 7.29 (5H, m, *H*-Ar); 5.28 (1H, s, –C═C*H*–CH); 4.54 (2H, m, CH–CH_2–CN); 4.52 (1H, m, *H*CNH); 3.13 (1H, m, *H*CCN); 2.54 (2H, s, CH_2–CO–); 2.21 (1H, m, ═CH–C*H*–CH_3); 1.59 (3H, s, CH_3–C═); 1.03 (3H, d, 7.2, CH–CH_3). ^{13}C{^{1}H} 172.5 (CH_2–*C*O–NH); 136.9 (*i*-Ph); 131.1 (CH═*C*–Me); 130.9 (*C*N); 129.7 (CHMe–*C*H═); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 126.6 (*p*-Ph); 45.5 (NH*C*H); 42.6 (Ph–*C*H_2); 31.8 (*C*HCN); 29.7 (CHCN–*C*H_2); 21.0 (CH–*C*H_3); 20.0 (CH_2–*C*H–Me).

4.5.9. 1-*N*-Phenylacetylamino-2-cyano-4,6-diethyl-5-cyclohexene (19). R_f=0.35. Yield: 70% (white solid), mp 96–97 °C. Calcd for $C_{19}H_{24}ON_2$: C, 76.99; H, 8.16; N, 9.45. Found: C, 77.34; H, 8.33; N, 9.63. IR (KBr): 3500–2920 m, 3278 w, 3157 m, 2939 w, 2184 s, 1688 s, 1704 s. MS (EI): 296 ([**19**]$^+$, 15%); 177 ([**19**–Ac]$^+$, 26%); 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 8.26 (1H, d, CO–N*H*–CH_2); 7.38–7.18 (5H, m, *H*-Ar); 5.44 (1H, s, –C═C*H*–CH); 4.46 (1H, m, *H*CNH); 3.11 (1H, m, *H*CCN); 2.51 (2H, s, CH_2–CO–); 1.56 (1H, m, ═CH–C*H*–Et); 1.53 (2H, t, 6.4, Me–CH_2–C═); 1.36 (2H, m, CH–CH_2–CN); 1.34 (2H, m, CH–CH_2–CH_3); 1.02 (3H, t, 7.8, CH_3–CH_2–C═); 0.91 (3H, m, CH–CH_2–CH_3). ^{13}C{^{1}H} 172.5 (CH_2–*C*O–NH); 136.9 (*i*-Ph); 129.7 (*C*N); 129.4 (*o*-Ph); 129.3 (CHEt–*C*H═); 128.5 (*m*-Ph); 126.6 (*p*-Ph); 121.3 (CH═*C*–Et); 44.2 (NH*C*H); 42.6 (Ph–*C*H_2); 36.4 (*C*HCN); 31.5 (CH_2–*C*H–Et); 28.2 (CHCN–*C*H_2); 27.6 (CH–*C*H_2–CH_3); 26.8 (═C–*C*H_2–CH_3); 12.5 (═C–CH_2–*C*H_3); 11.5 (CH–CH_2–*C*H_3).

4.5.10. 1-*N*-Phenylacetylamino-2-cyano-4-methyl-5-cyclohexene (20). R_f=0.4. Yield: 54% (white solid), mp 130–132 °C. Calcd for $C_{16}H_{18}ON_2$: C, 75.56; H, 7.13; N, 11.01. Found: C, 75.76; H, 7.28; N, 11.13. IR (KBr): 3460–2740 m, 3328 w, 2954 w, 2785 m, 1754 s, 1614 s. MS (EI): 254 ([**20**]$^+$, 15%); 135 ([**20**–Ac]$^+$, 23%; 119 ([Ac]$^+$, 100%). NMR (DMSO-d_6): ^{1}H 8.27 (1H, d, 7.9, CO–N*H*–CH); 7.22–7.06 (5H, m, *H*-Ar); 5.41 (1H, d, 4.2, –C═C*H*–CH); 4.52 (1H, m, *H*CNH); 3.09 (1H, m, *H*CCN); 2.39 (2H, s, CH_2–CO–); 1.86 (2H, m, CH–CH_2–CN); 1.51 (1H, m, ═CH–C*H*–CH_3); 0.93 (3H, d, 7.3, *Me*). ^{13}C{^{1}H} 172.5 (CH_2–*C*O–NH); 136.9 (*i*-Ph); 129.7 (*C*N); 129.4 (*o*-Ph); 128.5 (*m*-Ph); 126.6 (*p*-Ph); 124.4 (CH═*C*–CHNH); 124.3 (CHMe–*C*H); 44.2 (NH*C*H); 42.6 (Ph–*C*H_2); 30.6 (CH_2–*C*H–Me); 29.8 (*C*HCN); 29.7 (CHCN–*C*H_2); 20.6 (CH–*C*H_3).

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Hydrolytic and transglycosylation reactions of *N*-acyl modified substrates catalysed by β-*N*-acetylhexosaminidases

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Abstract—The hydrolytic and transglycosylation capabilities of 35 fungal β-*N*-acetylhexosaminidases with *p*-nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside and its four *N*-acyl derivatives (CH═O, $COCH_2OH$, $COCH_2CH_3$, $COCF_3$) as substrates were tested. The preparation of four novel *p*-nitrophenyl disaccharides from these unnatural substrates catalysed by enzymes from *Aspergillus oryzae*, *Penicillium oxalicum* and *Talaromyces flavus* represents a considerable extension of the synthetic potential of glycosidases.

1. Introduction

The recent dynamic development of glycosciences has brought about the increasing need for new glycostructures. Novel carbohydrate compounds can effectively be synthesized using enzymatic methods.[1] Glycosidases (EC 3.2), although they often exhibit a poor regioselectivity and sometimes low yields, are readily available from different sources. They tolerate environmental stress and thanks to their broad substrate specificity they are able to accept a wide range of donors with different aglycon moieties and acceptors.[2–6]

It has been revealed only recently that besides their natural substrates (glycons), glycosidases are capable of accepting substrates bearing various structural modifications. These modified substrates, often exhibiting important biological properties,[7] find applications in many areas, such as structure-activity relationship studies[8–10] or treatment of glycosidase-induced pathogenic states.[9]

The first papers dealing with the enzymatic recognition of substrates modified at the glycon moiety were published in the early 70s, mostly investigating the substrate specificity of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* isolated from a digestive amylase preparation Takadiastase®. Substrates bearing 3-*O*-methyl, 6-*O*-methyl, 3,4-di-*O*-methyl and 3,4,6-tri-*O*-methyl groups,[11] as well as a range of *N*-acyl modified substrates[12,13] were studied.

The affinity of various β-*N*-acetylhexosaminidases from mushrooms and marine vertebrates to substrates modified at C-2 amino group was thoroughly studied by Molodtsov and Vafina.[14–16] They isolated and characterised the 'β-*N*-glycylhexosaminidase' from the marine helminth *Chaetopterus variopedatus*[17] and the 'β-*N*-benzoylhexosaminidase' from the scallop *Mizuhopecten yessoensis*.[18] Besides, they detected a high tolerance to *N*-acyl modifications with the β-*N*-acetylhexosaminidase from the fungus *Hohenbuehelia serotina*.[19] A similar study was carried out by Leaback and Walker with the commercial pig epididymal β-*N*-acetylhexosaminidase.[20]

Probably the first use of modified substrates in a transglycosylation reaction was described by Wong and Takayama with *p*-nitrophenyl 6-oxo-β-D-galactopyranoside.[21] C-6 modifications were also studied by MacManus et al.[22] with a set of 10 *p*-nitrophenyl galactopyranosides bearing different functionalities at C-6, such as methyl, alkene, alkyne or fluorine. A β-*N*-acetylhexosaminidase was first used by Hušáková et al. in a reaction with a 6-*O*-acetylated glycosyl donor.[23] To our best knowledge, no case of transglycosylation with *N*-acyl modified substrates catalysed by glycosidases has yet been published.

In the present paper, we report the affinity of a library of 35 fungal β-*N*-acetylhexosaminidases to various *N*-acyl modified substrates from the viewpoint of their hydrolytic and transglycosylation potentials. Four novel *p*-nitrophenyl

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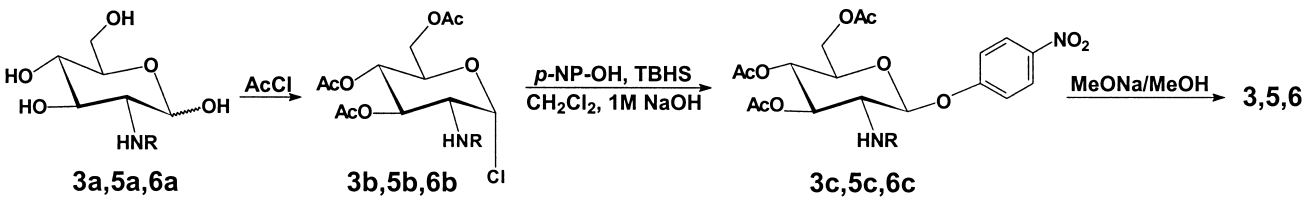


3: R = -CHO
5, **5a**: R = -$COCH_2OH$
5b, **5c**: R = -$COCH_2OAc$
6: R = -$COCH_2CH_3$

Scheme 1.

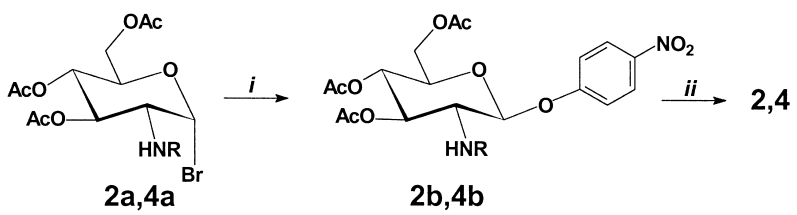
disaccharides were prepared in transglycosylation reactions with these new substrates. The experimental results are discussed regarding molecular models of modified substrates docked in the active centre of the β-*N*-acetylhexosaminidase from *A. oryzae* Culture Collection of Fungi 1066.

2. Results and discussion

Compounds **3**, **5** and **6** were prepared from the corresponding 2-acylamido-2-deoxy-D-glucopyranoses[24–26] using modified method[27] (Scheme 1). The selective formylation with *p*-nitrophenyl formiate[28] as well as those using HCOOH with Ac_2O[29,30] did not lead to the desired product, and so the formylation with a mixed acetic formic anhydride was performed.[24] Substrate **2** was synthesized using modified method[31] (Scheme 2). In the synthesis of substrate **4** (Scheme 2), 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride[32] was trifluoroacetylated[33] to give **4a**.

Substrates **2**–**6** were subjected to a screening for hydrolytic cleavage comprising 35 fungal β-*N*-acetylhexosaminidases from the genera of *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces* (Table 1).

Compounds **2** and **4** were poor substrates for all β-*N*-acetylhexosaminidases tested. On the other hand, substrates **6**, **3** and **5** (from the best to the worst, respectively) were cleaved by the majority of tested enzymes. The β-*N*-acetylhexosaminidase from *A. oryzae* Culture Collection of Fungi (CCF) 1066 did not substantially hydrolyse any of the modified substrates **2**–**6** at a comparable level to the standard substrate **1** (Table 1). These experimental results were evaluated with regard to the interaction energies calculated from the molecular model of this enzyme with substrates **1**–**6** (Table 2). The decrease of the interaction energy of an enzyme–substrate complex generally reflects better binding. Neither of the respective modified substrates exhibited any substantial steric conflict with the enzyme active centre, as could be seen from minor differences in the steric interaction energies. Surprisingly, the steric interaction energy slightly decreased with the size of the substituent (formyl→acetyl→propionyl, −71, −84 and −114 kJ/mol, respectively). More obvious differences were observed in the electrostatic interaction energies. Here, the standard substrate **1** displayed the strongest electrostatic interaction (−216 kJ/mol), whereas the electrostatic interaction energy of substrate **6** was significantly higher (−83 kJ/mol). Thus, the low cleavage of substrate **6** appears to be a result of a lower binding affinity. With substrates **2**–**5** the situation is different: it may be supposed from the interaction energy values that all these substrates bind well to the active site of the enzyme and that their low



Substrate	R	Reagents (*i*)	Reagents (*ii*)
2	-H	*p*-NP-OAg, dry MeCN, 12 h	MeONa/ MeOH, 3 h
4	-$COCF_3$	*p*-NP-OH, Fétison reagent, 2,6-dimethylpyridine, MeCN, 2 h	sat. NH_4OH: MeOH 1:10, 6 h

Scheme 2.

Table 1. Screening on the hydrolysis of substrates **2**–**6** with fungal β-*N*-acetylhexosaminidases

Source of enzyme	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
Acremonium persicinum CCF 1850	+	+++	−	+++[c]	***[c]
Aspergillus awamori CCF 763	−	+	+	+	*
A. caelatus CCF 3087	−	+	−	+	*
A. flavipes CCF 869	−	+	−	+	*
A. flavipes CCF 1895	+	+	−	+	nd
A. flavipes CCF 3067	−	+	−	+	nd
A. flavofurcatis CCF 107	−	+	−	+	*
A. flavus CCF 642	−	+	−	+	nd
A. flavus CCF 3056	−	+	−	+	*
A. niger CCIM K2	−	+	nd	+	nd
A. niveus CCF 3057	−	+	−	+	**
A. nomius CCF 3086	−	+	−	+	nd
A. oryzae CCF 147	−	++	−	+	**
A. oryzae CCF 1066	−	++	−	++	*
A. parasiticus CCF 141	−	+	−	+	nd
A. parasiticus CCF 1298	−	+	−	+	**
A. phoenicis CCF 61	−	+	−	++	*
A. sojae CCF 3060	+	+	−	+	*
A. tamarii CCF 1665	−	+	−	−	*
A. terreus CCF 2539	+	+++	−	+++[c]	***
A. terreus CCF 3059	nd	nd	nd	nd	*
A. versicolor CCF 2491	+	+	−	+	nd
Hamigera avellanea CCF 2923	+	+++	−	++	***
Penicillium brasilianum CCF 2155	−	+++	−	+++	***[c]
P. brasilianum CCF 2171	−	+++[c]	−	+++	nd
P. chrysogenum CCF 1269	−	++	−	++	nd
P. funiculosum CCF 1994	nd	nd	−	nd	**
P. multicolor CCF 2244	−	++	−	++	**
P. oxalicum CCF 1959	−	+++	−	++	**
P. oxalicum CCF 2315	−	++	−	++	***[c]
P. oxalicum CCF 2430	−	+++	−	+++	**
P. oxalicum CCF 3009	nd	nd	−	nd	**
P. pittii CCF 2277	−	+++[c]	−	+++	nd
P. spinulosum CCF 2159	−	+++	−	+++	nd
Talaromyces flavus CCF 2686	+	+++[c]	−	+++[c]	**

[a] Hydrolysis of substrates **2**–**5** was spectrophotometrically determined as the liberated *p*-nitrophenol: the ratio of hydrolysis rates of the respective modified substrate and substrate **1** was 24–12% (+++), 11–5% (++), 4–1% (+) or lower than 1% (−).

[b] Hydrolysis of substrate **6** was analysed by TLC: the ratio of hydrolysed substrates **6** and **1** was 100–50% (***), 50–25% (**) or lower than 25% (*). Both substrates were assayed at the starting concentration of 1 mM due to poor water solubility of substrate **6** (sat. solution 1.4 mM) and enzymatic activities were estimated according to TLC.

[c] Three best hydrolysing enzymes for substrates **3**, **5** and **6**.

conversion into the hydrolytic product is caused by the destabilisation of the oxazolinium reaction intermediate in the proposed reaction mechanism,[9] or at another level of the 'dynamic' part of the hydrolytic process.

The three best hydrolysing enzymes for each of substrates **3**, **5** and **6** (Table 1) were selected as potential candidates for catalysing transglycosylation reactions, namely β-*N*-acetylhexosaminidases from *Acremonium persicinum* CCF 1850 (substrates **5** and **6**), *A. terreus* CCF 2539 (**5**), *Penicillium brasilianum* CCF 2155 (**6**), *P. brasilianum* CCF 2171 (**3**), *P. oxalicum* CCF 2315 (**6**), *P. pittii* CCF 2277 (**3**) and *T. flavus* CCF 2686 (**3**, **5**). The β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 displayed the lowest hydrolytic potential of all tested enzymes with substrate **6** and, therefore, it was selected for the catalysis of a transglycosylation reaction with substrate **6** as a glycosyl acceptor.

Substrates **3**, **5** and **6** suffered from poor water solubility (saturated solutions at 35 °C: substrate **3**: 3.3 mM, substrate **5**: 10.0 mM, substrate **6**: 1.4 mM). To reach the optimum concentration of substrates for transglycosylation reactions (approx. 50 mM),[1] MeCN was used as a cosolvent in the reaction medium.[1,34,35] It was shown before that MeCN is well tolerated by respective β-*N*-acetylhexosaminidases.[36] The activities of the selected enzymes in MeCN (0–45% v/v) were tested (Table 3).

Table 3. Activity (%) of β-*N*-acetylhexosaminidases in MeCN

β-*N*-Acetylhexosaminidase	5% v/v MeCN[a]	45% v/v MeCN[a]
A. persicinum CCF 1850	84	0
A. terreus CCF 2539	77	33
A. oryzae CCF 1066	107	7
P. brasilianum CCF 2155	125	2
P. brasilianum CCF 2171	81	29
P. oxalicum CCF 2315	93	22
P. pittii CCF 2277	87	43
T. flavus CCF 2686	107	55

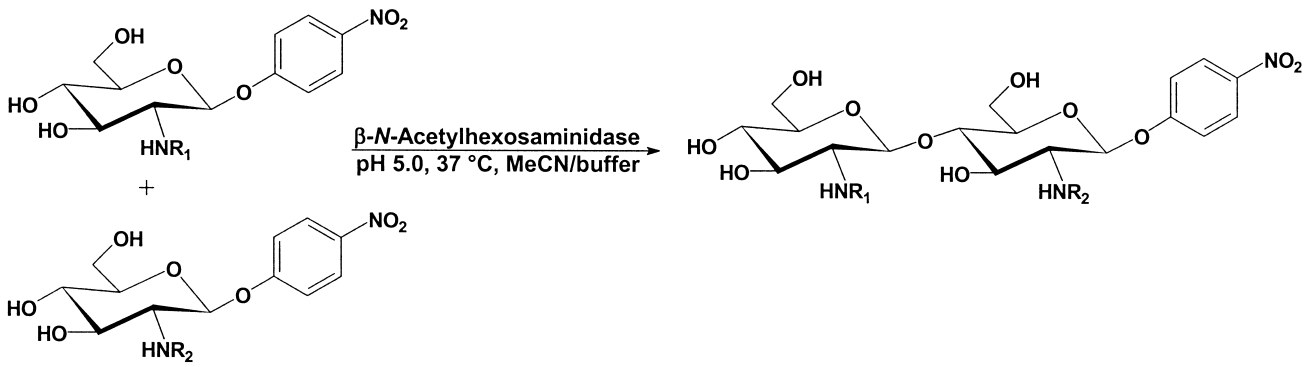
[a] Relative activities correlated to the values obtained in respective reactions without MeCN.

All β-*N*-acetylhexosaminidases were inhibited by 45% v/v MeCN, those from *A. persicinum* CCF 1850 and *P. brasilianum* CCF 2155 were almost totally inactivated. However, at 5% v/v, the cosolvent had a certain activation effect on the β-*N*-acetylhexosaminidases from *A. oryzae* CCF 1066, *P. brasilianum* CCF 2155 and *T. flavus* CCF 2686, the activity of which increased up to 125%.

To test the synthetic ability of β-*N*-acetylhexosaminidases with modified glycosyl donors and to optimise reaction conditions, reactions with substrates **3**, **5** and **6** were first tested on an analytical scale and monitored by TLC and HPLC. For each substrate, three best hydrolysing enzymes (Table 1) were tested and classified regarding the following criteria: (i) the most effective product formation; (ii) the lowest impairment of the enzymatic activity by MeCN. As a result, the following transglycosylation reaction schemes were proposed: (i) substrates **3** or **5** with the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 in 45 and 5% v/v MeCN, respectively; (ii) substrate **6** with the

Table 2. Interaction energies of substrates with the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066

Substrate	Total interaction energy (kJ/mol)	Steric contribution (kJ/mol)	Electrostatic contribution (kJ/mol)
1	−299	−84	−216
2	−251	−87	−164
3	−227	−71	−156
4	−226	−123	−103
5	−274	−111	−163
6	−194	−114	−83



Product	R	Source of enzyme	MeCN [%]	Yield [%]
7	$R_1 = R_2 =$ -CHO	*T. flavus* CCF 2686	45	16
8	$R_1 = R_2 =$ -$COCH_2OH$	*T. flavus* CCF 2686	5	78
9	$R_1 = R_2 =$ -$COCH_2CH_3$	*P. oxalicum* CCF 2315	45	24
10	R_1 = -$COCH_2CH_3$, R_2 = Ac	*A. oryzae* CCF 1066	45	1.8

Scheme 3.

β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2315 in 45% v/v MeCN; (iii) substrates **1** and **6** with the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 in 45% v/v MeCN.

The above reactions were performed on a semi-preparative scale (Scheme 3) under conditions optimised by HPLC analyses. Products **7**, **8** and **9** were obtained in reasonable yields (78% for product **8**) and with a high selectivity (exclusive formation of β(**1**→**4**) bonds). The formation of product **10** was unexpected. Due to the molar ratio of substrates **1**: **6**=1:2 and the low hydrolysis rate of substrate **6** by the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 we assumed the formation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(**1**→**4**)-2-deoxy-2-propionamido-β-D-glucopyranoside as a major product. However, the enzyme preferred to transfer the modified glycosyl moiety to *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and only products **9** and **10** were isolated from the reaction mixture. This demonstrates the high affinity of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 to the modified substrate **6**.

3. Conclusion

The acetamido group is a crucial structural feature of substrates for β-*N*-acetylhexosaminidases, which are quite sensitive to its modification. This study clearly demonstrates for the first time that besides cleavage, fungal β-*N*-acetylhexosaminidases are able to catalyse synthetic transglycosylation reactions with *N*-acyl modified substrates. They tolerate certain sterical changes at C-2 (shorter or longer acyls, a hydroxyl instead of a hydrogen). Nevertheless, they do not accept highly electronegative acyls, for example, trifluoroacetyl, nor the free amino group. Transglycosylation products were obtained selectively and in good yields. Moreover, the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 exhibited a higher affinity to the modified substrate **6** than to the standard substrate **1**. This concept widely extends the synthetic potential of β-*N*-acetylhexosaminidases.

4. Experimental

4.1. Materials

Citrate/phosphate buffer (Mc Ilvaine) pH 5.0 was prepared by mixing 0.1 M citric acid (24.3 mL) and 0.2 M Na_2HPO_4 (25.7 mL), by diluting with water to 100 mL and adjusting pH to 5.0. The fungal strains producing β-*N*-acetylhexosaminidases (EC 3.2.1.52) originated from the CCF, Department of Botany, Charles University, Prague, or from the Culture Collection of the Institute of Microbiology (CCIM), Prague. The strains were cultivated as described previously.[37,38] Flasks (500 mL) with medium (100 mL) were inoculated with the suspension of spores in 0.1% Tween 80 and cultivated on a rotary shaker at 28 °C. Three media were used. Mineral medium (for *A. oryzae* CCF 1066), [g/l]: yeast extract (0.5), KH_2PO_4 (3), $NH_4H_2PO_4$ (5), $(NH_4)_2SO_4$ (2), GlcNAc (5), NaCl (15), pH 6.0. Peptone medium (for *Aspergillus awamori* CCF 763, *A. niger* CCIM K2, *A. phoenicis* CCF 61 and *P. chrysogenum* CCF 1269), [g/l]: yeast extract (0.5), mycological peptone (5), KH_2PO_4 (3), $NH_4H_2PO_4$ (5), casein acid hydrolysate (7.5), pH 6.0. In inductor supplemented medium (for all other strains in Table 1), the casamino acids were replaced by crude chitin hydrolysate (2 g/l). After sterilisation each flask was supplemented with 0.5 mL 10% $MgSO_4 \cdot 7H_2O$. Enzymes were isolated by fractional $(NH_4)_2SO_4$ precipitation (20–80% sat.) and the precipitates were directly used for respective reactions.

4.2. Methods

4.2.1. Analytical methods. TLC was performed on pre-coated Merck silica gel DC-Alufolien Kieselgel 60 F_{254}

plates. The spots were visualised by charring with 5% H_2SO_4 in EtOH. Optical rotation was measured on a Perkin–Elmer 241 polarimeter at 589 nm. CD spectra were measured on Jobin–Yvon/Spex CD 6 spectrophotometer in the spectral region of 184–400 nm in H_2O (compounds **7**, **8**) or MeOH (compounds **9**, **10**). ^{1}H- and ^{13}C NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz and 100.55 MHz, respectively) at 30 °C in the indicated solvents. Chemical shifts are expressed in the δ-scale and were measured with digital resolution justifying the reported values to three or two decimal places, respectively. HMQC and HMBC readouts are accurate to one decimal place only. Residual solvent signals were used for referencing ($CDCl_3$: δ_H 7.265, δ_C 77.00; CD_3OD: δ_H 3.330, δ_C 49.30), internal acetone (δ_H 2.030, δ_C 30.50) was used for D_2O solutions. The reported assignment is based on gCOSY, HMQC, HMBC, and 1D-TOCSY experiments. Positive-ion electrospray (ESI) mass spectra were recorded on an LCQ^{DECA} ion trap mass spectrometer (Finnigan, San Jose, USA) equipped with an ESI ion source. Spray voltage was set at 5.0 kV, tube lens voltage was −10 V. Samples dissolved in 40% MeCN were continuously infused into the ion source via a linear syringe pump at a flow rate of 3 μL/min. Full scan spectra were acquired over the *m*/*z* range of 150–1000 Da. A 0.1% solution of Ultramark 1621 (PCR, Inc., Gainesville, FL) in MeCN was used to calibrate the *m*/*z* scale of the instrument. Analytical HPLC was carried out on a Spectra Physics modular analytical system (San Jose, USA) comprised of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/VIS detector. Preparative HPLC was performed on a Spectra Physics modular preparative system (San Jose, USA) comprised of an SP 8810 Ti pump, a Rheodyne injection port with a 100 μL sample load, a Spectra 100 variable wavelength UV/VIS detector and a ChromJet SP 4400 integrator. A Lichrospher 100-5 NH_2 column (Watrex, CR, 250×4 mm—analytical and 250×8 mm—preparatory) with a mobile phase MeCN–H_2O 79:21 was used at ambient temperature. Compounds were detected at 200 nm.

4.2.2. Molecular modelling. The primary sequence of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 was aligned with the known X-ray structures of the β-*N*-acetylhexosaminidases from *Serratia marcescens* and *Streptomyces plicatus*, extracted from the Brookhaven Protein Database (PDB entry: 1QBA and 1HP4, respectively). The sequence data are available in DDBJ/EMBL/GeneBank databases (http://www.ncbi.nlm.nih.gov/) under the accession AY091636. 3D models were generated by Modeller6 package.[40] For model refinement and minimisation, the SYBYL package with the TRIPOS force field (TRIPOS Associates Inc.) were used. The complete modelling including the alignment and energy minimisation was done exactly as described previously.[39] The docking of ligands was performed as described earlier.[39] The positioning of the ligand in the arbitrary site was done with the DOCK module included in SYBYL/MAXIMIN2 that calculates energies of interaction based on steric contributions from the TRIPOS force field and on electrostatic contributions from any atomic charges present in the ligand. Exact positioning of the ligand was done by a two step procedure, that is, energy minimisation followed by a molecular dynamics, in exactly the same manner for all ligands described.[39] The non-binding interaction energy between the model and the ligands within the optimised complex was calculated using the TRIPOS force field. This estimation of a real interaction energy neglects solvation and desolvation effects.

4.3. Synthesis of substrates

4.3.1. General procedure. Compound **3a**[24] (**5a**[25] or **6a**;[26] 22.0 mmol) was dissolved in acetyl chloride (15 mL) and refluxed under argon. After 12 h, the reaction mixture was diluted with CH_2Cl_2 (80 mL) and extracted with ice cold water (100 mL) and ice cold sat. $NaHCO_3$ (2 × 100 mL), all within 10–15 min. The organic phase was dried with Na_2SO_4 and evaporated in vacuo. The crude compound **3b** (**5b** or **6b**), tetrabutyl ammonium hydrogen sulphate (22.0 mmol) and *p*-nitrophenol (44.0 mmol), were dissolved in a mixture of CH_2Cl_2 (80 mL) and 1 M NaOH (80 mL) and stirred vigorously for 4 h. The reaction was monitored by TLC (AcOEt–hexane 4:1). The mixture was extracted with CH_2Cl_2 (400 mL), the organic phase was separated, washed with water (2×400 mL), dried with Na_2SO_4 and evaporated in vacuo, yielding the crude acetate **3c** (**5c** or **6c**), which was further purified.

4.3.2. *p*-Nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranoside (3). Following Section 4.3.1, compound **3c** (2.45 g, 5.39 mmol; 25%) was prepared from the starting material **3a** (4.56 g, 22.0 mmol) and purified by column chromatography on Merck silica gel 60 (40–63 μm) (AcOEt–hexane 3:1). The deacetylation according to Zemplén[41] yielded the title compound **3** (1.69 g, 5.15 mmol; 96%). White crystals; $[\alpha]_D^{23}=-27.7$ (*c* 0.141, MeOH) (Lit.[12] $[\alpha]_D^{18}=-24.2$ (DMF)); according to NMR it is a mixture of 73% *trans*- and 27% *cis*-amide. ^{1}H NMR (CD_3OD) *trans*-amide: δ 3.468 (dd, 1H, *J*=9.8, 8.7 Hz, H-4), 3.564 (ddd, 1H, *J*=9.8, 5.7, 2.3 Hz, H-5), 3.661 (dd, 1H, *J*=10.3, 8.7 Hz, H-3), 3.572 (dd, 1H, *J*=12.1, 5.7 Hz, H-6u), 3.954 (dd, 1H, *J*=12.1, 2.3 Hz, H-6d), 4.023 (ddd, 1H, *J*=10.3, 8.4, 1.1 Hz, H-2), 5.290 (d, 1H, *J*=8.4 Hz, H-1), 7.206 and 8.225 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 8.213 (d, 1H, *J*=1.1 Hz, CH=O); *cis*-amide: δ 3.472 (dd, 1H, *J*=10.4, 8.5 Hz, H-4), 3.473 (dd, 1H, *J*=10.3, 7.9 Hz, H-2), 3.551 (ddd, 1H, *J*=10.4, 5.5, 2.2 Hz, H-5), 3.552 (dd, 1H, *J*=10.3, 8.5 Hz, H-3), 3.757 (dd, 1H, *J*=12.1, 5.5 Hz, H-6u), 3.953 (dd, 1H, *J*=12.1, 2.2 Hz, H-6d), 5.182 (d, 1H, *J*=7.9 Hz, H-1), 7.237 and 8.242 (4H, AA′BB′, Σ*J*=9.2 Hz, *p*-NP), 8.138 (s, 1H, CH=O); ^{13}C NMR (CD_3OD, HMQC readouts) *trans*-amide: δ 55.6 (C-2), 61.9 (C-6), 71.1 (C-4), 74.7 (C-3), 77.9 (C-5), 99.2 (C-1), 117.0 (2×C-*ortho*), 125.9 (2×C-*meta*), 163.7 (CH=O); *cis*-amide: δ 59.6 (C-2), 62.9 (C-6), 71.1 (C-4), 74.7 (C-3), 77.9 (C-5), 99.1 (C-1), 117.0 (2×C-*ortho*), 126.0 (2×C-*meta*), 167.2 (CH=O); ESI-MS: *m*/*z* calcd for $C_{13}H_{16}N_2O_8Na$ $[M+Na]^+$ 351.1, found 351.3.

4.3.3. *p*-Nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranoside (5). Following Section 4.3.1, compound **5c** (3.20 g, 6.07 mmol; 28%) was prepared from the starting material **5a** (6.14 g, 22.0 mmol) and purified by column chromatography on silica gel (AcOEt–PE 2:1). White powder; ^{1}H NMR ($CDCl_3$): δ 2.070 (6H, 2×Ac), 2.086 (3H, Ac), 2.155 (3H, Ac), 3.937 (ddd, 1H, *J*=9.8, 5.6, 2.6 Hz, H-5), 4.192 (dd, 1H, *J*=12.3, 2.6 Hz, H-6u), 4.270 (ddd, 1H,

J=10.7, 8.6, 8.1 Hz, H-2), 4.290 (dd, 1H, J=12.3, 5.6 Hz, H-6d), 4.432 and 4.611 (2H, AB, J=15.5 Hz, CH_2O-), 5.168 (dd, 1H, J=9.8, 9.3 Hz, H-4), 5.370 (d, 1H, J=8.1 Hz, H-1), 5.397 (dd, 1H, J=10.7, 9.3 Hz, H-3), 6.275 (d, 1H, J=8.6 Hz, NH), 7.076 and 8.194 (4H, AA′BB′, ΣJ=9.3 Hz, *p*-NP); ^{13}C NMR ($CDCl_3$, HMQC and HMBC readouts): δ 20.5 (4×Ac), 54.2 (C-2), 61.9 (C-6), 62.7 (CH_2O-), 68.4 (C-4), 71.2 (C-3), 72.6 (C-5), 98.3 (C-1), 116.7 (2×C-*ortho*), 125.7 (2×C-*meta*), 143.2 (C-*para*), 161.3 (C-*ipso*), 167.6 (2-CO), 169.2 (4-CO), 169.5 (CH_2OCO), 170.3 (6-CO), 171.1 (3-CO); ESI-MS: *m/z* calcd for $C_{20}H_{24}N_2O_{12}Na$ $[M+Na]^+$ 549.1, found 549.2. The deacetylation according to Zemplén[41] and crystallisation from hot water yielded the title compound **5** (1.59 g, 4.43 mmol; 73%). White crystals; $[\alpha]_D^{23}=-28.9$ (*c* 0.135, H_2O).

4.3.4. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranoside (6). Following Section 4.3.1, compound **6c** (3.6 g, 7.5 mmol; 34%) was prepared from the starting material **6a** (5.18 g, 22.0 mmol) and purified by crystallisation from hot EtOH. The deacetylation according to Zemplén[41] and crystallisation from hot water yielded the title compound **6** (1.67 g, 4.7 mmol; 62%). White crystals; $[\alpha]_D^{23}=-17.3$ (*c* 0.196, MeOH) (Lit.[12] $[\alpha]_D^{18}=-19.7$ (DMF)); ^{1}H NMR (CD_3OD): δ 1.141 (t, 3H, J=7.7 Hz, Et), 2.227 (dq, 1H, J=16.7, 7.7 Hz), 2.278 (dq, 1H, J=16.7, 7.7 Hz), 3.453 (dd, 1H, J=9.8, 8.6 Hz, H-4), 3.537 (ddd, 1H, J=9.8, 5.7, 2.3 Hz, H-5), 3.626 (dd, 1H, J=10.4, 8.6 Hz, H-3), 3.744 (dd, 1H, J=12.1, 5.7 Hz, H-6u), 3.952 (dd, 1H, J=12.1, 2.3 Hz, H-6d), 3.982 (dd, 1H, J=10.4, 8.5 Hz, H-2), 5.229 (d, 1H, J=8.5 Hz, H-1), 7.195 and 8.226 (4H, AA′BB′, ΣJ=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD): δ 11.4 ($\mathbf{C}H_3CH_2$), 31.5 ($CH_3\mathbf{C}H_2$), 58.2 (C-2), 63.4 (C-6), 72.5 (C-4), 76.0 (C-3), 79.2 (C-5), 100.8 (C-1), 118.0 (2×C-*ortho*), 127.1 (2×C-*meta*), 144.9 (C-*para*), 164.5 (C-*ipso*), 178.3 (C=O); ESI-MS: *m/z* calcd for $C_{15}H_{20}N_2O_8Na$ $[M+Na]^+$ 379.1, found 379.2.

4.3.5. *p*-Nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside (2). 3,4,6-Tri-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (**2a**)[42] (8.98 g, 20.0 mmol) was dissolved in dry MeCN (150 mL), silver *p*-nitrophenolate (5 g, 20.3 mmol) was added and the mixture was stirred at ambient temperature overnight. The reaction was monitored by TLC (AcOEt–hexane 4:1). The insoluble materials were removed by filtration through Celite and the filtrate was evaporated in vacuo yielding the crude acetate **2b**, which was purified by column chromatography on silica gel (AcOEt–hexane 4:1). Acetate **2b** (3.2 g, 6.9 mmol; 35%) was deprotected according to Zemplén[41] and crystallised from hot MeOH affording the title compound **2** (991 mg, 3.3 mmol; 48%). Yellowish crystals; $[\alpha]_D^{23}=-91.1$ (*c* 0.214, H_2O) (Lit.[42] $[\alpha]_D^{20}=-42$ (*c* 0.4, MeOH) for hydrochloride); ^{1}H NMR (D_2O): δ 2.737 (dd, 1H, J=9.9, 8.2 Hz, H-2), 3.278 (m, 1H, H-3), 3.296 (m, 1H, H-4), 3.471 (ddd, 1H, J=9.6, 5.7, 2.3 Hz, H-5), 3.556 (dd, 1H, J=12.4, 5.7 Hz, H-6u), 3.730 (dd, 1H, J=12.4, 2.3 Hz, H-6d), 4.960 (d, 1H, J=8.2 Hz, H-1), 7.026 and 8.039 (4H, AA′BB′, ΣJ=9.4 Hz, *p*-NP); ^{13}C NMR (D_2O): δ 56.49 (C-2), 60.72 (C-6), 69.67 (C-4), 75.72 (C-3), 76.54 (C-5), 100.52 (C-1), 116.61 (2×C-*ortho*), 126.25 (2×C-*meta*), 142.72 (C-*para*), 161.92 (C-*ipso*); ESI-MS: *m/z* calcd for $C_{12}H_{16}N_2O_7Na$ $[M+Na]^+$ 323.1, found 323.2.

4.3.6. *p*-Nitrophenyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (4). Crude syrupy 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-trifluoroacetamido-∝-D-glucopyranosyl bromide (**4a**)[33] (318 mg, 0.685 mmol) was dried with 4A molecular sieve (250 mg) in vacuo for 2 h. Dry *p*-nitrophenol (191.6 mg, 1.4 mmol) was mixed with molecular sieve A4 (290 mg), the mixture was suspended in dry MeCN (4 mL) under argon and stirred for 10 min. 2,6-Dimethylpyridine (160 μL) was added dropwise and after 10 min of stirring, Fétison reagent (50% Ag_2CO_3/celite; 221 mg, 0.368 mmol) was added. After 10 more minutes the solution of **4a** in MeCN (3×2 mL) was added via canula. The reaction was monitored by TLC (AcOEt–hexane 1:1). After 2 h the reaction mixture was diluted with CH_2Cl_2 (50 mL), extracted with water (50 mL), 0.02 M H_2SO_4 (50 mL) and with water (3×50 mL) again. The organic phase was dried with Na_2SO_4, concentrated in vacuo and recrystallised twice from hot EtOH yielding *p*-nitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**4b**) (142 mg, 0.272 mmol; 40%). White crystals; ^{1}H NMR ($CDCl_3$): δ 2.075 (s, 3H, 4-Ac), 2.086 (s, 3H, 3-Ac), 2.097 (s, 3H, 6-Ac), 3.975 (ddd, 1H, J=9.8, 5.6, 2.6 Hz, H-5), 4.177 (dd, 1H, J=12.4, 2.6 Hz, H-6u), 4.304 (dd, 1H, J=12.4, 5.6 Hz, H-6d), 4.313 (ddd, 1H, J=10.6, 8.8, 8.1 Hz, H-2), 5.182 (dd, 1H, J=9.8, 9.2 Hz, H-4), 5.336 (d, 1H, J=8.1 Hz, H-1), 5.405 (dd, 1H, J=10.6, 9.2 Hz, H-3), 6.706 (d, 1H, J=8.8 Hz, 2-NH), 7.076 and 8.204 (4H, AA′BB′, ΣJ=9.3, *p*-NP); ^{13}C NMR ($CDCl_3$): δ 20.37 (4-Ac), 20.50 (3-Ac), 20.64 (6-Ac), 54.63 (C-2), 61.87 (C-6), 68.14 (C-4), 70.95 (C-3), 72.44 (C-5), 97.58 (C-1), 114.42 (CF_3, $J_{C,F}$=287.9 Hz), 116.63 (2×C-*ortho*), 125.78 (2×C-*meta*), 143.29 (C-*para*), 157.48 (2-C=O, $J_{C,F}$=37.9), 161.09 (C-*ipso*), 169.30 (4-C=O), 170.51 (6-C=O), 170.99 (3-C=O); ESI-MS: *m/z* calcd for $C_{20}H_{21}F_3N_2O_{11}Na$ $[M+Na]^+$ 545.1, found 545.2. Compound **4b** (142 mg, 0.272 mmol) was mixed with sat. NH_4OH–MeOH 1:10 (5 mL) and left for 6 h at ambient temperature (TLC MeOH–CH_2Cl_2 1:2).[43] The evaporation in vacuo afforded **4** (107 mg, 0.270 mmol; 99%). Yellowish crystals; $[\alpha]_D^{23}=-9.2$ (*c* 0.229, H_2O) (Lit.[13] $[\alpha]_D^{25}=-10.8$ (*c* 0.157, DMF)).

4.4. Solubility of the *N*-acyl modified substrates 3, 5 and 6

The solubility of substrates **3**, **5** and **6** in MeCN/buffer mixture was measured by a gradual addition of MeCN to a suspension of substrates (1–2 mg) in citrate/phosphate buffer (pH 5.0) at 37 °C, so that the final substrate concentration would be about 50 mM and the MeCN concentration would not exceed 50% v/v, which is generally the highest possible concentration tolerated by most fungal β-*N*-acetylhexosaminidases. Substrates **3** (50 mM) and **6** (46 mM) were soluble in 45% v/v MeCN, whereas for substrate **5** (50 mM), 5% v/v MeCN was sufficient.

4.5. Enzymatic methods

4.5.1. Enzyme activity assay.[2] The reaction mixture containing substrate **1** (2 mM, starting concentration) and

β-*N*-acetylhexosaminidase (0.02–0.03 U) in buffer was incubated with shaking at 35 °C for 10 min. Liberated *p*-nitrophenol was determined spectrophotometrically (420 nm) under alkaline conditions (0.1 M Na_2CO_3). One unit of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the above conditions. The activity towards substrates **2**–**5** was determined analogously, with the amount of enzyme being 0.07–0.15 U. In the case of substrate **6**, reaction mixtures containing 1 mM substrate **6** and β-*N*-acetylhexosaminidase (0.07 U) in buffer at 35 °C were analysed by TLC after 10 min (AcOEt–MeOH–sat. NH_4OH 7:3:1). In the enzymatic screening, the enzymes were classified according to the ratio of hydrolysis rates of the respective modified substrate and substrate **1** assayed under the same conditions.

4.5.2. Analytical transglycosylation reactions. The reaction mixture contained 11–13 mg of substrate **3**, **5** (starting concentration 50 mM) or **6** (starting concentration 46 mM) in MeCN/buffer (45% v/v for substrates **3**, **6**; 5% v/v for substrate **5**). The reaction was started by the addition of β-*N*-acetylhexosaminidase (0.8–8.2 U) and was incubated at 37 °C with shaking for 24 h. Aliquots were analysed by TLC (AcOEt–MeOH–sat. NH_4OH 7:3:1) and by HPLC (reactions with substrates **3** and **5**).

4.5.3. *p*-Nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-formamido-β-D-glucopyranoside (7). Substrate **3** (82 mg, 0.250 mmol) was dissolved in 45% v/v MeCN/buffer (5 mL) and after the addition of the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (13.6 U) the mixture was shaken at 37 °C. After 3 h the reaction was stopped by diluting with MeOH and boiling for 5 min. The reaction mixture was evaporated and chromatographed on a silica gel column (AcOEt–MeOH–sat. NH_4OH 7:3:1.5) yielding the title compound **7** (10.5 mg, 0.020 mmol; 16%). White crystals; $[\Theta]$=+35.0[†] (194 nm), −4.01 (220 nm),+1.25 (280 nm), −0.662 (340 nm). According to NMR the ratio of *trans*-/*cis*-amide isomers was 74:26, however, four species containing all possible combinations were distinguished, albeit not completely; ^{1}H NMR (D_2O) *trans*-/*trans*-amide: δ 3.271 (dd, 1H, *J*=10.2, 10.1 Hz, H-3′), 3.306 (ddd, 1H, *J*=8.3, 5.3, 2.2 Hz, H-5′), 3.415 (dd, 1H, *J*=10.2, 8.3 Hz, H-4′), 3.512 (dd, 1H, *J*=12.2, 4.5 Hz, H-6u), 3.545 (dd, 1H, *J*=12.2, 5.3 Hz, H-6′u), 3.559 (ddd, 1H, *J*=8.4, 4.5, 3.2 Hz, H-5), 3.606 (dd, 1H, *J*=10.5, 10.2 Hz, H-3), 3.626 (ddd, 1H, *J*=10.1, 8.4, 0.7 Hz, H-2′), 3.679 (dd, 1H, *J*=10.5, 8.4 Hz, H-4), 3.691 (dd, 1H, *J*=12.2, 3.2 Hz, H-6d), 3.724 (dd, 1H, *J*=12.2, 2.2 Hz, H-6′d), 3.915 (ddd, 1H, *J*=10.2, 8.4, 0.6 Hz, H-2), 4.473 (d, 1H, *J*=8.4 Hz, H-1′), 5.114 (d, 1H, *J*=8.4 Hz, H-1), 6.965 and 8.022 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 8.004 (d, 1H, *J*=0.6 Hz, 2-CH═O), 8.056 (d, 1H, *J*=0.7 Hz, 2′-CH═O); *cis*-/*cis*-amide: δ 3.120 (dd, 1H, *J*=10.2, 8.0 Hz, H-2′), 3.267 (dd, 1H, *J*=9.7, 8.6 Hz, H-4′), 3.306 (ddd, 1H, *J*=9.7, 5.3, 2.2 Hz, H-5′), 3.391 (dd, 1H, *J*=10.2, 8.6 Hz, H-3′), 3.444 (dd, 1H, *J*=10.0, 8.2 Hz, H-2), 3.547 (dd, 1H, *J*=12.2, 5.3 Hz, H-6′u), 3.725 (dd, 1H, *J*=12.2, 2.2 Hz, H-6′;d), 4.438 (d, 1H, *J*=8.3 Hz, H-1′), 5.126 (d, 1H, *J*=8.5 Hz, H-1), 7.000 and 8.033 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 7.946 (s, 2H, 2×CH═O); *cis*-/*trans*-amide and *trans*-/*cis*-amide: δ 3.920 (dd, 1H, *J*=10.4, 8.3 Hz, H-2), 4.479 (d, 1H, *J*=8.3 Hz, H-1′), 5.151 (d, 1H, *J*=8.5 Hz, H-1), 8.027 (4H, AA′BB′, Σ*J*=8.6 Hz, *p*-NP), 7.818 (s, 2H, 2×CH═O); ^{13}C NMR (D_2O) *trans*-/*trans*-amide: δ 53.64 (C-2), 54.54 (C-2′), 60.04 (C-6), 60.72 (C-6′), 69.88 (C-3′), 71.98 (C-5), 73.38 (C-4′), 75.03 (C-3), 76.09 (C-5′), 78.79 (C-4), 98.30 (C-1), 101.20 (C-1′), 116.71 (2×C-*ortho*), 126.22 (2×C-*meta*), 142.89 (C-*para*), 161.74 (C-*ipso*), 165.02 (CH═O), 165.08 (CH═O); *cis*-/*cis*-amide: δ 58.32 (C-2), 59.24 (C-2′), 59.67 (C-6), 60.68 (C-6′), 69.74 (C-3′), 71.83 (C-3), 73.11 (C-4′), 75.03 (C-5), 75.09 (C-5′), 78.46 (C-4), 97.85 (C-1), 100.65 (C-1′), 116.71 (2×C-*ortho*), 126.22 (2×C-*meta*), 142.92 (C-*para*), 161.48 (C-*ipso*), 168.43 (CH═O), 168.52 (CH═O); long-range coupling between aldehyde protons and the respective H-2 protons was observed in the *trans*-series. CH═O also showed coupling to C-2 and H-2 to the aldehydic carbonyl (HMBC); ESI-MS: *m*/*z* calcd for $C_{20}H_{27}N_3O_{13}Na$ $[M+Na]^+$ 540.1, found 540.3.

4.5.4. *p*-Nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-glycoloylamido-β-D-glucopyranoside (8). Substrate **5** (41 mg, 0.114 mmol) was dissolved in 5% v/v MeCN/buffer (2.3 mL), the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (3.16 U) was added and the mixture was shaken at 37 °C. After 100 min the reaction was stopped by diluting with MeOH and boiling for 5 min. The reaction mixture was evaporated and chromatographed on a silica gel column (AcOEt–MeOH–sat. NH_4OH 7:3:1.5) yielding the title compound **8** (25.6 mg, 0.044 mmol; 78%). Colourless syrup; $[\Theta]$=+36.2 (194 nm), −4.52 (220 nm), +1.11 (280 nm), −0.534 (340 nm); ^{1}H NMR (D_2O): δ 3.276 (dd, 1H, *J*=9.9, 8.4 Hz, H-4′), 3.317 (m, 1H, H-5′), 3.478 (dd, 1H, *J*=12.3, 2.6 Hz, H-6u), 3.479 (dd, 1H, *J*=10.4, 8.4 Hz, H-3′), 3.548 (dd, 1H, *J*=12.3, 5.6 Hz, H-6′u), 3.553 (m, 1H, H-5), 3.567 (dd, 1H, *J*=9.4, 8.3 Hz, H-4), 3.627 (dd, 1H, *J*=10.4, 8.4 Hz, H-2′), 3.682 (dd, 1H, *J*=12.3, 10.9 Hz, H-6d), 3.728 (dd, 1H, *J*=12.3, 2.1 Hz, H-6′d), 3.734 (dd, 1H, *J*=10.5, 8.3 Hz, H-3), 3.867 and 3.893 (2H, AB, *J*=16.6 Hz, CH_2O-), 3.920 (dd, 1H, *J*=10.5, 8.4 Hz, H-2), 3.936 and 3.961(2H, AB, *J*=16.6 Hz, CH_2O–), 4.506 (d, 1H, *J*=8.4 Hz, H-1′), 5.170 (d, 1H, *J*=8.4 Hz, H-1), 6.959 and 8.025 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (D_2O): δ 54.71 (C-2), 55.63 (C-2′), 60.20 (C-6), 60.89 (C-6′), 61.28 (2×CH_2O–), 70.08 (C-4′), 72.19 (C-3), 73.58 (C-3′), 75.20 (C-5), 76.23 (C-5′), 79.21 (C-4), 98.51 (C-1), 101.42 (C-1′), 116.85 (2×C-*ortho*), 126.36 (2×C-*meta*), 143.02 (C-*para*), 161.87 (C-*ipso*), 175.86 (C═O), 176.03 (C═O); ESI-MS: *m*/*z*$[M+Na]^+$ 600.2, found 600.3.

4.5.5. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-propionamido-β-D-glucopyranoside (9). Substrate 6 (60.8 mg, 0.171 mmol) was dissolved in 45% v/v MeCN/buffer (3.7 mL) and the β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2315 (51 U) was added. The mixture was shaken at 37 °C. After 16 h the substrate was practically consumed and the reaction was stopped by diluting with MeOH (10 mL) and boiling for 5 min. The solids were filtered off; the reaction mixture was evaporated to dryness, dissolved in water and extracted into a solid phase on Amberlite XAD-4 resin (BDH Chemicals,

[†] Molar elipticity $[\Theta]$ is reported in units of 10^3 deg cm^2/dmol.

Ltd). After washing with water, *p*-nitrophenyl glycosides were eluted with MeOH, the eluent was evaporated and separated on Sephadex LH-20 (25–100 μm, Pharmacia) with a mobile phase of MeOH–H_2O 4:1 and a flow rate of 22 mL/h, which afforded the title compound **9** (11.8 mg, 0.021 mmol; 24%). White crystals; $[\Theta]$=+56.6 (194 nm), −5.95 (220 nm),+1.83 (280 nm), −0.669 (340 nm); ^{1}H NMR (CD_3OD): δ 1.136 (t, 3H, *J*=7.6 Hz, 2-Et), 1.189 (t, 3H, *J*=7.6 Hz, 2′-Et), 2.232 (dq, 1H, *J*=15.2, 7.6 Hz, 2-CH_2-u), 2.246 (dq, 1H, *J*=15.2, 7.6 Hz, 2-CH_2-d), 2.305 (dq, 1H, *J*=15.2, 7.6 Hz, 2′-CH_2-u), 2.330 (dq, 1H, *J*=15.2, 7.6 Hz, 2′-CH_2-d), 3.335 (dd, 1H, *J*=9.8, 8.3 Hz, H-4′), 3.390 (ddd, 1H, *J*=9.8, 6.4, 2.3 Hz, H-5′), 3.479 (dd, 1H, *J*=10.3, 8.3 Hz, H-3′), 3.601 (ddd, 1H, *J*=9.7, 4.6, 2.0 Hz, H-5), 3.674 (dd, 1H, *J*=11.9, 4.6 Hz, H-6′u), 3.674 (dd, 1H, *J*=9.7, 8.2 Hz, H-4), 3.695 (dd, 1H, *J*=12.2, 4.6 Hz, H-6u), 3.777 (dd, 1H, *J*=10.3, 8.5 Hz, H-2′), 3.786 (dd, 1H, *J*=10.4, 8.2 Hz, H-3), 3.874 (dd, 1H, *J*=12.2, 2.0 Hz, H-6d), 3.943 (dd, 1H, *J*=11.9, 2.2 Hz, H-6′d), 4.059 (dd, 1H, *J*=10.4, 8.4 Hz, H-2), 4.556 (d, 1H, *J*=8.5 Hz, H-1′), 5.220 (d, 1H, *J*=8.4 Hz, H-1), 7.181 and 8.221 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD): δ 10.2 (Et), 10.5 (Et′), 30.5 (CH2′), 30.6 (CH2), 56.4 (C-2), 57.3 (C-2′), 61.8 (C-6), 62.7 (C-6′), 72.3 (C-4′), 74.0 (C-3), 76.0 (C-3′), 77.1 (C-5), 78.2 (C-5′), 81.0 (C-4), 100.2 (C-1), 103.3 (C-1′), 118.0 (2×C-ortho), 126.9 (2×C-meta), 144.3 (C-para), 163.7 (C-ipso), 177.2 (2′-C=O), 177.8 (2-C=O); ESI-MS: *m/z* calcd for $C_{24}H_{35}N_3O_{13}Na$ $[M+Na]^+$ 596.2, found 596.5.

4.5.6. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (10). Substrates **1** (40.4 mg, 0.118 mmol) and **6** (80.6 mg, 0.226 mmol) were dissolved in 45% v/v MeCN/buffer (4.9 mL) and the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 (48 U) was added. The mixture was shaken at 37 °C. The enzyme was added twice more (24 U after 7 h and 12 U after 13 h). After 15.5 h substrate **1** was consumed and the reaction was stopped by diluting with MeOH (10 mL) and boiling for 5 min. The solids were filtered off and the reaction mixture was evaporated to dryness, dissolved in water and loaded onto Amberlite XAD-4 resin. After washing with water, *p*-nitrophenyl glycosides were eluted with MeOH, the eluent was evaporated and separated on Sephadex LH-20 (mobile phase MeOH–H_2O 4:1, flow rate 24 mL/h) affording a mixture of **9** and **10**, which was further separated by preparative HPLC. Besides product **9** (2.9 mg, 0.005 mmol; yield 4.3% referred to substrate **1**), the title compound **10** (1.2 mg, 0.002 mmol; yield 1.8%) was obtained. White crystals; $[\Theta]$=+91.8 (194 nm), −9.25 (220 nm),+2.75 (280 nm), −1.23 (340 nm); ^{1}H NMR (CD_3OD): 1.188 (t, 3H, *J*=7.6 Hz, Et), 1.986 (s, 3H, Ac), 2.293 (dq, 1H, *J*=15.1, 7.6 Hz, half of CH_2), 2.338 (dq, 1H, *J*=15.1, 7.6 Hz, half of CH_2), 3.335 (dd, 1H, *J*=9.6, 8.3 Hz, H-4′), 3.393 (ddd, 1H, *J*=9.6, 6.2, 2.2 Hz, H-5′), 3.479 (dd, 1H, *J*=10.3, 8.3 Hz, H-3′), 3.600 (ddd, 1H, *J*=9.7, 4.6, 2.0 Hz, H-5), 3.674 (dd, 1H, *J*=11.9, 6.2 Hz, H-6′u), 3.676 (dd, 1H, *J*=9.7, 8.2 Hz, H-4), 3.691 (dd, 1H, *J*=12.2, 4.6 Hz, H-6u), 3.778 (dd, 1H, *J*=10.3, 8.5 Hz, H-2′), 3.780 (dd, 1H, *J*=10.3, 8.2 Hz, H-3), 3.869 (dd, 1H, *J*=12.2, 2.0 Hz, H-6d), 3.945 (dd, 1H, *J*=11.9, 2.2 Hz, H-6′d), 4.047 (dd, 1H, *J*=10.3, 8.5 Hz, H-2) 4.556 (d, 1H, *J*=8.5 Hz, H-1′), 5.224 (d, 1H, *J*=8.5 Hz, H-1). 7.186 and 8.223 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD, HMQC and HMBC readouts): δ 10.4 ($\mathbf{C}H_3CH_2$), 23.0 (Ac), 30.6 ($CH_3\mathbf{C}H_2$), 56.7 (C-2), 57.5 (C-2′), 61.8 (C-6), 62.8 (C-6′), 72.4 (C-4′), 74.2 (C-3), 76.1 (C-3′), 77.1 (C-5), 78.5 (C-5′), 81.1 (C-4), 100.2 (C-1), 103.4 (C-1′), 118.0 (2×C-*ortho*), 126.9 (2×C-*meta*), 144.7 (C-*para*), 164.2 (C-*ipso*), 174.2 ($CH_3\mathbf{C}O$), 177.8 ($CH_3CH_2\mathbf{C}O$); ESI-MS: *m/z* calcd for $C_{23}H_{33}N_3O_{13}Na$ $[M+Na]^+$ 582.2, found 582.4.

4.5.7. NMR characterisation of transglycosylation products. All prepared disaccharides showed the expected pseudomolecular ions in the MS spectra. According to $J_{1,2}$ values (^{1}H NMR), the anomeric configuration was β in all cases. H-1 and H-1′ protons were differentiated on the basis of the coupling of the former to C-*ipso* of *p*-NP. The (**1→4**)-linkage was inferred from heteronuclear couplins of H-1′ to C-4 or H-4 to C-1′ observed in HMBC. The downfield shift of C-4 with respect to the parent compound provided a supporting argument. The attachment of a substituent to C-2 is apparent from the coupling of H-2 and side chain protons to the same carbonyl.

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Adrenaline profiling of lipases and esterases with 1,2-diol and carbohydrate acetates

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Abstract—The adrenaline test for enzymes is a general back-titration procedure to detect 1,2-diols, 1,2-aminoalcohols and α-hydroxyketones reaction products of enzyme catalysis by colorimetry. The method was used to profile a series of esterases and lipases for their esterolytic activity on a series of carbohydrate and polyol acetates. Substrates were prepared by peracetylation and used for parallel microtiter-plate analysis of enzyme activities. This method can be used to achieve a rapid and automated characterization of a set of enzymes during HTS screening.

1. Introduction

Enzyme assays suitable for high-throughput screening are essential for the exploration of biological diversity in search for novel enzymes,[1] and the use of biocatalysis in industrial processes is rapidly growing.[2] The vast majority of processes are based on hydrolytic enzymes, in particular lipases and esterases, which are essential tools in fine chemical synthesis. Several thousand different lipases and esterases have been described, yet there is almost no information available in the literature that allows comparing these different enzymes in terms of their reactivities and selectivities. We have described recently a general procedure for generating activity fingerprints of hydrolytic enzymes using arrays of fluorogenic and chromogenic substrates.[3] The method was based on an indirect release strategy including an oxidation step with $NaIO_4$, followed by a β-elimination leading to a fluorescent product, such that various enzyme classes could be assayed including alcohol dehydrogenases,[4] aldolases,[5] proteases,[6] and lipases.[7] The diversity of reactivity patterns observed even between closely related enzymes suggested that a large body of differential reactivity information can indeed be established by such procedures.[8] Nevertheless each of the substrates used in this study had to be individually synthesized by asymmetric multistep synthesis, and was available only in limited quantities. We wanted to explore the possibility of using more readily available substrates such that the method would become more broadly available even for laboratories not familiar with organic synthesis. Herein we report that acetate derivatives of commercially available carbohydrates and diols provide a readily available substrate family for profiling lipases and esterases based on the chromogenic adrenaline test for enzymes.

2. Results and discussion

The adrenaline test is a back-titration procedure which measures the concentration of periodate-sensitive reaction products formed from periodate-resistant substrates by an enzymatic transformation.[9] The test solution is treated with a measured amount of sodium periodate, which rapidly reacts with oxidizable functional groups presents, in particular 1,2-diols, 1,2-aminoalcohols or α-hydroxyketones released from the hydrolysis of ester, amide, phosphate or epoxide precursors used as substrates for the corresponding enzymes. The unreacted periodate reagent is then revealed by addition of adrenaline **1**, which undergoes an instantaneous oxidation with periodate to give adrenochrome **2**, a cationic orthoquinone dye with a red absorption maximum in the visible spectrum (Scheme 1). We had demonstrated that this colorimetric procedure provided off-the-shelf endpoint assays for lipases using vegetal oils as substrates, phytases using phytic acid as substrate, and epoxide hydrolases using epoxides as substrates.

In the perspective of fine chemical synthesis, kinetic resolution of chiral alcohols by enantioselective lipases is one of the most popular and reliable methods for producing enantiomerically pure products. The preferred reactions are the formation or hydrolysis of acetate esters of these

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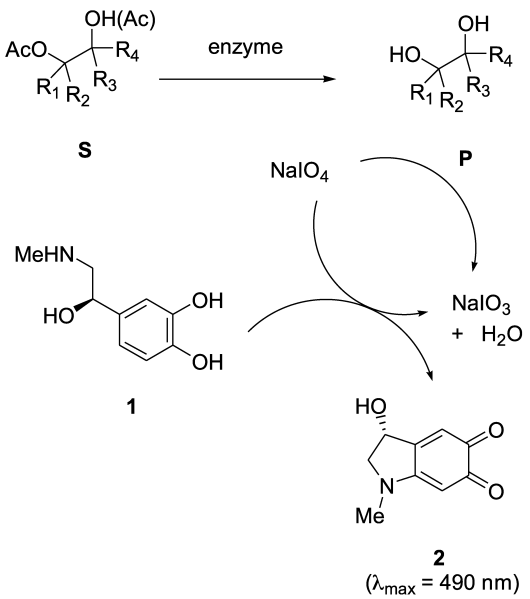

Scheme 1. Principle of the adrenaline test for esterolysis of 1,2-diol acetates.

alcohols. However many enzymes show only very poor reactivities towards acetates, and a preliminary screening against such substrates is a necessary first step for assessing the value of any enzyme collection in view of such applications. It was shown recently that a multi-enzyme detection kit for acetic acid can be used to test such reactivities.[10]

We envisioned that our adrenaline test for enzymes would provide an interesting opportunity for profiling such reactivities with an array of 1,2-diol and carbohydrate acetates. Indeed these substrates could be easily prepared by peracetylation of the parent polyols. The resulting substrate family was structurally diverse, such that the resulting reactivity profiles would be useful for selecting enzymes on the basis of their reactivity. Peracetate derivatives were prepared by exhaustive acetylation with acetic anhydride in pyridine. The products were purified simply by liquid extraction and their structure confirmed by NMR-analysis. The series was extended with a few commercially available di- and triacetates to give a total of 35 different acetates (Fig. 1).

The polyol substrates and their acetate derivatives were conditioned as 10 mM stock solutions in water/acetonitrile mixtures for the assay procedure. First, the reactivity of the compounds against periodate in the adrenaline assay was calibrated by testing serial dilutions of the products against periodate (1 mM) followed by addition of **1** (1.5 mM). The ester derivatives showed either full signal or only a weak decrease of adrenochrome absorption with the adrenaline test, in agreement with the absence of any free 1,2-diol functionality in these derivatives. By contrast, the corresponding polyols showed the expected reactivity, and the results are shown in Table 1. The adrenaline procedure efficiently detected most polyol in the expected range of $100\ \mu M < EC_{50} < 750\ \mu M$ based on the expected stoichiometry. Free hexitols and hexoses were detected with the highest sensitivity in agreement with the fact that these

Table 1. Detection of free polyols by back-titration of sodium periodate with adrenaline. EC_{50} is the product concentration that inhibits 50% of the color reaction. Data calculated from OD decrease at 490 nm by increasing polyol molar concentration, in the presence of 1 mM $NaIO_4$ in 50 mM aqueous borate buffer pH 8.0 for 1 h at 37 °C, followed by reaction with 1.5 mM of **1** for 5 min at 26 °C

Alcohol	Acetates	EC_{50} (mM)
D-Mannitol	**37**	0.09
D-Glucose	**28**	0.10
D-Lactose	**31**	0.10
D-Sorbitol	**32**	0.11
D-Mannose	**24**	0.12
D-Maltose	**36**	0.12
D-Galactose	**34**	0.13
D-Fructose	**29**	0.13
D-Xylose	**25**	0.14
D-Fucose	**34**	0.15
L-Arabinose	**35**	0.15
D-Arabinose	**30**	0.16
D-Ribose	**23**	0.18
D,L-Glyceraldehyde	**13**	0.26
2,3-Butanediol	**10**	0.52
Glycerol	**8, 9**	0.55
1,2-Dodecanediol	**7**	0.65
Ethyleneglycol	**3**	0.74
1,2-Propanediol	**4**	0.74
1,2-Butanediol	**5**	0.74
Cis-1,2-cyclopentanediol	**18**	0.74
Cis-1,2-cyclohexanediol	**19**	0.78
1,2-Octanediol	**6**	0.79
(1*R*,2*R*)-*trans*-1,2-cyclohexanediol	**21**	0.81
3,3-Dimethyl-1,2-butanediol	**11**	1.00
(*S*)-(−)-1,1-Diphenyl-1,2-propanediol	**22**	1.35
Pinacol	**12**	2.24
D-Sucrose	**26**	2.24
D-Trehalose	**27**	2.24

carbohydrates undergo fivefold oxidation with periodate. Sucrose, trehalose, and pinacol gave EC_{50} values in the 2 mM range, probably because their 1,2-diol functional groups only react very slowly with periodate. This calibration showed that all substrates were in principle suited to generate a colorimetric signal upon complete deacetylation. In general however, any partial deacetylation liberating at least one 1,2-diol functionality would lead to a decrease in the colorimetric signal in the adrenaline test.

A series of 33 lipases and esterases was assayed with the peracetate substrate array shown in Figure 1, comprising 21 commercial lipases and 12 proprietary enzymes derived from culture collections at Protéus (Table 2). These proprietary enzymes have been obtained during HTS screening programs dedicated to the discovery of thermophilic esterases and lipases that can be used in the industrial synthesis of fine chemical compounds at high temperature. These enzymes, originating from strains isolated in either deep sea hydrothermal vents, oil fields or hot terrestrial soil, were characterized for their acyl chain length specificity (ranging from C2 to C18).

The enzymes were incubated under a standard microtiter-plate assay procedure consisting in incubating each of the 35 substrates at 1 mM in 100 mL of 50 mM aqueous borate buffer pH 8.0, 5% DMF cosolvent, with 1 mg/mL enzyme and 1 mM $NaIO_4$ at 37 °C for 1 h, followed by treatment with **1** for 5 min. The red color was quantitated at 490 nm with a microtiter-plate reader. The relative signal intensity

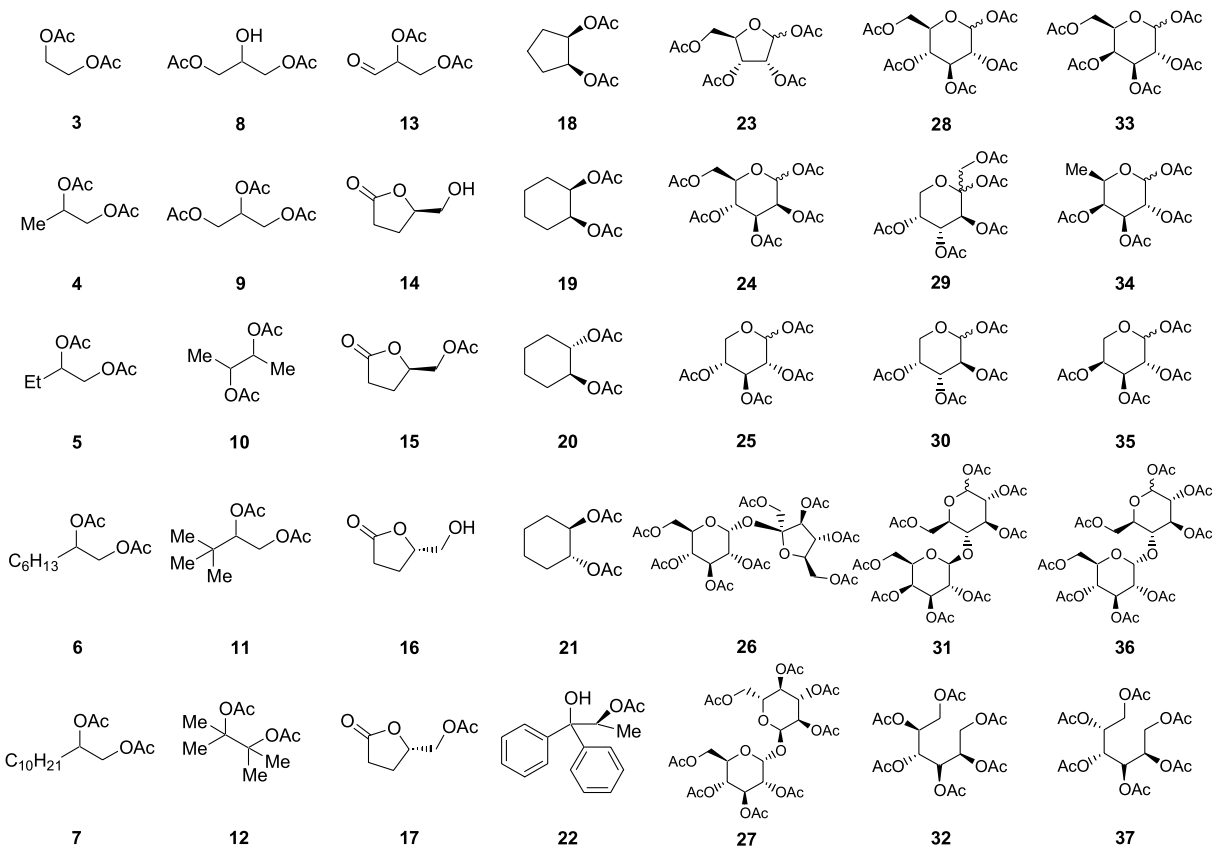

Figure 1. Array of polyol acetates used for adrenaline profiling.

was then corrected by subtracting the blank measurement, which concerned mainly the acetylated fructose derivative **29**. The net signal thus obtained was then used to generate activity patterns in grayscale, which is a convenient display form for data analysis. The results are shown in Figure 2.

The reactivity patterns obtained were strongly dependent on the enzyme used. The reactivities observed should be compared with the blank data observed without enzyme, as well as with the values generated by reaction in the presence of bovine serum albumin, which may be considered as a measure of the non-specific reactivity of the substrates. These blank values show an unusually high reactivity of peracetylated fructose **29** and mannitol hexaacetate **37** and may be associated with a spontaneous partial deacetylation or intrinsic chemical unstability of such substrates. The intrinsic reactivity of the substrates was tested by treatment at higher temperatures under conditions where complete solubility of all substrates was ensured (Fig. 3). Most carbohydrate derivatives were labile under these conditions, while simple aliphatic diols not containing further oxygenated substituents were clearly the most stable. The higher reactivity of carbohydrate acetates compared to less substituted alcohol esters might be related to their higher polarity as well as to the lower pK_a of their hydroxyl groups, which are acidified by inductive effects of the neighbouring oxygen atoms. Indeed the simple effect of stoichiometry (Table 1) is not sufficient to explain the reactivity difference between the two ester classes.

Remarkably, the reactivity with the enzymes did not parallel that of the blank data, showing that the specific enzyme reactivities reflected selective interactions with the enzymes rather than non-specific acceleration of the chemical hydrolysis by the enzymes. Most enzymes showed the strongest signal with acetylated carbohydrates in agreement with the fact that these derivatives release the highest number of periodate-sensitive 1,2-diols. All enzymes tested showed individually differentiated reactivity patterns. These patterns were reproducible and could thus be considered as showing the intrinsic selectivity pattern of each enzyme. The broad array of patterns observed illustrates the enormous diversity in reactivities available in esterolytic enzymes, despite of the fact that all of them are mostly naturally selected to hydrolyze triglycerides. There was no detectable association of the reactivity pattern observed with the association of the enzyme to a lipase or esterase type reactivity, in agreement with the fact that this classification is problematic and sometimes grounded in historical reasons.

As for the proprietary enzymes, PE6 clearly resembled the famous CAL-B enzyme often used for kinetic resolutions. Remarkably, PE3 and PE4 displayed a high reactivity on the hindered secondary ester **22** which was not found in any of the other enzymes tested. PE7 was highlighted by a strong and highly enantioselective reaction with the (*R*,*R*)-enantiomer of *trans*-cyclohexanediol **21**. This selectivity was clearly more pronounced than that of ACE and PLE2 on the **20/21** enantiomeric pair. These interesting reactivities are under further investigation.

Table 2. Enzymes tested using the acetylated substrates array

Code	Enzyme[a]	Specific activity[b] (U/mg)	Specific periodate activity on 23[c] (mU/mg)	Specific periodate activity on 9[d] (mU/mg)
PE1	Proteus esterase number 1	–	5.5	<0.1
PE2	Proteus esterase number 2	–	3.4	0.3
PE3	Proteus esterase number 3	–	31.5	8.2
PE4	Proteus esterase number 4	–	6.8	1.0
PE5	Proteus esterase number 5	–	5.3	5.2
PE6	Proteus esterase number 6	–	5.5	5.4
PE7	Proteus esterase number 7	–	3.7	0.4
PE8	Proteus esterase number 8	–	9.8	0.7
PE9	Proteus esterase number 9	–	0.1	0.1
PE10	Proteus esterase number 10	–	0.1	0.7
PE11	Proteus esterase number 11	–	2.4	<0.1
PE12	Proteus esterase number 12	–	5.5	0.1
ACE	*Electrophorus electricus* AChE (F-01022)	850[e]	14.2	14.2
ASL	*Aspergillus* lipase (F-84205)	0.5[f]	14.4	1.4
BAE	*Bacillus* sp. esterase (F-46062)	0.1[g]	0.7	3.2
BSE	*Bacillus stearothermophilus* esterase (F-46051)	0.4[h]	10.5	2.1
BTE	*Bacillus thermoglucosidasius* esterase (F-46054)	0.1[g]	10.3	2.4
CAL	*Candida antarctica* lipase (F-62299)	3[i]	8.0	<0.1
CAL B	*Candida antarctica* lipase B		5.6	14.7
CCL 1	*Candida cylindracea* lipase (F-62316)	2[i]	13.1	4.8
CCL 2	*Candida cylindracea* lipase (F-62302)	20[i]	12.1	3.7
CLE	*Candida lipolytica* esterase (F-46056)	0.1[j]	13.8	3.5
HKA	Hog kidney acylase I (F-01821)	16.5[k]	14.7	3.5
HLE	Horse liver esterase (F-46069)	0.7[l]	2.8	1.8
HPL	Hog pancreatic lipase (F-62300)	25[m]	14.7	<0.1
MML	*Mucor miehei* lipase (F-62298)	1[i]	1.8	1.4
PCL	*Pseudomonas cepacia* lipase (F-62309)	50[i]	9.0	1.1
PFL	*Pseudomonas fluorescens* lipase (F-62312)	36[i]	10.8	4.8
PLE 1	Pig liver esterase (S-E3019)	15[m]	13.9	<0.1
PLE 2	Pig liver esterase (F-46058)	130[g]	14.8	14.8
RAL	*Rhizopus arrhizus* lipase (F-62305)	10[n]	7.9	0.9
RNL	*Rhizopus niveus* lipase (F-62310)	1.5[o]	13.9	2.6
WGL	Wheat germ lipase (S-L3001)	10[p]	13.1	0.8

[a] Product reference from Fluka (F) or Sigma (S).
[b] Activity given by the provider in units per milligram of powder; definition given in footnote.
[c] Activity given in milliunits per milligram of powder of commercial samples, or per milligram of protein of proprietary enzyme samples; 1 U corresponds to the amount of enzyme which liberates 1 μmol diol per minute at pH 8.0 and 37 °C, peracetylated D-ribose **23** as substrate.
[d] Activity given in milliunits per milligram of powder of commercial samples, or per milligram of protein of proprietary enzyme samples; 1 U corresponds to the amount of enzyme which liberates 1 μmol diol per minute at pH 8.0 and 37 °C, triacetin **9** as substrate.
[e] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol acetylcholine per minute at pH 8.0 and 37 °C.
[f] 1 U corresponds to the amount of enzyme which liberates 1 μmol acetic acid per minute at pH 7.4 and 40 °C (triacetin as substrate).
[g] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of ethyl valerate per minute at pH 8.0 and 25 °C.
[h] 1 U corresponds to the amount of enzyme which releases 1 μmol 4-nitrophenol per minute at pH 7.0 and 65 °C (4-nitrophenyl-*n*-caproate as substrate).
[i] 1 U corresponds to the amount of enzyme which liberates 1 μmol oleic acid per minute at pH 8.0 and 40 °C (triolein as substrate).
[j] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of α-methyl-DL-phenylalanine-*O*-methylester per minute at pH 7.5 and 25 °C.
[k] 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol of *N*-acetyl-L-methionine per minute at pH 7.0 and 25 °C.
[l] 1 U will hydrolyze 1 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 and 25 °C.
[m] 1 U corresponds to the amount of enzyme which liberates 1 μmol fatty acid from triglycerides per minute at pH 8.0 and 37 °C (olive oil as substrate).
[n] 1 U corresponds to the amount of enzyme which liberates 1 μmol butyric acid per minute at pH 8.0 and 40 °C (tributyrin as substrate).
[o] 1 U corresponds to the amount of enzyme which liberates 1 μmol fatty acid from a triglyceride per minute at pH 7.7 and 40 °C (olive oil as substrate).
[p] 1 U will hydrolyze 1 μequiv. of fatty acid from a triglyceride in 1 h at pH 7.7 and 37 °C.

3. Conclusion

Esterases and lipases are involved in industrial processes for the synthesis of regioisomers of polyfunctional compounds, resolutions of secondary alcohols and asymmetric synthesis from prochiral or *meso*-compounds. The commercially available enzymes are not robust and efficient enough to investigate the feasibility of carrying out industrially interesting syntheses, leading to increasing requests from industry for novel enzymes. During the discovery processes of such enzymes (which include microbial screening, HTS gene isolation, and recombinant expression), the key point is to be compatible with the timelines of the industry. To fit with these timelines, robust and versatile HTS screening tests that mimic the final industrial substrates and conditions, while lowering the level of false positive must be used.[11] At the end of the discovery process, novel enzymes are validated for their intrinsic biochemical properties. This step is generally cumbersome and time consuming.

The experiments above address this issue and demonstrate that enzyme reactivity patterns can be readily obtained using the adrenaline test and acetate esters of commercially available diols and carbohydrates. Such reactivity profiling can be readily established in any laboratory since the synthetic chemistry involved is very limited and the measurement method only uses inexpensive and simple reagents. This biochemical fingerprint can be automated using usual liquid handling systems. Comparison of reactivity patterns of novel enzymes should help early

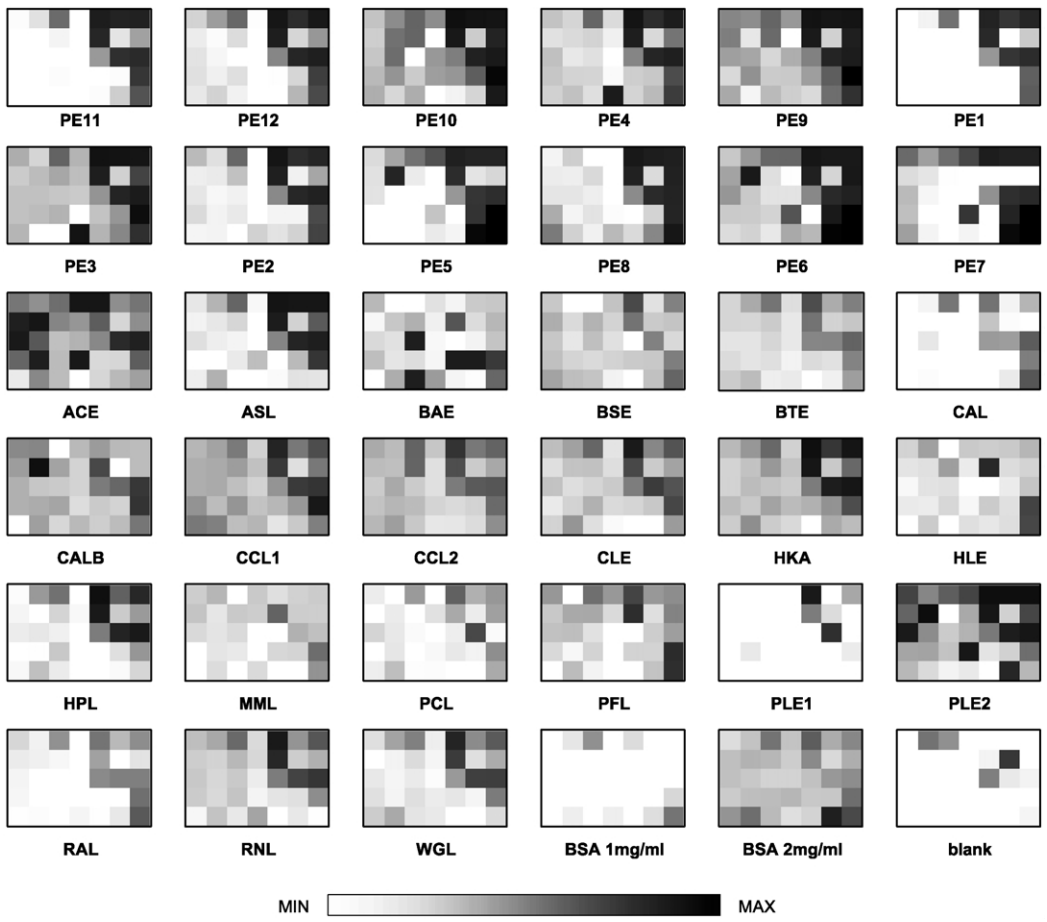

Figure 2. Activity of esterases and lipases towards polyacetates **3**–**37** as determined by the adrenaline test. Each grayscale square corresponds to one ester substrate according to the layout in Figure 1, and shows the percent reduction of OD_{490} from white (0%, no activity) to black (100%, maximum activity), after correction from blank values. Conditions: (1) Enzyme in 50 mM aq. borate pH 8.0, 1 mM $NaIO_4$, 1 mM substrate, 60 min at 37 °C; (2) 1.5 mM **1**, 5 min, 26 °C. Key for enzyme samples are given in Table 2. Commercial enzyme samples were tested at 1 mg/ml, proprietary esterases and lipases samples were used at one tenth of crude enzyme extracts filtered through size-exclusion chromatography columns.

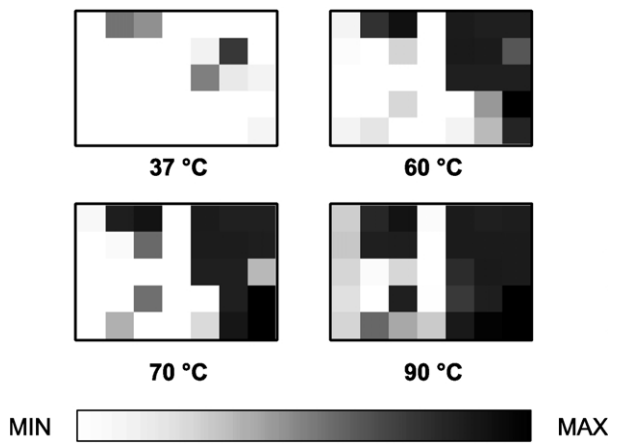

Figure 3. Intrinsic hydrolytic lability of acetate substrates without enzyme. Each grayscale square corresponds to one ester substrate according to the layout in Figure 1, and shows the percent reduction of OD_{490} from white (0%, no degradation) to black (100%, total consumption of sodium periodate). Conditions: (1) 1 mM substrate in 50 mM aq. borate pH 8.0, 1 mM $NaIO_4$, 60 min at the indicated temperature; (2) 1.5 mM adrenaline, 5 min, 26 °C.

validation of discoveries and guide research efforts towards those new enzymes displaying the most interesting and novel reactivity patterns. In that respect, the fact that the proprietary enzymes tested here all display patterns that differ strongly from standard esterases and lipases highlights the validity of pursuing biodiversity mining as a source for new enzymes with novel selectivity properties.

4. Experimental

4.1. General

NMR spectra were recorded on a Bruker Avance 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) with a Bruker auto-sampler B-ACS 120. Deuterated chloroform with the chemical shifts ^{1}H: 7.26 ppm, ^{13}C: 77.7 ppm, or deuterated methanol with the chemical shifts ^{1}H: 3.31, 4.79 ppm, ^{13}C: 49.0 ppm were used as solvents. All chemical shifts were expressed in ppm according to the reference solvent peaks. Coupling constants (J) are given in Hertz (Hz). Atoms of first acetate residue were quoted with $'$, atoms of the second acetate residue with $''$, etc. The reactions were monitored by thin layer chromatography (TLC) using plastic sheets with silica

gel 60 F_{254} (Merck). The melting points were determined with a Büchi Type S melting point apparatus and are uncorrected.

Pyridine as the solvent for synthesis was purified and dried before use. Polyols were obtained from Sigma-Aldrich-Fluka or Lancaster. Diacetin, triacetin and ethyleneglycol diacetate, as commercial polyacetate substrates, were obtained from Fluka.

4.2. General procedure for the preparation of compounds 4–7, 10–11, 13, 15, 17–21, 24–25, 28, 30, and 32–35

To an ice-bath cooled, stirred mixture of polyol in dry pyridine were added 2 molar equiv. of acetic anhydride per alcohol function. The reaction mixture was stirred overnight, the temperature coming back slowly to room temperature. The reaction was stopped by dilution with water and ethylacetate, and the mixture washed with water and brine. After separation and drying over magnesium sulfate, the organic phase was concentrated to dryness. Coevaporation under vacuum with pyridine and toluene of the residue afforded pure polyacetate. Products were characterized and validated by NMR analysis (proton and carbon), and their homogeneity verified by TLC. Yields obtained ranged from 83 to 99%.

4.3. General procedure for the preparation of compounds 12, 22 and 29

To an ice-bath cooled, stirred mixture of polyol in dry pyridine were added 2 molar equiv. of acetic anhydride and 0.1 molar equiv. of 4-dimethylamino-pyridine per alcohol function. The reaction mixture was stirred overnight, the temperature coming back slowly to room temperature. The reaction was stopped by dilution with water and ethylacetate, and the mixture washed with water and brine. After separation and drying over magnesium sulfate, the organic phase was concentrated to dryness. Coevaporation under vacuum with pyridine and toluene of the residue afforded pure polyacetate. Products were characterized and validated by NMR analysis (proton and carbon), and their homogeneity verified by TLC. Yields obtained ranged from 54 to 78%.

4.3.1. 1,2-Propanediol diacetate 4.[12] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.23 (d, 3H, J=6.4 Hz, C3H_3), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.02 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.15 (dd, 1H, J=11.8, 3.6 Hz, C1HH), 5.11 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=17.1 (C3), 21.4 (C2′), 21.8 (C2″), 66.7 (C1), 68.9 (C2), 171.1 (C=O′), 171.4 (C=O″).

4.3.2. 1,2-Butanediol diacetate 5.[13] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.91 (t, 3H, J=7.4 Hz, C4H_3), 1.60 (m, 2H, C3H_2), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.03 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.21 (dd, 1H, J=11.8, 3.4 Hz, C1HH), 4.99 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=10.1 (C4), 21.4 (C2′), 21.7 (C2″), 24.4 (C3), 65.4 (C1), 73.4 (C2), 171.2 (C=O′), 171.4 (C=O″).

4.3.3. 1,2-Octanediol diacetate 6.[14] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.86 (t, 3H, J=6.6 Hz, C8H_3), 1.22 (m, 8H, C4H_2, C5H_2, C6H_2, C7H_2), 1.54 (m, 2H, C3H_2), 2.04 (s, 3H, C2′H_3), 2.05 (s, 3H, C2″H_3), 4.01 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.21 (dd, 1H, J=12.0, 3.0 Hz, C1HH), 5.05 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=14.7 (C8), 21.4 (C2′), 21.7 (C2″), 23.2 (C7), 25.7 (C6), 29.7 (C5), 31.3 (C4), 32.3 (C3), 65.8 (C1), 72.2 (C2), 171.3 (C=O′), 171.4 (C=O″).

4.3.4. 1,2-Dodecanediol diacetate 7.[15] Yellow syrup. ^{1}H NMR (300 MHz): δ=0.83 (t, 3H, J=6.4 Hz, C12H_3), 1.20 (m, 16H, C4H_2, C5H_2, C6H_2, C7H_2, C8H_2, C9H_2, C10H_2, C11H_2), 1.51 (m, 2H, C3H_2), 2.01 (s, 3H, C2′H_3), 2.02 (s, 3H, C2″H_3), 3.97 (dd, 1H, J=11.8, 6.6 Hz, C1HH), 4.17 (dd, 1H, J=11.9, 3.2 Hz, C1HH), 5.01 (m, 1H, C2H_1). ^{13}C NMR (75 MHz): δ=14.7 (C12), 21.3 (C2′), 21.6 (C2″), 23.3 (C11), 25.7 (C10), 29.8 (C9), 29.9 (C8), 30.0 (C7), 30.1 (C6), 30.2 (C5), 31.3 (C4), 32.5 (C3), 65.8 (C1), 72.3 (C2), 171.6 (C=O′), 171.7 (C=O″).

4.3.5. 2,3-Butanediol diacetate 10.[16] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.19 (m, 3H, C1H_3), 1.23 (m, 3H, C4H_3), 2.04 (s, 3H, C2′H_3), 2.06 (s, 3H, C2″H_3), 4.98 (m, 2H, C2H_1, C3H_1). ^{13}C NMR (75 MHz): δ=8.3 (C1), 9.3 (C4), 14.4 (C2′), 14.5 (C2″), 64.6 (C2), 64.7 (C3), 163.7 (C=O′, C=O″).

4.3.6. 3,3-Dimethyl-1,2-butanediol diacetate 11. Yellow syrup. ^{1}H NMR (300 MHz): δ=0.94 (m, 9H, C4H_3, C5H_3, C6H_3), 2.01 (s, 3H, C2′H_3), 2.07 (s, 3H, C2″H_3), 3.98 (dd, 1H, J=11.7, 8.9 Hz, C1HH), 4.37 (dd, 1H, J=11.7, 2.4 Hz, C1HH), 4.93 (dd, 1H, J=8.9, 2.4 Hz, C2H_1). ^{13}C NMR (75 MHz): δ=21.5 (C2′), 21.6 (C2″), 26.6 (C4, C5, C6), 34.2 (C3), 64.2 (C1), 67.4 (C2), 171.3 (C=O′), 171.6 (C=O″).

4.3.7. Pinacol diacetate 12.[17] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.20 (m, 12H, C1H_3, C4H_3, C5H_3, C6H_3,), 2.05 (m, 6H, C2′, C2″H_3). ^{13}C NMR (75 MHz): δ=22.4 (C1, C4, C5, C6), 25.6 (C2′, C2″), 75.3 (C2, C3), 172.1 (C=O′, C=O″).

4.3.8. D,L-Glyceraldehyde diacetate 13.[18] White solid. Mp 142–144 °C. ^{1}H NMR (300 MHz): δ=2.08 (s, 3H, C2′H_3), 2.14 (s, 3H, C2″H_3), 4.15 (dd, 1H, J=12.4, 3.0 Hz, C3HH), 4.27 (dd, 1H, J=12.5, 4.7 Hz, C3HH), 5.76 (d, 1H, J=7.9 Hz, C2H_1). ^{13}C NMR (75 MHz): δ=21.4 (C2′), 21.6 (C2″), 62.3 (C3), 75.0 (C2), 169.3 (C=O′), 171.2 (C=O″).

4.3.9. (*R*)-(−)-Dihydro-5-(hydroxymethyl)-2(3*H*)-furanone acetate 15.[19] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.10 (s, 3H, C2′H_3), 2.35 (m, 2H, C3H_2), 2.58 (m, 2H, C2H_2), 4.14 (dd, 1H, J=12.2, 5.6 Hz, C5HH), 4.30 (dd, 1H, J=12.2, 3.2 Hz, C5HH), 4.73 (m, 1H, C4H_1). ^{13}C NMR (75 MHz): δ=21.4 (C2′), 24.6 (C3), 28.8 (C2), 66.0 (C5), 77.9 (C4), 171.2 (C=O′), 177.1 (C=O).

4.3.10. (*S*)-(+)-Dihydro-5-(hydroxymethyl)-2(3*H*)-furanone acetate 17.[19] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.09 (s, 3H, C2′H_3), 2.34 (m, 2H, C3H_2), 2.59 (m, 2H, C2H_2), 4.13 (dd, 1H, J=12.2, 5.6 Hz, C5HH), 4.30 (dd, 1H, J=12.2, 3.2 Hz, C5HH), 4.73 (m, 1H, C4H_1). ^{13}C NMR

(75 MHz): δ=21.4 (C2′), 24.6 (C3), 28.8 (C2), 66.0 (C5), 77.9 (C4), 171.2 (C=O′), 177.1 (C=O).

4.3.11. *cis*-1,2-Cyclopentanediol diacetate 18.[20] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.80 (m, 12H, C3H$_2$, C4H$_2$, C5H$_2$, C2′, C2″H$_3$), 5.12 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=19.8 (C4), 21.6 (C2′, C2″), 28.8 (C3, C5), 74.8 (C1, C2), 171.1 (C=O′, C=O″).

4.3.12. *cis*-1,2-Cyclohexanediol diacetate 19.[21] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 5.00 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 22.3 (C4, C5), 28.3 (C3, C6), 71.6 (C1, C2), 171.1 (C=O′, C=O″).

4.3.13. (1*S*,2*S*)-*trans*-1,2-Cyclohexanediol diacetate 20.[22] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 4.78 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 24.1 (C4, C5), 30.8 (C3, C6), 74.4 (C1, C2), 171.1 (C=O′, C=O″).

4.3.14. (1*R*,2*R*)-*trans*-1,2-Cyclohexanediol diacetate 21.[23] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.70 (m, 14H, C3H$_2$, C4H$_2$, C5H$_2$, C6H$_2$, C2′H$_3$, C2″H$_3$,), 4.78 (m, 2H, C1H$_1$, C2H$_1$). ^{13}C NMR (75 MHz): δ=21.8 (C2′, C2″), 24.0 (C4, C5), 30.8 (C3, C6), 74.4 (C1, C2), 171.3 (C=O′, C=O″).

4.3.15. (S)-(−)-1,1-Diphenyl-1,2-propanediol 2-acetate 22.[24] White solid. Mp 137–139 °C. ^{1}H NMR (300 MHz): δ=1.09 (d, 3H, *J*=6.4 Hz, C3H$_3$), 1.87 (s, 3H, C2′H$_3$), 5.91 (q, 1H, *J*=6.4 Hz, C2H$_1$), 7.28 (m, 10H, C$_{Arom}$). ^{13}C NMR (75 MHz): δ=15.1 (C3), 21.8 (C2′), 74.5 (C2), 80.2 (C1), 126.2, 126.5, 127.7, 128.9, 129.0, 143.8, 146.1 (C$_{Arom}$), 170.8 (C=O′).

4.3.16. D-Mannose pentaacetate 24.[25] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.99 (s, 3H, C2′H$_3$), 2.03 (s, 3H, C2″H$_3$), 2.07 (s, 3H, C2‴H$_3$), 2.15 (s, 3H, C2⁗H$_3$), 2.16 (s, 3H, C2⁗′H$_3$), 4.09 (m, 2H, C5H$_1$, C6*H*H), 4.26 (dd, 1H, *J*=12.2, 4.7 Hz, C6*H*H), 5.28 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.06 (m, 1H, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 62.7 (C6), 66.2 (C4), 69.0 (C2), 69.4 (C3), 71.2 (C5), 91.2 (C1), 168.7 (C=O′), 170.1 (C=O″), 170.3 (C=O‴), 170.6 (C=O⁗), 171.2 (C=O⁗′).

4.3.17. D-Xylose tetraacetate 25.[26] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.02 (s, 3H, C2′H$_3$), 2.04 (s, 3H, C2″H$_3$), 2.05 (s, 3H, C2‴H$_3$), 2.17 (s, 3H, C2⁗H$_3$), 3.71 (dd, 1H, *J*=11.0 Hz, C5*H*H), 3.93 (dd, 1H, *J*=11.0, 5.9 Hz, C5*H*H), 5.02 (m, 2H, C2H$_1$, C4H$_1$), 5.46 (dd, 1H, *J*=9.5 Hz, C3H$_1$), 5.47 (m, 1H, C3H$_1$), 6.25 (d, 1H, *J*=3.7 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′), 21.3 (C2″), 21.4 (C2‴), 21.5 (C2⁗), 61.3 (C5), 69.3 (C2, C4), 70.0 (C3), 89.9 (C1), 169.7 (C=O′), 170.4 (C=O″), 170.5 (C=O‴), 170.8 (C=O⁗).

4.3.18. D-Glucose pentaacetate 28.[27] White solid. Mp 100–102 °C. ^{1}H NMR (300 MHz): δ=2.01 (s, 3H, C2′H$_3$), 2.04 (s, 3H, C2″H$_3$), 2.05 (s, 3H, C2‴H$_3$), 2.06 (s, 3H, C2⁗H$_3$), 2.09 (s, 3H, C2⁗′H$_3$), 4.09 (m, 2H, C5H$_1$, C6*H*H), 4.26 (dd, 1H, *J*=12.5, 4.1 Hz, C6*H*H), 5.11 (m, 2H, C2H$_1$, C4H$_1$), 5.41 (m, 1H, C3H$_1$), 6.32 (d, 1H, *J*=3.7 Hz, C1H$_1$).

4.3.19. D-Fructose pentaacetate 29.[28] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.04 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.08 (s, 3H, C2‴H$_3$), 2.09 (s, 3H, C2⁗H$_3$), 2.10 (s, 3H, C2⁗′H$_3$), 4.16 (m, 2H, C5H$_2$,), 4.75 (m, 2H, C6H$_2$), 5.38 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 62.3 (C5), 63.6 (C2), 64.1 (C3), 67.2 (C4), 68.8 (C6), 103.2 (C1), 170.2 (C=O′, C=O″, C=O‴, C=O⁗, C=O⁗′).

4.3.20. D-Arabinose tetraacetate 30.[26] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.02 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.12 (s, 3H, C2‴H$_3$), 2.15 (s, 3H, C2⁗H$_3$), 4.10 (m, 2H, C5H$_2$), 5.34 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.34 (d, 1H, *J*=2.9 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′), 21.4 (C2″), 21.6 (C2‴), 21.7 (C2⁗), 63.4 (C5), 67.3 (C2), 67.7 (C3), 69.1 (C4), 90.9 (C1), 169.8 (C=O′), 170.6 (C=O″), 170.8 (C=O‴), 171.0 (C=O⁗′).

4.3.21. D-Sorbitol hexaacetate 32.[29] White solid. Mp 87–89 °C. ^{1}H NMR (300 MHz): δ=2.05 (s, 3H, C2′H$_3$), 2.06 (s, 3H, C2″H$_3$), 2.07 (s, 3H, C2‴H$_3$), 2.08 (s, 3H, C2⁗H$_3$), 2.09 (s, 3H, C2⁗′H$_3$), 2.13 (s, 3H, C2⁗″H$_3$), 4.01 (dd, 1H, *J*=12.1, 6.2 Hz, C1*H*H), 4.12 (dd, 1H, *J*=12.1, 5.2 Hz, C6*H*H), 4.24 (dd, 1H, *J*=12.1, 3.7 Hz, C1*H*H), 4.36 (dd, 1H, *J*=12.1, 4.3 Hz, C6*H*H), 5.05 (m, 1H, C2H$_1$), 5.24 (m, 1H, C5H$_1$), 5.42 (m, 2H, C3H$_1$, C4H$_1$). ^{13}C NMR (75 MHz): δ=21.2 (C2′, C2″, C2‴, C2⁗, C2⁗′, C2⁗″), 62.1 (C1), 62.5 (C6), 69.0 (C2), 69.2 (C5), 69.3 (C3), 70.0 (C4), 170.4 (C=O′), 170.5 (C=O″), 170.6 (C=O‴), 170.7 (C=O⁗), 171.1 (C=O⁗′), 171.2 (C=O⁗″).

4.3.22. D-Galactose pentaacetate 33.[30] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.98 (s, 3H, C2′H$_3$), 1.99 (s, 3H, C2″H$_3$), 2.02 (s, 3H, C2‴H$_3$), 2.03 (s, 3H, C2⁗H$_3$), 2.14 (s, 3H, C2⁗′H$_3$), 4.07 (m, 2H, C5H$_1$, C6*H*H), 4.32 (dd, 1H, *J*=12.1, 5.6 Hz, C6*H*H), 5.31 (m, 2H, C2H$_1$, C4H$_1$), 5.48 (m, 1H, C3H$_1$), 6.35 (m, 1H, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗, C2⁗′), 61.9 (C6), 67.1 (C4), 68.0 (C2), 68.1 (C3), 69.4 (C5), 90.3 (C1), 169.5 (C=O′), 170.0 (C=O″), 170.5 (C=O‴), 170.8 (C=O⁗), 171.0 (C=O⁗′).

4.3.23. D-Fucose tetraacetate 34.[31] Yellow syrup. ^{1}H NMR (300 MHz): δ=1.13 (d, 3H, *J*=6.4 Hz, C6H$_3$), 1.98 (s, 3H, C2′H$_3$), 1.99 (s, 3H, C2″H$_3$), 2.16 (s, 3H, C2‴H$_3$), 2.17 (s, 3H, C2⁗H$_3$), 4.25 (m, 1H, C3H$_1$), 5.31 (m, 3H, C2H$_1$, C4H$_1$, C5H$_1$), 6.31 (d, 1H, *J*=2.8 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C6), 21.5 (C2′, C2″, C2‴, C2⁗), 63.4 (C5), 66.8 (C2), 66.9 (C3), 68.0 (C4), 91.6 (C1), 169.4 (C=O′), 170.1 (C=O″), 170.4 (C=O‴), 170.6 (C=O⁗).

4.3.24. L-Arabinose tetraacetate 35.[32] Yellow syrup. ^{1}H NMR (300 MHz): δ=2.01 (s, 3H, C2′H$_3$), 2.02 (s, 3H, C2″H$_3$), 2.12 (s, 3H, C2‴H$_3$), 2.14 (s, 3H, C2⁗H$_3$), 4.05 (m, 2H, C5H$_2$), 5.30 (m, 3H, C2H$_1$, C3H$_1$, C4H$_1$), 6.32 (d, 1H, *J*=2.6 Hz, C1H$_1$). ^{13}C NMR (75 MHz): δ=21.3 (C2′, C2″, C2‴, C2⁗), 63.4 (C5), 67.3 (C2), 67.7 (C3), 69.1 (C4), 90.8 (C1), 169.8 (C=O′), 170.5 (C=O″), 170.8 (C=O‴), 170.9 (C=O⁗).

4.4. Enzyme measurements

Substrates were diluted from 10 mM stock solutions in

water/acetonitrile mixtures. Enzymes were diluted from 10 mg mL^{-1} stock solutions in aqueous borate buffer (50 mM, pH 8.0). $NaIO_4$ was added as a freshly prepared 10 mM stock solution in water. Adrenaline (as. HCl salt) was added as a 15 mM stock solution in water. Assays (0.1 mL) were conducted in individual wells of 96-well flat-bottom half-area polystyrene microtiter-plates (Costar) as described in the figure legends. The OD was recorded using a Spectramax 190 Microplate Spectrophotometer (Molecular Devices, λ=490 nm).

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The use of a thermostable signature amidase in the resolution of the bicyclic synthon (*rac*)-γ-lactam

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Abstract—The resolution of the bicyclic synthon (*rac*)-γ-lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) is an important step in the synthesis of a group of chemotherapuetic agents known as carbocyclic nucleosides. The archaeon *Sulfolobus solfataricus* MT4 produces a thermostable γ-lactamase that has a high sequence homology to the signature amidase family of enzymes. It shows similar inhibition patterns of amidases towards benzonitrile, phenylmethylsulfonyl fluoride and heavy metals such as Hg^{2+}, and is activated by thiol reagents. The enzyme selectively cleaves the (+)-enantiomer from a racemic mix of γ-lactam. It also exhibits general amidase activity by cleaving linear and branched aliphatic and aromatic amides. The enzyme catalyses the synthesis of benzoic hydrazide from benzamide preferentially to benzamide cleavage in the presence of excess hydrazine. This enzyme has potential for use in industrial biotransformations in the production of both carbocyclic nucleosides and hydrazides.

1. Introduction

The search for and development of new therapeutic agents drives the need for the synthesis of new enantiomerically pure compounds. Partially purified enzymes or whole cells have been used industrially in the production of fine chemicals and pharmaceuticals. Enzymes are especially useful in the production of fine chemicals and pharmaceuticals due to their chemo-, regio- and more particularly their enantioselectivity.[1]

Carbocyclic nucleosides, where the ribose oxygen of the nucleoside is replaced by methylene, are valuable as chemotherapeutic agents such as cardiac vasodilators and in the treatment of viral infection.[2] γ-Lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) is a bicyclic lactam that is well established as an important synthon in the synthesis of carbocyclic nucleosides such as the anti-HIV agent (−)-carbovir.[2,3] The resolution of (*rac*)-γ-lactam by enantioselective cleavage using enzymes is an economical means of obtaining single enantiomers of the bicyclic lactam.

The use of whole cell preparations and impure enzymes in the resolution of (*rac*)-γ-lactam has been reported previously.[2–4] γ-Lactamases from *Rhodococcus* sp (ENZA1) and an *Aureobacterium* sp (ENZA25) cleave the [1(*R*),4(*S*)]-(−)-enantiomer, while enzymes from *Pseudomonas solanacearum* (ENZA20) *P. cepaecia* and *P. fluorescens* (ENZA22) cleave the [1(*S*),4(*R*)]-(+)-enantiomers of γ-lactam.[2,3] A (+)-γ-lactamase enzyme was isolated from *Comomonas acidovorans* and cloned and over-expressed in *Escherichia coli*.[3] The sequence of this protein showed 63% identity to a formamidase from *Methylophilus methylotropus*[5] and 56% identity to an acetamidase from *Mycobacterium smegmatis*[6] a family of enzymes for which there is currently no structural information available. This enzyme has been crystallised and preliminary X-ray data has been obtained.[7] A (−)-γ-lactamase was isolated from an *Aureobacterium* sp which has been cloned and over-expressed in *E. coli*.[8] This enzyme has a high sequence identity (68%) to two cofactor free haloperoxidase enzymes from *Streptomyces aureofaciens*.[9,10]

No thermophilic γ-lactamase has been described to date and it was considered that a more stable form of this enzyme would be useful for industrial processes.[11] This paper describes the cloning, purification and use of a γ-lactamase from the thermophilic archaeon *Sulfolobus solfataricus* MT4 and a description of its potential use in biotransformation reactions.

Keywords: β-Lactamase; Signature amidase; *Sulfolobus solfataricus*; Biotransformations.

Abbreviations: γ-Lactam, 2-azabicyclo[2.2.1]hept-5-en-3-one; DAN, diazoacetyl-norleucine methyl ester; PMSF, phenylmethylsulfonyl fluoride.

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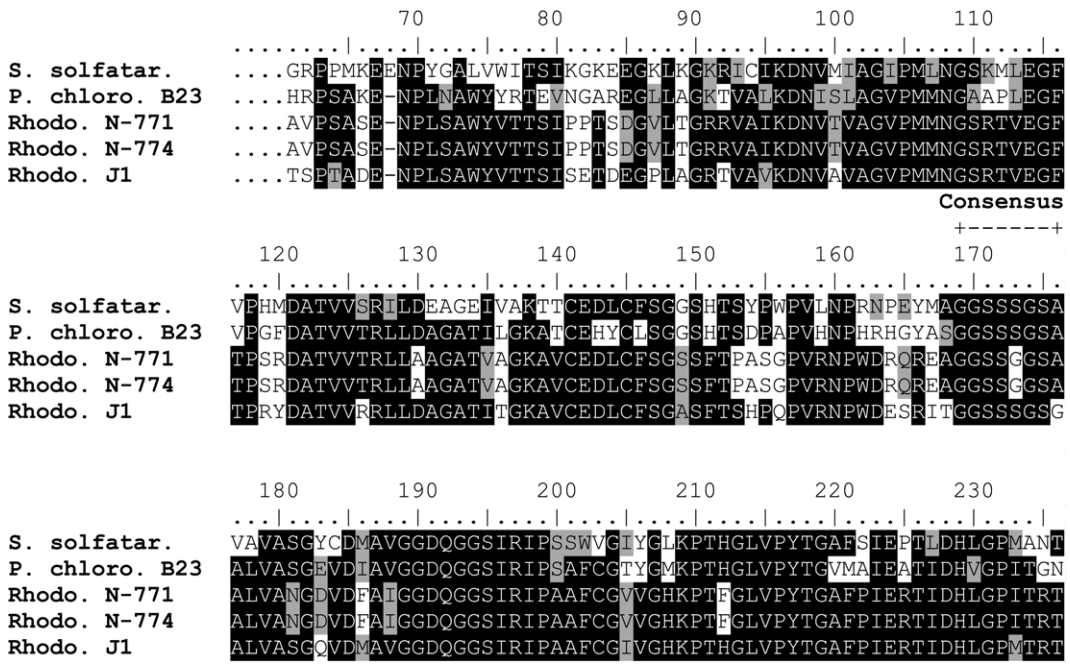


Figure 1. Partial alignment of the more conserved regions of the γ-lactamase/amidase from *Sulfolobus solfataricus* MT4 (*S. solfat.* MT4) with 4 other amidases. Residues boxed in black are entirely conserved. The alignment was created using ClustalW.[27] Consensus=the conserved GGSS(S/G)SA sequence of signature amidases. *P. chloro.* B23=*Pseudomonas chlororaphis* B23;[13] *Rhodo.* N-771=*Rhodococcus* sp. N-771 (Accession number BAA36596); *Rhodo.* N-774=*R. erythropolis* N-774;[28] *Rhodo.* J1=*R. rhodochrous* J1.[15]

2. Results

2.1. γ-Lactamase sequence

The thermostable amidase from *S. solfataricus* has been described previously.[12] Figure 1 shows an alignment of the amino acid sequences of the γ-lactamase from *S. solfataricus* MT4 with four other amidases from *Pseudomonas chlororaphis* B23,[13] *Rhodococcus* sp. N-771 (Accession number BAA36596), *R. erythropolis* N-774[28] and *R. rhodochrous* J1.[15] The γ-lactamase shows a 41–44% sequence identity to these amidases.

2.2. General properties

The γ-lactamase was purified to homogeneity as detected by SDS-PAGE (results not shown). The molecular mass of the monomer was estimated to be 55 kDa by SDS-PAGE which is consistent with the calculated molecular mass of 55.7 kDa. Figure 2 shows the temperature profile of the γ-lactamase. The highest activity was achieved at pH 7.0 at 85 °C with the substrate γ-lactam. This high optimal temperature is not surprising as *Sulfolobus* is a thermophilic archaeon. This enzyme is known to have a half life of 25 h at 80 °C.[12]

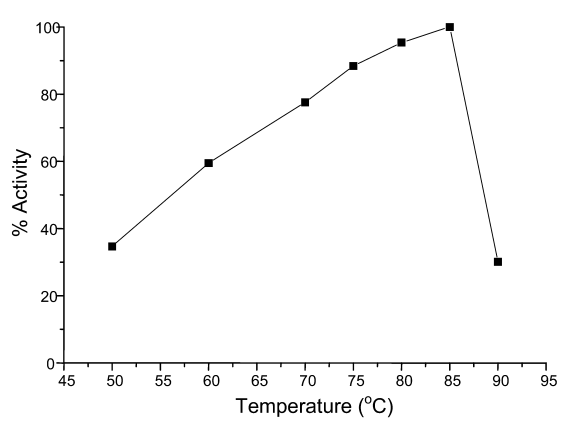


Figure 2. Temperature profile of γ-lactamase with 20 mM (*rac*)-lactamide in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0.

2.3. Inhibitors

Table 1 shows the effect of inhibitors on γ-lactamase. The enzyme was significantly inhibited by benzonitrile which has been suggested to be an amidase dead-end inhibitor.[14] The serine protease inhibitors PMSF and benzamidine

Table 1. Effect of inhibitors on the activity of γ-lactamase

Inhibitor	Class of inhibition	Activity remaining (%)
10 mM Benzonitrile	Amidase dead-end inhibitor	48.1
1 mM PMSF	Serine protease	38.4
1 mM Benzamidine	Serine protease	44.5
1 mM Dithiothreitol	Cysteine protease	145.5
1 mM Iodoacetic acid	Cysteine protease	120.9
10 μM Hg^{2+}	Binds to thiols	53.6
10 μM Pb^{2+}	Binds to thiols	79.2
10 μM Cu^{2+}	Binds to thiols	28.1
10 μM Fe^{3+}	Binds to thiols	111.3
10 mM EDTA	Metallo-enzyme inhibitor	105.8
1 mM *o*-Phenanthroline	Metallo-enzyme inhibitor	84.0
100 μM Pepstatin	Aspartic protease	99.5
10 mM DAN[a]	Aspartic protease	52.2

Activity was determined by the benzamide HPLC method as described.
[a] DAN=diazoacetylnorleucine methyl ester.

partially inhibited the enzyme, while the sulfhydryl reagents dithiothreitol (DTT) and iodoacetic acid (IAA) significantly activated the enzyme. Heavy metals such as Hg^{2+}, Pb^{2+} and Cu^{2+} were inhibitory.

The metal chelators *o*-phenanthroline and EDTA had slight to no inhibitory effect, respectively. There was no inhibition by the aspartic protease inhibitor pepstatin, but significant inhibition by the aspartate-modifying reagent diazoacetyl-norleucine methyl ester (DAN).

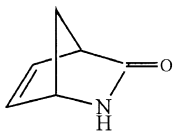
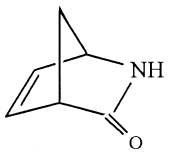
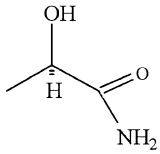
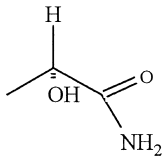
2.4. Substrate specificity

Table 2 shows the specific activity of the γ-lactamase towards a variety of amide substrates. The highest activity and enantioselectivity were detected with the (+)-enantiomer of γ-lactam. It had a slightly lower activity towards the substrate (*R*)-(+)-lactamide and a more relaxed enantioselectivity. The enzyme also exhibits activity towards a range of aliphatic and aromatic amides cleaved by other signature amidases[15,16] though with a much lower specificity. The enzyme had a low specificity towards acrylamide, urea and *N*-substituted amides.

The γ-lactamase achieved 50% conversion of industrial grade (*rac*)-γ-lactam at 10 g l^{-1} at 85 °C, which corresponds to about 100% conversion of (+)-γ-lactam. No reaction was detectable with (−)-γ-lactam.

The γ-lactamase was able to synthesize benzoic hydrazide

Table 2. Substrate specificity of γ-lactamase

Substrate	Structure	Specific activity (μmol/min/mg)
(+)-γ-Lactam		21.5
(−)-γ-Lactam		0.0
(*R*)-(+)-Lactamide		18.9
(*S*)-(−)-Lactamide		2.4
Formamide	$HCONH_2$	0.0
Acetamide	CH_3-CONH_2	0.2
Propionamide	$CH_3-CH_2-CONH_2$	0.8
Isobutyramide	$(CH_3)_2CH-CONH_2$	4.6
Trimethylacetamide	$(CH_3)_3C-CONH_2$	1.7
Butyramide	$CH_3-CH_2-CH_2-CONH_2$	1.4
Valeramide	$CH_3-CH_2-CH_2-CH_2-CONH_2$	3.1
Benzamide	$(C_6H_5)-CONH_2$	7.4
N,*N*-Dimethyl formamide	$HCON(CH_3)_2$	0.0
N,*N*-Dimethyl acetamide	$CH_3-CON(CH_3)_2$	0.4
Acrylamide	$CH_2=CH-CONH_2$	0.3
Urea	$(NH_2)_2CO$	0.1
Thiourea	$(NH_2)_2CS$	0.0

Activity towards γ-lactam and benzamide was determined by the HPLC methods described. Activity with all other substrates was determined by the ammonia detection method as described.

from benzamide and excess hydrazine (specific activity=8.6 μmol/min/mg) with negligible benzamide cleavage. In comparison, the enzyme cleaved benzamide to benzoic acid in the absence of hydrazine with a slightly lower specific activity of 7.4 μmol/min/mg. γ-Lactamase did not possess nitrile hydratase or nitrilase activity towards benzonitrile (results not shown). This is not surprising as benzonitrile is an inhibitor of the enzyme. It also had no detectable aminoacylase, protease or esterase activity (results not shown).

3. Conclusion

We have described the cloning, sequencing and characterization of a thermostable γ-lactamase/amidase from *S. solfataricus* MT4. It belongs to the signature amidase family which all contain the sequence GGSS(S/G)GS located in residue positions 173–179 (Fig. 1 numbering). The amino acid sequence of γ-lactamase contains the highly conserved putative catalytic residues Asp191 and Ser195, but not the highly conserved Cys207 residue (Asp194, Ser199 and Cys207 in Figure 1). Kobayashi et al., showed that the residues Asp191 and Ser195, and to a lesser extent Cys203, in signature amidases are important for catalytic activity.[15,17] The inhibition pattern of *S. solfataricus* amidase shows partial inhibition by PMSF and benzamidine, similar to the amidase from *R. rhodochrous* J1. There was also a significant inhibition by the aspartate-modifying reagent DAN. However, this is not a key indicator of the presence of a catalytic aspartate residue as the amidase from *R. rhodochrous* J1 was not inhibited by DAN.[15]

This class of amidases has a mixed response to the presence of thiol reagents. *S. solfataricus* and *R. rhodochrous* M8[18] amidases are activated by DTT while the amidase from *R. rhodochrous* J1 which contains the highly conserved Cys203 (valine in γ-lactamase) was inhibited by thiol reagents.[15] The lack of significant inhibition to metal chelators suggests that metal ions are not required for catalysis.

The highest activity and enantioselectivity were detected with γ-lactam as it specifically cleaves only the (+)-enantiomer. This makes it an industrially useful enzyme as it can be used at one stage in the synthesis of carbocyclic nucleosides. This is an example of the usefulness of enzymes as biocatalysts as their specificity can increase the yield of enantiomerically pure biochemicals and pharmaceuticals.

The more relaxed enantioselectivity towards lactamide could be due to the more flexible structure of lactamide compared to the bicyclic lactam allowing the unfavourable enantiomer to bind significantly to the enzyme. Activity towards aliphatic amides, such as acetamide and valeramide, showed that as the length of the hydrocarbon chain increased, the specific activity increased. The enzyme was over 3-fold more active towards isobutyramide than butyramide suggesting that branched-chained substrates are more favourable. The specific activity increased further with the aromatic substrate benzamide.

Compounds such as isoniazid (isonicotinic acid hydrazide) are widely used as anti-tuberculosis drugs.[17] The amidase from *R. rhodochrous* J1 was found to be capable of synthesizing hydrazides. Similarly, γ-lactamase was able to synthesize benzoic hydrazide from benzamide and excess hydrazine with negligible benzamide cleavage. The competing amidase reaction, cleavage of benzamide, occurred significantly only in absence of hydrazine. Thus, there is potential to develop processes using amidases in the synthesis of hydrazides in industry.

Protein crystals grew in droplets using 16–20% polyethylene glycol 4000 as the precipitant in 100 mM sodium acetate buffer pH 4.5–5.0. The crystals were small needles with dimensions 0.05×0.05×0.5 mm^3. These are not yet of sufficient quality for X-ray structural studies, however, they grow readily. These crystals are ideal for use after being chemically cross-linked with glutaraldehyde to further stabilize the enzyme and enable its easy recovery and potential reuse for commercial biotransformation applications.

The thermophilic archaeal γ-lactamase described in this paper belongs to the signature amidase class of enzyme which is different to the γ-lactamase enzymes previously described.[2,3,7,8] Due to its thermostability and substrate specificity the enzyme has use for commercial biotransformation applications.

4. Experimental

4.1. Cloning of the γ-lactamase

A search of the complete genomic sequence of the hyperthermophile *Sulfolobus solfataricus* strain P2[19] revealed the presence of a putative amidase (Swiss-Prot accession number P95896). Total genomic DNA was prepared from *Sulfolobus solfataricus* strains MT4 and P1 (obtained from CAMR, Salisbury) by the method described by Taylor et al.[3] The open reading frames encoding an amidase from each strain were amplified by the polymerase chain reaction (PCR). Restriction endonuclease cleavage sites for *Sph*I and *Bam*HI were incorporated within the 5′ and 3′ oligonucleotide primers, respectively. Seven open reading frames were detected and sequenced by Cambridge Bioscience (Cambridge, UK) and found to be identical. The PCR fragments were digested with *Sph*I and *Bam*HI (New England Biolabs, Essex), ligated into pUCCER11 expression vector[3] and transformed into *Escherichia coli* strain DH5 (Dibco BRL, Scotland).

4.2. Production and purification of recombinant γ-lactamase

The *E. coli* strain DH5 harboring the γ-lactamase/amidase was grown in a 2 l ST Applikon bioreactor, as described by Toogood et al.[20] and a cell-free enzyme extract from 10 g of cell paste was prepared by sonication. The enzyme was precipitated with 40% (saturated) ammonium sulfate and recovered by centrifugation at 11,000*g* for 30 min at 4 °C. The precipitate was resuspended and dialysed against 10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0, then loaded onto a

60 ml phenyl Sepharose Fast Flow column (Amersham Pharmacia, Buckinghamshire) pre-equilibrated in 20 mM Tris–HCl pH 8.0 containing 1 M ammonium sulfate. Protein was eluted with a gradient of equilibration buffer to 20 mM Tris–HCl pH 8.0, followed by a gradient of the latter buffer to 20 mM Tris–HCl pH 8.0 containing 50% ethylene glycol. The active fractions were concentrated and equilibrated in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 by ultrafiltration using a Centriprep-10 (Amicon, Gloucestershire) concentrator. The enzyme was loaded onto a HiLoad Superdex 200 gel filtration column (Amersham Pharmacia, Buckinghamshire) and eluted with 50 mM Tris–HCl pH 8.0 containing 100 mM NaCl.

4.3. Protein determination

The protein concentration was determined by a modification of the Bradford assay method[21] kit supplied by BioRad (Hertfordshire, UK). BSA was used as a standard in the range of 0–100 μM.

4.4. Determination of molecular mass

Enzyme extract samples were run on SDS-PAGE[22] to determine their degree of purity and molecular mass. Protein bands were stained using a coomassie staining method.[23] Molecular weight standards in the range of 14.4–97 kDa were obtained from Amersham Pharmacia (Buckinghamshire). Native molecular mass of the purified enzyme was determined by gel filtration using a Superdex 200 Hiload 16/60 column (Pharmacia). The column was equilibrated with 10 mM Tris–HCl pH 8.0 buffer at 0.3 ml/min and calibrated with the standards ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

4.5. Enzyme assays

All enzyme assays were carried out with 0.05–0.5 μM enzyme. Activity towards γ-lactam was determined by incubating the enzyme in 1 ml of 10 g l^{-1} (91 mM) (*rac*)-γ-lactam in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. The reaction was terminated by adding 100 μl of the reaction to 900 μl of 50:50 methanol:10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0. Analysis was performed by HPLC as described by Gonsalvez et al.[7]

General amidase activity was determined by incubating the enzyme in 1 ml of 20 mM amide substrates in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 50 °C. The amount of ammonia produced in the reaction was determined by the phenol/hypochlorite method of Silman et al.[24] Activity towards benzamide was determined with 10–20 mM substrate in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. The reaction was terminated by adding 100 μl of the reaction to 900 μl of 50:50 methanol:10 mM NaH_2PO_4/Na_2HPO_4 pH 7.0. Analysis was performed by HPLC as described by Kobayashi et al.[15] Nitrilase and nitrile hydratase activity with benzonitrile was determined according to the method of Kobayashi et al.[25] Synthesis of benzoic acid hydrazide from benzamide and hydrazine was carried out according to the method of Kobayashi et al.[17]

Proteolytic activity was determined by incubating the enzyme in 1 ml of 0.2% azocasein in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C. Detection of the cleavage products was performed by the trichloroacetic acid (TCA) precipitation method of Peek et al.[26] Esterase activity with *p*-nitrophenyl acetate was determined by incubating the enzyme in 1 ml of 0.4 mM substrate in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 containing 0.8% (w/v) Triton X-100 and 10% (v/v) acetonitrile with continuous monitoring of the absorbance at 400 nm for 1–3 min at 30–50 °C. Aminoacylase activity was determined in 1 ml of 10 mM *N*-acetyl-DL-tryptophan in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h at 85 °C according to the method of Toogood et al.[20]

4.6. Optimization of reaction conditions

The optimal temperature was determined by reacting the γ-lactamase in 1 ml of 20 mM (*rac*)-lactamide in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 for 1 h in the temperature range of 50–90 °C using the ammonia detection method of Silman et al.[24] To determine the optimal pH, the γ-lactamase was reacted as above for 10 min at 85 °C in acetate (pH 3.0–4.5), phosphate (pH 4.5–8.5) and borate (pH 8.5–10.0) buffers.

4.7. Inhibitor studies

To determine the effect of inhibitors on activity the enzyme was incubated with the inhibitors for 2 h at 25 °C, then assayed for remaining activity using the above benzamide substrate. The concentration of the inhibitors in the assay was the same as in the preincubation with the enzyme.

4.8. Crystallisation

Crystallisation experiments were carried out using either the sitting or hanging drop vapour diffusion method. The purified protein was concentrated to 10 mg/ml using a Vivaspin 2 (Sartorius AG, Germany) and diluted with an equal volume of well precipitant (16–20% polyethylene glycol 4000 in sodium acetate buffer pH 4.5–5.0). The crystallization dish was incubated at 17 °C.

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Use of hydrolases for the synthesis of cyclic amino acids

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Abstract—The synthesis of several cyclic amino acids that have all the necessary structural features to make them ideal scaffolds for use in medicinal chemistry is described. A key step in each synthesis is the use of hydrolase enzymes to define a chiral centre. In order to elaborate these building blocks into more complex molecules, crystallization and asymmetric hydrogenation were used to define further stereocentres.

1. Introduction

One can analyse the mainstream chemical literature to determine the habits of practicing chemists in their application of enzymes for (predominantly) chiral synthetic methodology. In doing this, one is immediately drawn to the conclusion that hydrolases dominate this field; they did in 1990 and continue to do so today. Scanning a few sample years of *Tetrahedron: Asymmetry* for use of different enzymes illustrates this point clearly, as shown in Figure 1 below. Since the mid-1990's hydrolases have been used at over twice the rate of non-hydrolases.

In particular, it is the lipase and esterase family of enzymes that dominate the hydrolases, and a more detailed snapshot of enzyme usage in 2002 is given in Figure 2.

The reasons for the popularity of these enzymes are well documented, as for example in 'Hydrolases in Organic Synthesis' by Uwe Bornscheuer and Romas Kazlauskas.[1] Some of these reasons include their relative commercial availability, the reasonable number that are available for screening, a lack of need for expensive or demanding cofactors, versatility for synthetic or hydrolytic usage, and the ability to perform reactions in aqueous or solvent based media. From an industrial perspective they often admirably meet the essential criteria for 'industrial readiness', such as availability, performance (turnover, selectivity, volume efficiency) and freedom from intellectual property issues. However, the main area of use of hydrolase enzymes has been in kinetic resolution chemistry, and a drawback of this approach is the maximum 50% theoretical yield obtained of the desired enantiomer. In recent years, approaches have

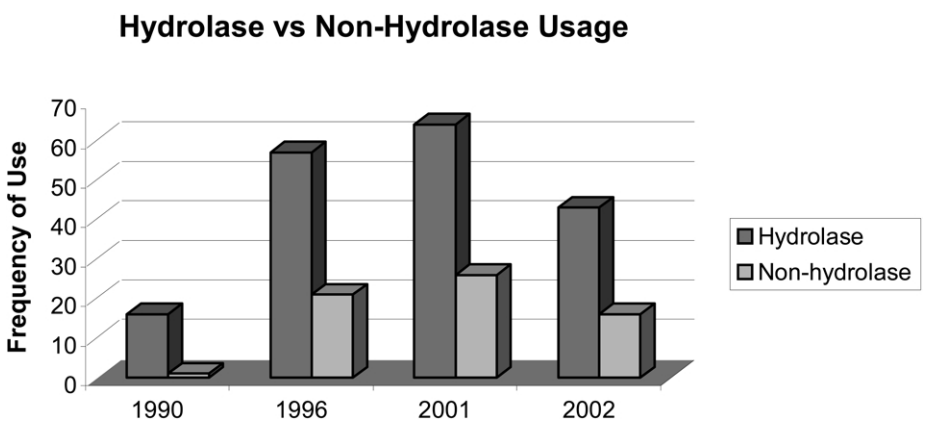

Figure 1. Reported use of hydrolase enzymes versus non-hydrolase enzymes. (Source: papers published in *Tetrahedron: Asymmetry* in years specified).

Keywords: Biocatalysis; Amino acids; Stereochemistry.
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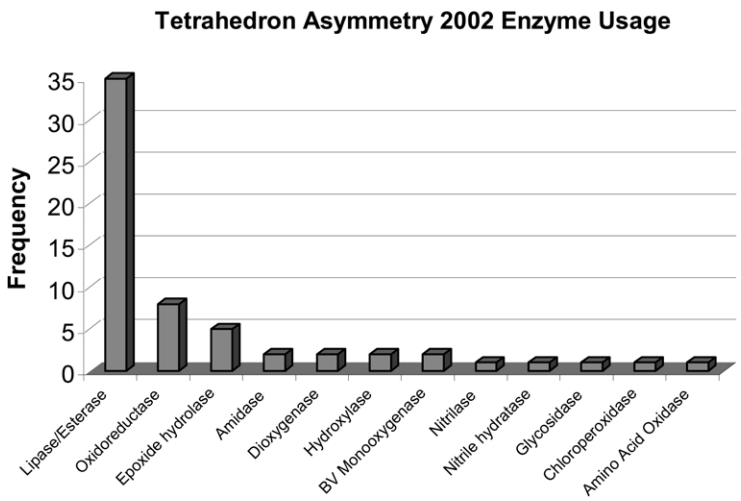


Figure 2. Breakdown of biocatalysis papers published in *Tetrahedron: Asymmetry* by enzyme class in 2002.

been made to overcome this, and hydrolases have now been employed in dynamic kinetic resolutions[2] in conjunction with racemase enzymes,[3] organometallic hydrogen transfer catalysts[4] or a more judicious selection of substrate.[5]

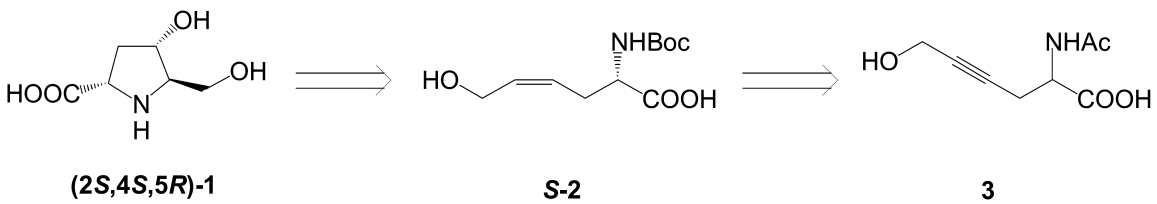
It is against this background that we have continued to develop and exploit hydrolases generally for chiral synthetic routes to more complex, conformationally rigid molecules, where these molecules are expected to usefully contribute in medicinal chemistry. Although Lipinski's rules[6] do not mention rigidity as a desirable function in drug-like molecules, recent studies have indicated that the number of rotatable bonds is a crucial parameter that should be considered.[7] However, putting extra chiral complexity into molecules means that one must call upon additional skills and approaches, such as discovery and development of new hydrolase enzymes, and the practice of complementing biocatalysis with other chiral technologies such as crystallisation and chemocatalysis. In this paper we describe some of our work that has allowed us to elevate the use of hydrolases for more sophisticated molecules.

2. Resolution of α-amino acids using acylases and subsequent elaboration to more complex molecules

2.1. Bulgecinine

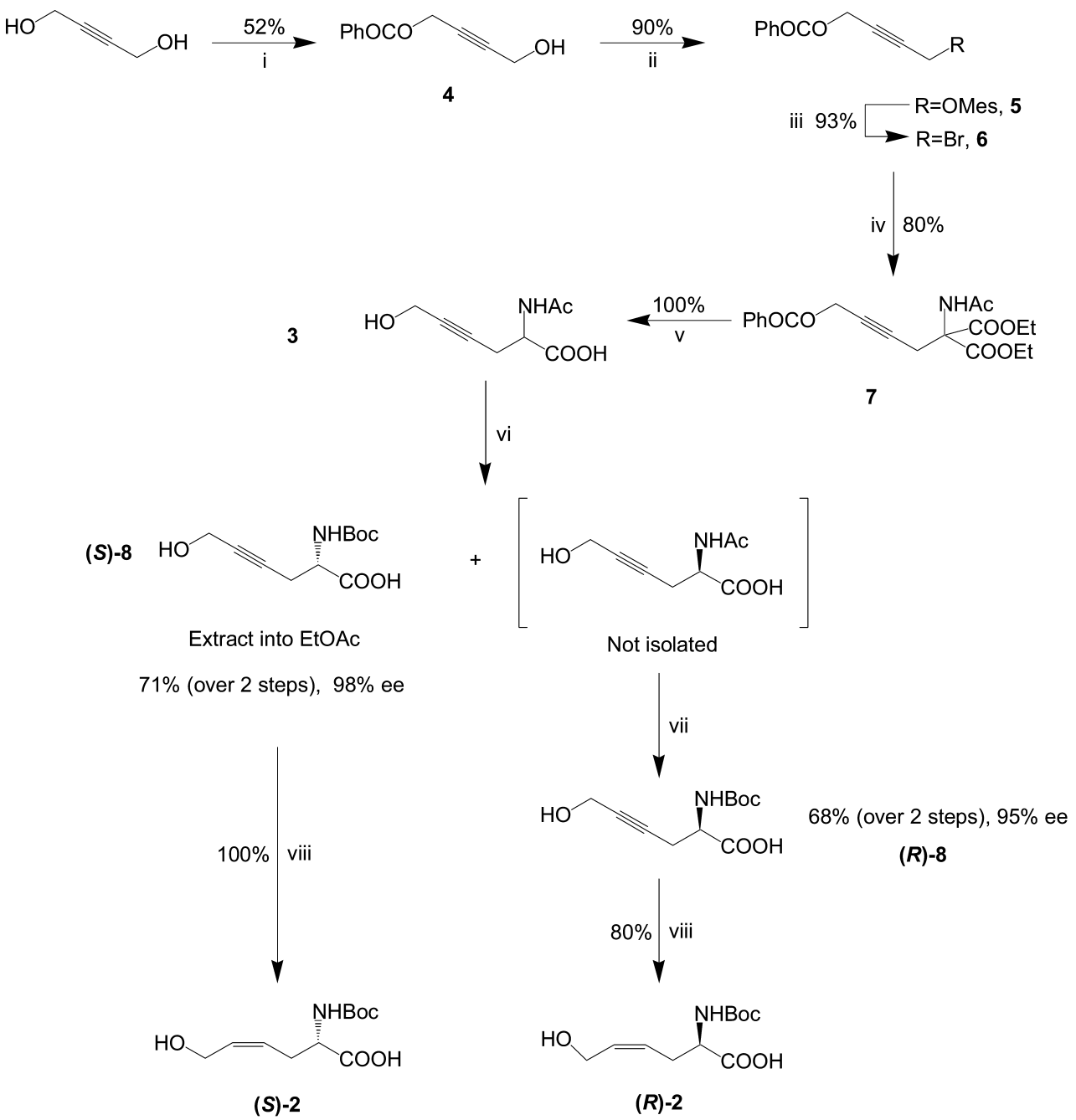
Acylases are commonly used for the synthesis of enantiopure amino acids.[8] We have successfully used proprietary *Thermococcus litoralis* L-aminoacylase[9] (extremophile) and *Alcaligenes* sp. D-aminoacylase[10] enzymes for the synthesis of a wide range of unnatural α-amino acids. When approaching the synthesis of cyclic amino acids, however, this approach has not been so successful. (−)-Bulgecinine, (2*S*,4*S*,5*R*)-**1**, is a cyclic amino acid with three stereocentres isolated in various glycosylated forms from *Pseudomonas acidophila* and *Pseudomonas mesoacidophila*.[11] Its rigid structure and multiple functionality make it an ideal candidate for use as a scaffold around which compound libraries may be designed: when used in conjunction with β-lactam antibiotics the natural glycosylated forms (bulgecins) enhance the antibiotic effect. Previous workers identified the *cis*-allylic alcohol *S*-**2** (Scheme 1) as a vital intermediate for the synthesis of **1**.[12] However, the functionality on the side-chain of *S*-**2** makes it a challenging synthetic target that has previously only been accessed using chemistry that we did not think would be scaleable. We targeted the racemic *N*-acetyl amino acid **3** as the key intermediate. It was anticipated that **3** would be a suitable substrate for resolution using acylases, which would provide a mild environment to introduce the chirality whilst protecting the functionality of the molecule. Following resolution, the key intermediate *S*-**2** would then be readily available, and the route could be carried out on 100 g scale.[13]

The *N*-acetyl amino acid **3** was prepared from the readily available 2-butyne-1,4-diol as shown in Scheme 2. Sequential use of L-acylase followed by D-acylase allowed both enantiomers of the *N*-Boc amino acid **8** to be prepared in a one pot procedure. Lindlar hydrogenation[14] of **8** was used to access both enantiomers of **2**. Halolactonisation of *S*-**2** gave the lactone (2*S*,4*S*,5*S*)-**9** with good diastereoselectivity,[12] and the modified conditions of Oppolzer[15] were then used to convert lactone (2*S*,4*S*,5*S*)-**9** to *N*-Boc-(−)-bulgecinine, *N*-Boc-(2*S*,4*S*,5*R*)-**1** (Scheme 3). Elaboration of *R*-**2** in a similar fashion gave the *N*-Boc-(2*R*,4*R*,5*S*) diastereomer. It can be seen that the two additional chiral centres introduced by cyclisation are both induced by the stereochemistry of

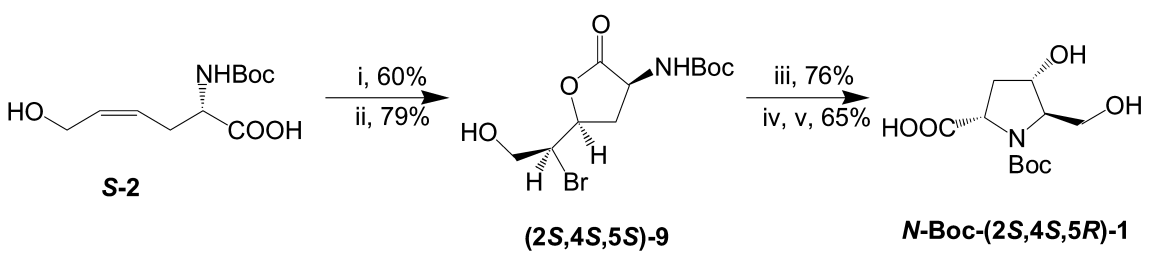


(2*S*,4*S*,5*R*)-1 **S-2** **3**

Scheme 1.



Scheme 2. *Reagents and conditions*: (i) PhCOCl (1 equiv.), Py (1 equiv.), DCM, 10 °C to RT, o/n; (ii) Et_3N (1.1 equiv.), DMAP (0.025 equiv.), MesCl (1.1 equiv.), DCM, 0–5 °C, 2 h; (iii) LiBr (2 equiv.), acetone, 10–15 °C, 1.5 h; (iv) Diethyl acetamidomalonate (1.4 equiv.), KO^{t}Bu (1.4 equiv.), THF, reflux, 22 h; (v) NaOH (3 equiv.), MeOH, reflux, 3.5 h, then acidify to pH 3.5 with conc. HCl, reflux, 22 h; (vi) L-acylase (200 U/g substrate), 30 mM KH_2PO_4, pH 7, 65 °C, 2.5 h, then Boc_2O (0.5 equiv. cf. racemic *N*-Ac acid), THF, 5 M NaOH to maintain pH at 10. RT, 3.5 h; (vii) D-Acylase (40 U/g substrate), aqueous NaOH to adjust to pH 8, RT, 21 h, then Boc_2O (0.5 equiv. cf. racemic *N*-Ac acid), THF, 5 M NaOH to maintain pH at 10, RT, o/n; (viii) 10 wt% Lindlar catalyst (5 wt% Pd on $CaCO_3$ poisoned with Pb), MeOH, 1 bar hydrogen, 20 °C, 1.5 h.



Scheme 3. *Reagents and conditions*: (i) NBS (1.1 equiv.), THF, −10–0 °C, 20 min; (ii) 40% EtOAc/60% heptane slurry, RT, 15 min–0.5 h; (iii) *p*TsOH·H_2O (2 equiv.), EtOAc, RT, 1 h; (iv) H_2O, 1 M LiOH to pH 9, RT, 16 h; (v) Boc_2O (1 equiv.), THF, 1 M LiOH to maintain pH 9, RT, 4.5 h.

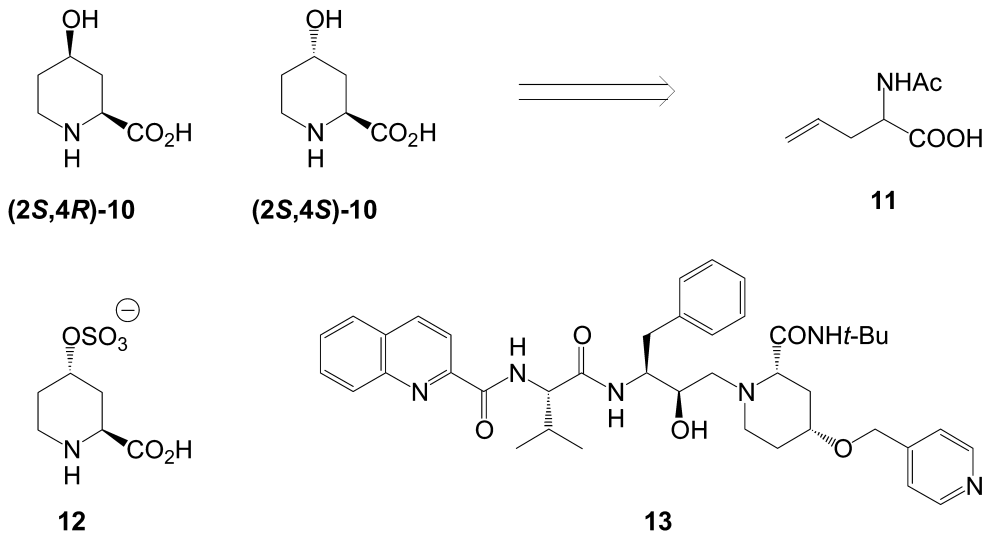


Figure 3. Source and use of 4-hydroxypipecolic acids.

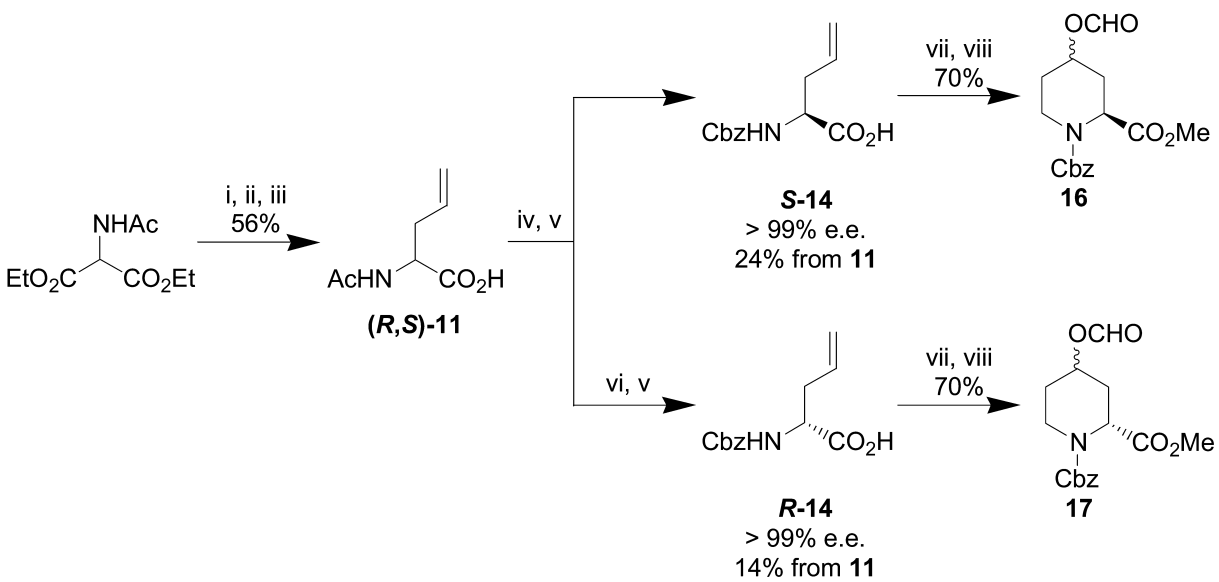
the amino acid **2**, which was, in turn, introduced to the synthesis by the choice of acylase enzyme.

2.2. 4-Hydroxypipecolic acids

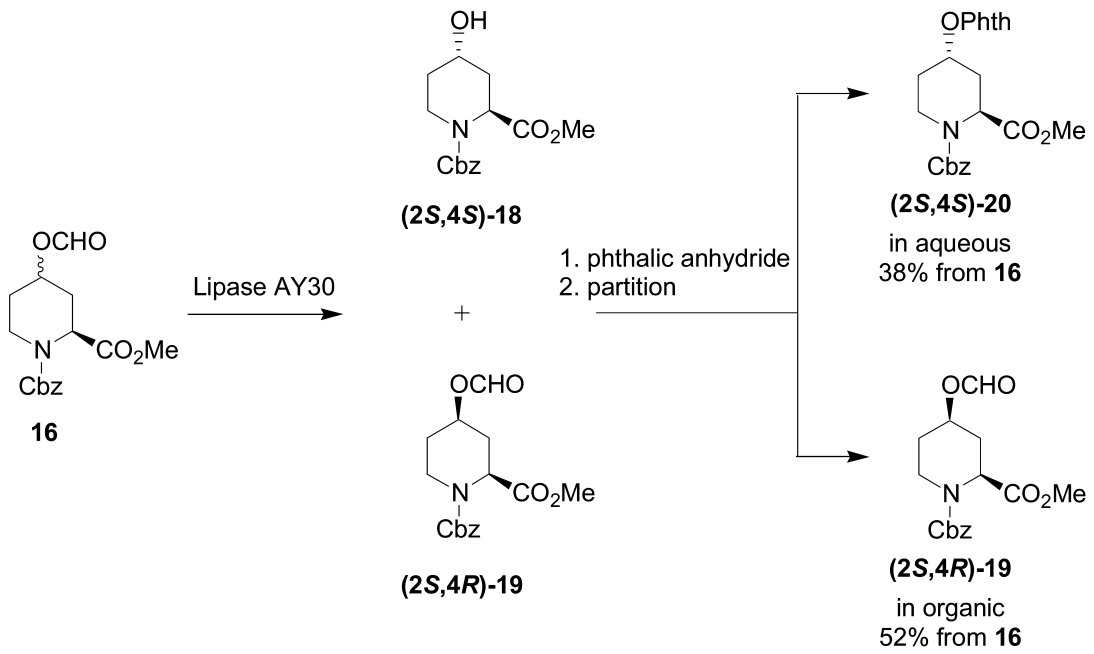
A similar approach was applied to the synthesis of all four diastereoisomers of 4-hydroxypipecolic acid **10**.[16] The (2*S*,4*RS*)-diastereomers are naturally occurring non-proteinogenic amino acids that have been isolated from the leaves of *Calliandra pittieri*, *Strophantus scandeus* and *Acacia oswaldii*.[17] Much like isomers of bulgecinine, these compounds possess all the necessary criteria for use as scaffolds in medicinal chemistry programmes. Indeed, molecules derived from both (2*S*,4*S*)-**10** and (2*S*,4*R*)-**10** have been demonstrated to possess biological activity: the naturally occurring sulphate **12**[18] is a NMDA receptor agonist[19] and (2*S*,4*R*)-**10** is a constituent of the synthetic HIV protease inhibitor palinavir **13** (Fig. 3).[20]

Previous work had identified the enantiomers of allylglycine, an ideal acylase substrate, as starting materials for the synthesis of the pipecolic acid core.[21] The racemic starting material, *N*-acetylallylglycine **11** (Fig. 3) was prepared from diethylacetamidomalonate and allyl bromide,[22] and proved to be an excellent substrate for both of our proprietary L- and D-acylase enzymes allowing for efficient resolution of both enantiomers (Scheme 4). The next key step in the synthesis, the acyliminium ion cyclisation of the methyl ester of **14**,[23] gave the 1:1 mixtures of diastereomers at C-4, **16** (from *S*-**14**) and **17** (from *R*-**14**), which had previously only been separated by chromatography, which we deemed unsatisfactory for a scaleable synthesis.

Selective hydrolysis of one of the formate esters in the mixtures **16** and **17** using lipases allowed the differentiation of the diastereomers in such a way they could then be



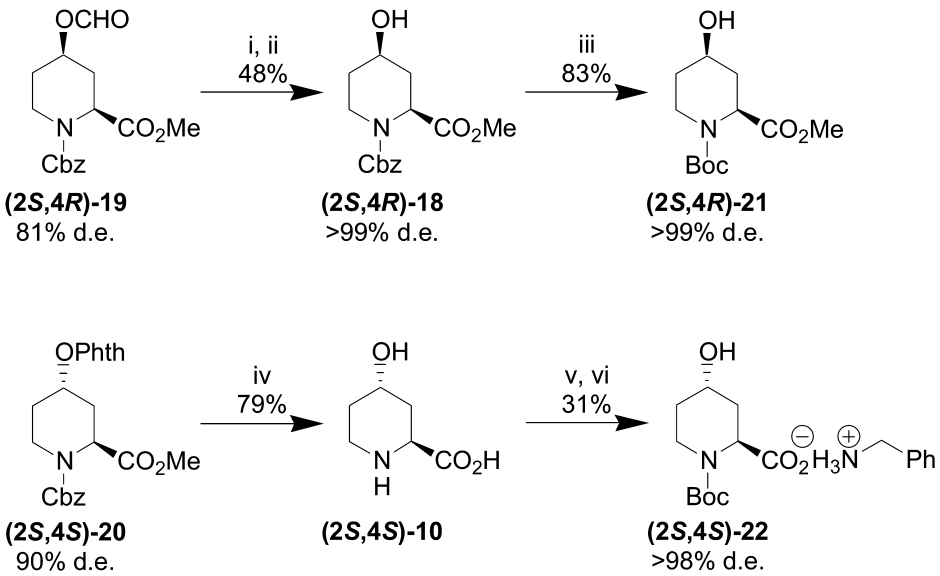
Scheme 4. *Reagents and conditions* (i) *t*-BuOK, allyl bromide (ii) NaOH (iii) HCl (iv) L-acylase, pH 8.0, 65 °C (v) Cbz-Cl, pH 8.0 (vi) D-acylase, pH 8.0 (vii) $SOCl_2$, MeOH (viii) paraformaldehyde, HCO_2H.



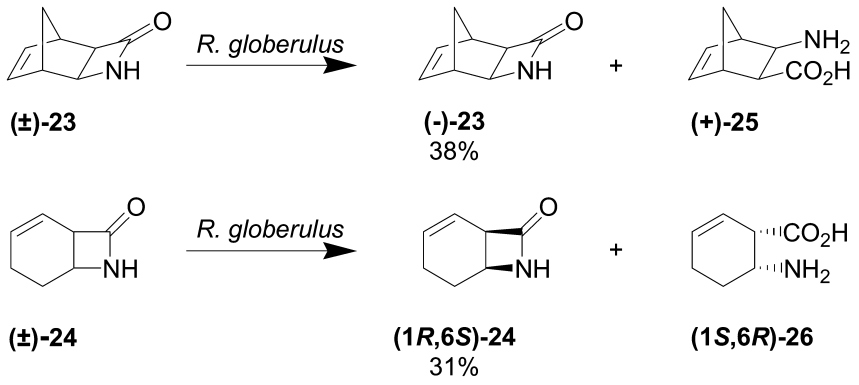
Scheme 5.

separated.[16] Lipase AY30 (*Candida rugosa*, Amano) selectively hydrolysed the formate ester of the (2*S*,4*S*)-diastereomer of mixture **16** (Scheme 5) to yield the 4-hydroxypipecolic acid methyl ester (2*S*,4*S*)-**18** (90% d.e.) and the 4-formyloxypipecolic acid methyl ester (2*S*,4*R*)-**19** (81% d.e.). The components of this mixture were separated by in situ derivatisation of (2*S*,4*S*)-**18** to the hemiphthalate ester derivative (2*S*,4*S*)-**20**, and partitioning between toluene and saturated ammonium carbonate solution.[24] Concentration of the toluene layer gave (2*S*,4*R*)-**19**. The hemiphthalate ester (2*S*,4*S*)-**20** was recovered by acidification of the aqueous layer and extraction into toluene. A similar approach was used for the separation of mixture **17**, using Chirazyme L9 (*Mucor miehei*, Roche) in the biotransformation, which preferentially hydrolysed the formate ester of the (2*R*,4*S*)-diastereomer of the mixture.

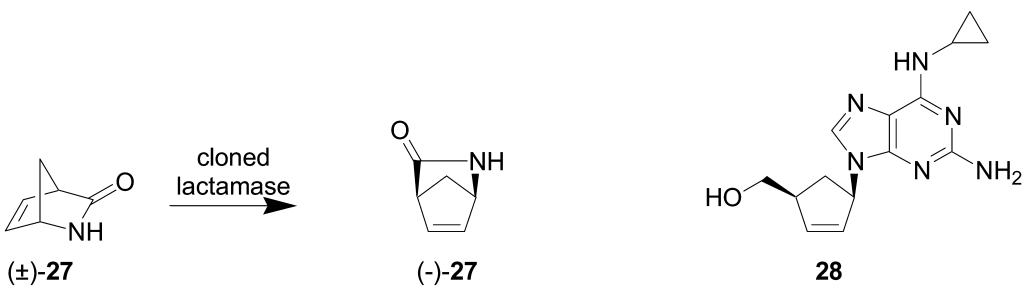
Although the lipases were able to separate the diastereomers, the products still lacked the required optical purity, which was gained using crystallisation. Optically pure *cis*-4-hydroxypipecolate (2*S*,4*R*)-**21** was obtained via (2*S*,4*R*)-**18** (Scheme 6), a known crystalline intermediate.[25] In the preparation of *trans*-4-hydroxypipecolic acid (2*S*,4*S*)-**10**, no suitable crystalline intermediate could be found, and thus was crystallised to diastereomeric purity as the *N*-Boc benzylamine salt (2*S*,4*S*)-**22**. Identical routes were used to prepare the enantiomeric compounds (2*R*,4*S*)-**21** and (2*R*,4*R*)-**22** from (2*R*,4*S*)-**19** and (2*R*,4*R*)-**20**, respectively. The syntheses of all four compounds demonstrate the usefulness of crystallisation as a dovetailing technology that greatly augments the power of the previously used chemistry. In this example, neither biocatalysis nor crystallisation alone was sufficient to separate the pairs of



Scheme 6. *Reagents and conditions* (i) K_2CO_3, MeOH (ii) recrystallise (iii) H_2, 10% Pd/C, Boc_2O (iv) 2 M HCl, then Amberlite IRA-93 (v) Boc_2O, Et_3N (vi) $BnNH_2$, recrystallise.



Scheme 7.

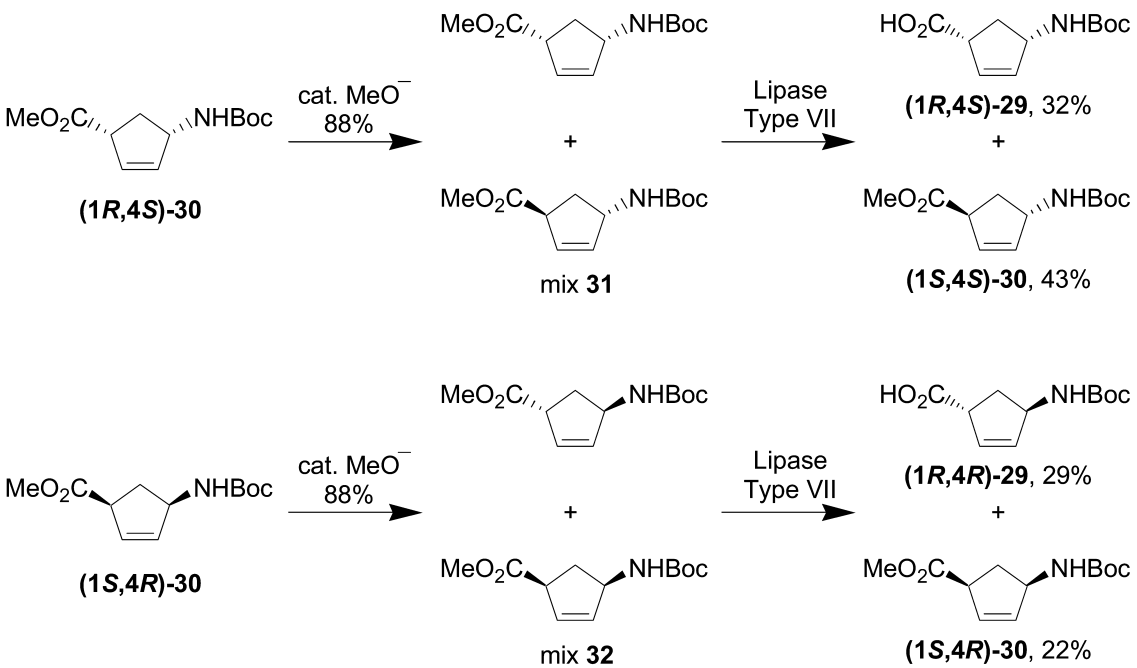


Scheme 8.

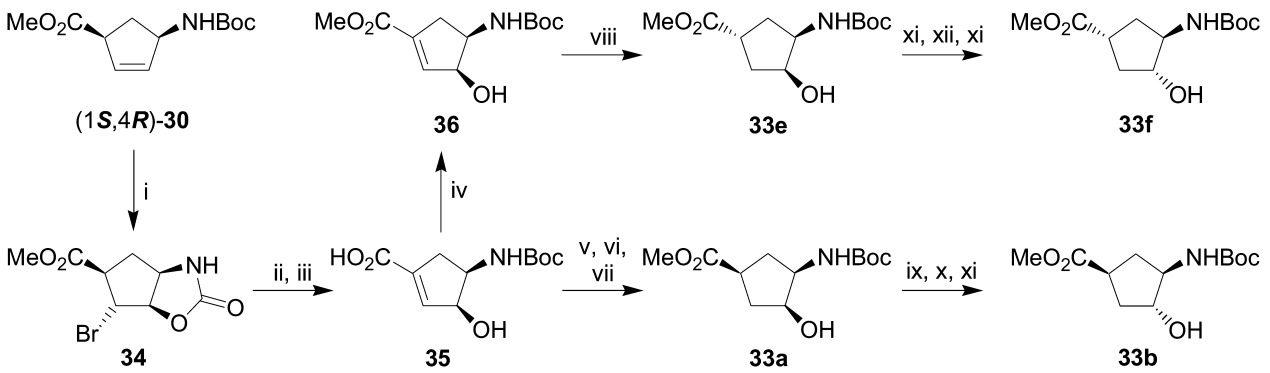
diastereomers; however, used in tandem they have provided an effective solution to this problem.

3. Use of lactamases for the preparation of β-and γ-amino acids

Many lactams possess biological activity, most notably the β-lactam class of antibiotics.[26] In addition to their own medicinal potential, lactams are a very useful source of amino acids. For example, a number of enantiopure cyclic β-amino acids have been prepared using lipase-catalysed kinetic resolutions of suitably protected β-lactams.[27] When condensed to form β-peptides, their properties differ greatly from α-peptides, especially when the $C_\alpha - C_\beta$ bond is constrained in a small ring.[28] In addition to this, β-peptide bonds appear far more stable than α-peptide bonds in a physiological environment as they are more resistant to protease enzymes.[29] We prepared 3-aza-tricyclo[4.2.1.$0^{2,5}$]-non-7-en-4-one **23** and 7-aza-bicyclo[4.2.0]oct-4-en-8-one



Scheme 9.



Scheme 10. *Reagents and conditions*: i, NBS, THF–H_2O (10:1), RT, 18 h, 95%; ii, KOH, MeOH–H_2O (1:1), 90 °C, 3 days; iii, Boc_2O, H_2O–THF (5:1), pH 10.5, 5 °C to RT, 18 h, 84% (overall for steps ii and iii); iv, MeOCOCl, Et_3N, MeOH, 5 °C then RT, 18 h, 81%; v, H_2 (35 psi), Pd/C, MeOH, RT, 18 h, (de 76%, 98%); vi, *tert*-butylamine, MTBE, recrystallise; vii, MeOCOCl, Et_3N, MeOH, 5 °C then RT, 18 h, 86%; viii, H_2 (75 psi), [(*R*,*R*)-Me-DuPHOS)-Rh(COD)]BF_4, MeOH, RT, (de >97%, 100%); ix, $MeSO_2Cl$, Et_3N, DMAP, CH_2Cl_2, 5 °C, 3 h, 97%; x, KOAc, DMF, 60 °C, 48 h, 85%; xi, NaOMe, MeOH, 5 °C, 7 h, 98%; xii, KOAc, DMF, 60 °C, 8 days, 88%.

24 as racemates from chlorosulphonylisocyanate and either 2,5-norbornadiene or 1,3-cyclohexadiene, respectively.[30] These were successfully resolved using a lactamase present in the whole cells of *Rhodococcus globerulus* (NCIMB 41042). The resolutions were carried out in phosphate buffer at pH 7 for 24 h, yielding (−)-**23** of >98% e.e. and (1*R*,6*S*)-**24** of >95% e.e. (Scheme 7). This stereochemistry was assigned by comparison of the optical rotation with that of its enantiomer.[27b]

We have previously reported the resolution of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one **27** using a cloned lactamase[31] in a process that has been used to produce tonne quantities of (−)-**27**, an intermediate for the industrial synthesis of carbocyclic nucleosides, for example abacavir, **28** (Scheme 8).

Derivatives of the products of these resolutions of **27** can be used in the synthesis of a range of compounds with biological activity. An example of this is the synthesis of both enantiomers of *trans*-4-aminocyclopent-2-ene-1-carboxylic acid, (1*S*,4*S*)-**29** (as the methyl ester (1*S*,4*S*)-**30**) and (1*R*,4*R*)-**29**. It was previously shown that the racemic amino acid inhibited the uptake of GABA in rat brain (Scheme 9).[32]

Treatment of the Boc-protected *cis*-amino acid methyl ester (1*R*,4*S*)-**30** with catalytic sodium methoxide in methanol at 0 °C led to an epimerisation at C-1 to give the 1:1 mixture of diastereomers **31**. Although separation of these compounds was not possible by crystallisation, a screen of **31** against **5** commercially available lipases revealed Lipase Type VII (*Candida rugosa*, Sigma) as a highly competent differentiating agent that preferentially hydrolysed the ester of the *cis*-isomer. Interestingly, during the synthesis of the opposite enantiomer, (1*R*,4*R*)-**29**, we found that Lipase Type VII was again the enzyme of choice for the separation of the diastereomer mix **32**, but this time preferentially hydrolysing the *trans*-isomer. This demonstrates the very high selectivity the enzyme displays for the C-1 chiral centre: in each case it is the *R*-centre that is hydrolysed, in spite of the steric constraints placed on the molecule by both the change in relative stereochemisty and the steric bulk of the other pendant functional group, the *N*-Boc.

The products of the bioresolution of (±)-**27** have also been used as precursors of the cyclopentane series of scaffolds **33a**–**33h** shown in Figure 4[33] Starting from the *N*-Boc protected amino acid methyl ester (1*S*,4*R*)-**30**, treatment with NBS yielded the oxazolidinone **34**, which upon treatment with base, eliminated and rearranged to give the allylic alcohol **35** in excellent yield (Scheme 10). Palladium on charcoal hydrogenation of **35** gave an all *cis*-compound in 76% d.e. which could be crystallised to diastereomeric purity as the *tert*-butylamine salt. Cracking of this salt followed by esterification gave **33a**. If, however, **35** was esterified, it became an excellent substrate for hydrogenation using the [(*R*,*R*)-Me-DuPHOS)-Rh(COD)]BF_4 catalyst, which gave **33e** directly (>97% d.e.). A mesylation-acetate inversion sequence on **33a** and **33e** provided the diastereomers **33b** and **33f**, respectively.

The other four diastereomers were obtained from the enantiomeric starting material (1*R*,4*S*)-**30**. The only

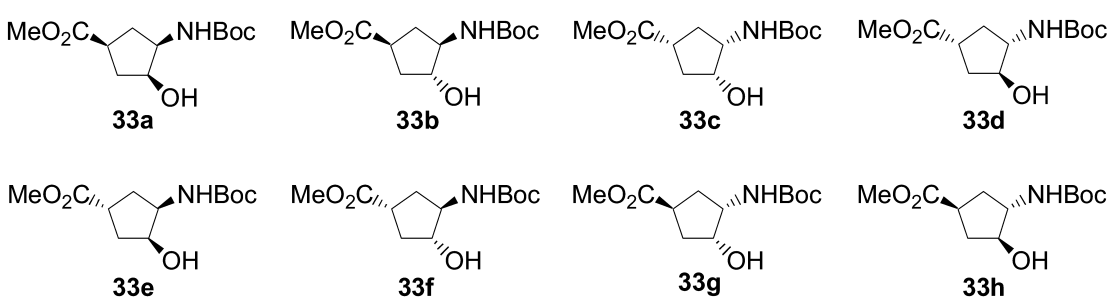


Figure 4. The eight diastereomers of 3-*tert*-butoxycarbonylamino-4-hydroxy-cyclopentanecarboxylic acid methyl ester.

difference in the sequence was that to obtain **33g**, the [(*S*,*S*)-Me-DuPHOS)-Rh(COD)]BF_4 catalyst was used for the hydrogenation. These scaffolds can be used for the synthesis of molecules with potential biological activity, for example in structures that are analogues of natural products.[34,35]

4. Conclusion

In the examples discussed in this paper, hydrolase enzymes have been used to resolve enantiomers and differentiate diastereomers such that they can be easily separated and provide a mild environment to enable manipulation of highly functionalised molecules. The nature of such chemistry allows for use on large scale. This broad spectrum of use clearly demonstrates why they are so widely used in synthetic chemistry today. However, to elaborate simple compounds into the more complex, rigid compounds described has emphasised the necessity for combining chiral technologies. In most of the syntheses described, although the biocatalytic step has been crucial for introducing at least one of the chiral centres in the target compound, others have been introduced by a compatible technology.

5. Experimental

5.1. General

All reagents and solvents were from commercially available sources and used as received. Proton NMR spectra were obtained on a Bruker Avance 400 spectrometer operating at 400 MHz for proton (^{1}H) and 100 MHz for carbon (^{13}C). Chemical shifts are reported in ppm using Me_4Si or residual nondeuterated solvent as reference. Coupling constants (*J*) are measured in Hz. GC-MS data were obtained using a HP 5890 Series 2 GC fitted with a J and W Scientific DB5 column, attached to a HP 5972 series Mass Selective Detector using electron impact ionisation. GC d.e. and e.e. data of final compounds were obtained using a Perkin Elmer Autosystem Gas Chromatograph fitted with a Varian Chirasil-Dex CB column (25 m×0.25 mm). HPLC data were obtained using a Gilson HPLC system fitted with a Luna Phenylhexyl column. HPLC e.e. data were recorded using a Phenomenex D-penicillamine (150×4.6 mm) column. Optical rotation data were obtained using a Perkin Elmer Polarimeter 341 instrument.

5.1.1. 4-Benzoyloxybut-2-yne-1-ol 4.[36] 2-Butyne-1,4-diol (500 g, 5.81 mol) was dissolved in CH_2Cl_2 (1 L) and pyridine (470 mL, 5.81 mol) and cooled to 5 °C under N_2. Benzoyl chloride (608 mL, 5.25 mol) in CH_2Cl_2 (1 L) was added over a 3 h period, keeping the reaction temperature below 10 °C. The resultant yellow suspension was allowed to warm to room temperature and stirred overnight. The reaction mixture was washed with 1 M H_2SO_4 (3×600 mL) and H_2O (2×600 mL) and organic phase concentrated to 900 mL volume. EtOH (750 mL) was added, and the solution placed in a freezer overnight. Any precipitate was removed by filtration and washed with EtOH (100 mL). The filtrate was concentrated under reduced pressure to give **4**, an orange oil (519 g, 52%). ^{1}H NMR ($CDCl_3$) 8.06 (2H, m), 7.57 (1H, m), 7.44 (2H, m), 4.96 (2H, t, *J*=2 Hz), 4.34 (2H, t, *J*=2 Hz), 2.97 (1H, br s). NMR also showed presence of ~8 mol% dibenzoate ester. ^{13}C NMR ($CDCl_3$) 166.4, 134.2, 130.9, 130.2, 128.8, 85.4, 81.4, 53.1, 51.5. *m*/*z* 190 (M^+), 173, 105, 77, 51.

5.1.2. 4-Benzoyloxybut-2-yne-1-ol, methanesulphonate ester 5.[37] **4** (278 g, 1.46 mol) in CH_2Cl_2 (2.1 L) was cooled to 0 °C under N_2 Triethylamine (224 mL, 1.61 mol) and DMAP (4.45 g, 36 mmol) were added, followed by methanesulphonyl chloride (125 mL, 1.61 mol) in CH_2Cl_2 (100 mL) dropwise over 30 min period, keeping the reaction temperature below 5 °C. The reaction was stirred at 0 °C for 2 h, when H_2O (1.5 L) was added. After stirring for 5 min, the layers were separated, and the organic layer washed with sat. $NaHCO_3$ (1.5 L) and 1 M HCl (1.5 L), dried ($MgSO_4$) and concentrated under reduced pressure to give **5**, a brown oil (350 g, 90%). ^{1}H NMR ($CDCl_3$) 8.04 (2H, m), 7.57 (1H, m), 7.45 (2H, m), 4.96 (2H, m), 4.89 (2H, t, *J*=2 Hz), 3.12 (3H, s). ^{13}C NMR ($CDCl_3$) 166.1, 134.1, 130.6, 130.1, 128.8, 84.5, 79.3, 57.9, 53.0, 39.5. *m*/*z* No M^+, 173, 105, 77, 51.

5.1.3. 1-Bromo-4-benzoyloxybut-2-yne 6.[37] A solution of **5** (345 g, 1.29 mol) in acetone (1.7 L) was cooled to 10 °C under N_2. LiBr (223 g, 2.57 mol) was added portionwise, keeping the temperature below 15 °C, and the reaction stirred at this temperature for 90 min. The mixture was filtered through celite, and the filtrate concentrated under reduced pressure. EtOAc (2 L) was added to the residue, and this solution washed with H_2O (2×1 L), dried ($MgSO_4$) and concentrated under reduced pressure to yield **6**, a brown oil (303 g, 90%). ^{1}H NMR ($CDCl_3$) 8.07 (2H, m), 7.57 (1H, m), 7.46 (2H, m), 4.99 (2H, m), 3.96 (2H, m). ^{13}C NMR ($CDCl_3$) 166.2, 133.8, 130.2, 129.9, 128.7, 82.2, 81.1, 53.1. *m*/*z* No M^+, 173, 105, 77, 51.

5.1.4. 1,1-Dicarboethoxy-1-acetamido-5-benzoyloxy-pent-3-yne 7. A suspension of diethylacetamidomalonate (258 g, 1.19 mol) and KO^{t}Bu (146 g, 1.30 mol) in THF (3 L) was heated to reflux for 1 h. To this refluxing mixture, a stream of **6** (300 g, 1.19 mol) in THF (500 mL) was added over a 10 min period. After 2.5 h, the reaction had not gone to completion, so a further 100 g (0.47 mol) diethyl-acetamidomalonate and KO^{t}Bu (58 g, 0.47 mol) were added and the mixture refluxed overnight. The reaction mixture was cooled and filtered through celite, and the filtrate concentrated under reduced pressure to a volume of 1 L. EtOAc (2 L) was added and the solution washed with 0.1 M HCl (1 L) and H_2O (1 L). The organic phase was concentrated under reduced pressure to yield **7**, a brown oil (365 g, 79%). ^{1}H NMR ($CDCl_3$) 8.05 (2H, m), 7.58 (1H, m), 7.46 (2H, m), 6.96 (1H, br s), 4.85 (2H, t, *J*=2 Hz), 4.25 (4H, m), 3.33 (2H, t, *J*=2 Hz), 2.04 (3H, s), 1.24 (6H, m). ^{13}C NMR (d_6-DMSO) 170.0, 166.6, 165.3, 134.0, 129.6, 129.5, 129.2, 81.8, 77.8, 65.6, 62.4, 53.0, 22.4, 14.1. *m*/*z* 389 (M^+), 345, 316, 274, 194, 174, 152, 124, 105, 77, 51.

5.1.5. *N*-Acetyl-(4-hydroxybut-2-ynyl)glycine 3. To a stirred solution of **7** (300 g, 0.77 mol) in EtOH (1.5 L) and H_2O (1 L) NaOH (92.5 g, 2.31 mol) was added in H_2O (500 mL) as a thin stream. The mixture was heated to reflux for 4 h, cooled and the EtOH removed under reduced pressure. The resultant aqueous solution was washed with

EtOAc (1 L), and then carefully acidified to pH 3.5 using conc. HCl, and heated to reflux for 24 h. After cooling, the solution was acidified to pH 2.5 with conc. HCl and extracted with EtOAc (1.5 L). The pH of the aqueous phase was then adjusted to 6.0 with 46–48% NaOH solution and concentrated under reduced pressure. The residue was slurried in MeOH (500 mL) and filtered through celite, after which decolourising charcoal (25 g) was added to the filtrate and this mixture heated to 50 °C for 20 min. After filtration through celite, the filtrate was concentrated under reduced pressure to give **3**, an orange oil (149 g, 100% crude yield). ^{1}H NMR (D_2O) 4.27 (1H, t, J=6 Hz), 4.15 (2H, t, J=2 Hz), 2.67 (2H, m), 2.01 (3H, s). ^{13}C NMR (d_6-DMSO) 172.5, 168.6, 82.7, 80.9, 53.3, 49.6, 23.3, 22.8.

5.1.6. Resolution of (±)-3. 3 (125 g, 0.67 mol) in 30 mM KH_2PO_4 (1.7 L) at pH 7 was treated with L-acylase solution (200 U/g substrate) and the solution stirred at 65 °C and pH 7 until ^{1}H NMR of an evaporated sample showed >40% conversion (44 h). Boc_2O (47 g, 0.27 mol) in THF (200 mL) was then added at RT, and the reaction maintained at pH 10 until the pH was static. The mixture was extracted with MTBE (500 mL), then the remaining aqueous acidified to pH 3 with $KHSO_4$ and extracted with EtOAc (3×1 L). The EtOAc portions were combined, dried ($MgSO_4$), filtered and evaporated in vacuo to give (*S*)-*N*-Boc-(4-hydroxybut-2-ynyl)glycine *S*-**8**, a pale yellow oil (44 g, 36%, 98% e.e.). ^{1}H NMR (d_6-DMSO) 12.6 (1H, br s), 7.05 (d, 1H, J=8 Hz), 5.07 (1H, br), 4.03 (2H, m), 2.55 (2H, m), 1.37 (9H, s). ^{13}C NMR (d_6-DMSO) 172.6, 155.6, 80.7, 78.6, 53.1, 54.0, 49.4, 28.5, 21.8. The remaining aqueous solution from above was then adjusted to pH 8 with 46/48% NaOH, and D-acylase solution added (40 U/g substrate). The reaction mixture was stirred at RT and pH maintained at pH 8 until the reaction was complete by ^{1}H NMR (21 h). The pH was then adjusted to 10 and the mixture treated with Boc_2O (54 g, 0.31 mol) and worked up as above to give (*R*)-*N*-Boc-(4-hydroxybut-2-ynyl)glycine *R*-**8**, an orange oil (48 g, 34%, 95% e.e.). ^{1}H NMR (d_6-DMSO): 12.6 (1H, br s), 7.05 (d, 1H, J=8 Hz), 5.07 (1H, br), 4.03 (2H, m), 2.55 (2H, m), 1.37 (9H, s). ^{13}C NMR (d_6-DMSO) 172.6, 155.6, 80.7, 78.6, 53.1, 54.0, 49.4, 28.5, 21.8.

5.1.7. *S*-*N*-Boc-(*Z*-4-hydroxybut-2-enyl)glycine *S*-2.[38] Lindlar's catalyst (3.0 g) was added to a degassed solution of *S*-**8** (30 g) in MeOH (600 mL), and the resultant mixture sealed in a 2 L Parr pressure vessel. After purging with N_2 and H_2, the vessel was charged to 1 bar with H_2 and the reaction mixture stirred at 20 °C until the H_2 pressure above remained constant. The remaining H_2 was released, and the vessel purged with N_2. The reaction mixture was filtered through celite and concentrated under reduced pressure to give *S*-**2** (30 g, 100%). This material can be purified by flash chromatography (eluent 5:4:1 EtOAc/heptane/AcOH) if required. ^{1}H NMR (d_6-DMSO): 7.02 (1H, d, J=8 Hz), 5.55 (1H, m), 5.34 (1H, m), 3.94 (2H, d, J=6), 3.86 (1H, m), 2.35 (2H, m), 1.36 (9H, s).

5.1.8. Resolution of 3-aza-tricyclo [4.2.1.0^{2,5}] non-7-en-4-one (±)-23. (±)-**23** (2 g, 15 mmol) and a whole cell preparation of *Rhodococcus globerulus* NCIMB 41042 (2 g, 1 wt equiv.) were suspended in 50 mM KH_2PO_4, pH 7 (40 mL). The resulting mixture was stirred at 25 °C for 24 h. The cells were then spun off by centrifuge (10 min, 3400 rpm) and the pellet was washed with H_2O (20 mL) and respun. The combined supernatants were extracted with EtOAc (3×50 mL) and the organic extracts dried over $MgSO_4$. Removal of solvent under reduced pressure yielded (−)-**23** (770 mg, 38%, e.e. (GC) >98%), a white solid. ^{1}H NMR ($CDCl_3$) 6.19–6.30 (1H, m), 6.00–6.18 (2H, m), 3.44–3.53 (1H, m), 2.98–3.10 (1H, m), 2.91–2.96 (1H, br s), 2.82–2.91(1H, br s), 1.81 (1H, d, J=10 Hz), 1.65 (1H, d, J=10 Hz). ^{13}C NMR ($CDCl_3$): 171.0, 138.6, 136.3, 58.6, 53.5, 44.0, 41.1, 39.1. *m*/*z* No M$^+$, 107, 91, 70, 66. $[\alpha]_D^{20}$ (*c* 0.01, MeOH) −91.

5.1.9. Resolution of racemic 7-aza-bicyclo [4.2.0]oct-4-en-8-one 24. 24 (1.49 g, 12 mmol) and a whole cell preparation of *Rhodococcus globerulus* NCIMB 41042 (1.4 g, 1 wt equiv.) were suspended in 50 mM KH_2PO_4, pH 7 (40 mL). The resulting mixture was stirred at 30 °C for 22 h. The cells were then spun off by centrifuge (10 min, 3400 rpm) and the pellet was washed with H_2O and respun. The combined supernatants were extracted with EtOAc (3×50 mL) and the organic extracts dried over $MgSO_4$. Removal of solvent under reduced pressure yielded (1*R*,6*S*)-**24**, (460 mg, 31%, e.e. (GC) >95%), an off-white solid. ^{1}H NMR ($CDCl_3$): 6.19–6.10 (1H, m), 6.07 (1H, br s), 5.98–5.90 (1H, m), 4.03 (1H, t, J=5 Hz), 3.55–3.48 (1H, m), 2.16–2.02 (3H, m), 1.68–1.55 (1H, m). ^{13}C NMR ($CDCl_3$) 172.3, 135.0, 126.3, 50.2, 44.7, 22.2, 22.1. *m*/*z* no M$^+$, 103, 80, 79. $[\alpha]_D^{25}$ (*c* 0.13, $CHCl_3$) −154.

5.1.10. Epimerisation (1*R*,4*S*)-*N*-Boc-4-aminocyclopent-2-ene-1-carboxylic acid, methyl ester, (1*R*,4*S*)-30. A solution of (1*R*,4*S*)-**30** (1.82 g, 7.55 mmol) in MeOH (30 mL) was cooled to 0 °C and NaOMe (25 wt% in MeOH, 2 mL, pre-cooled to 0 °C) was added. The resulting mixture was stirred for 120 min, maintaining the temperature at 0 °C throughout. After this time, it was quenched using glacial acetic acid (4 mL). The solvent was removed under reduced pressure to yield a yellow oil (1.6 g) which solidified on standing. GC–MS analysis indicates that the solid consists of a 1:1 mixture of (1*R*,4*S*) (*cis*) and (1*S*,4*S*) (*trans*) epimers (mixture **31**) in addition to a small amount (<10%) of the conjugated adduct. GC–MS: Retention time 16.59 min (*cis*-epimer, *m*/*z*: 185, 168, 141, 126, 82), 17.16 min (*trans*-epimer *m*/*z*: 185, 168, 141, 126, 82), 17.91 min (conjugated adduct).

5.2. Screening for selective hydrolysis of mixtures 31 and 32

50 mg of substrate (either **31** or **32**) was placed into a scintillation vial with 50 mM KH_2PO_4, pH 7 (5 mL) and enzyme (20 mg) and the vial agitated at 26 °C in a water bath/shaker After 18 h the reaction was extracted with EtOAc (5 mL). The organic extracts were dried over $MgSO_4$ and analysed by GC–MS. Enzymes used: Porcine pancreatic lipase (Sigma), Lipase Type VII (Sigma), Lipase PS (Amano), Chirazyme L9 (Roche) and Chirazyme L2 (Roche). Analysis revealed that Lipase Type VII gave the greatest selectivity for both mixtures, hydrolysing the *cis*-epimer of **31** and the *trans*-epimer of **32**.

5.2.1. Scale up of the separation of mixture 31. 31 (0.6 g,

2.5 mmol) was suspended in 50 mM phosphate buffer, pH 7 (60 mL) and Lipase type VII (250 mg) added The mixture was left to shake in a water bath/shaker at 26 °C for 24 h and extracted with EtOAc (3×50 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (50 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure to yield (1*S*,4*S*)-**30**, an off-white solid (260 mg, 43%). GC-MS confirmed the structure of the recovered material. To recover the *cis*-epimer, the aqueous phase was acidified with 1.2 M HCl and extracted with EtOAc (3×50 mL). The combined extracts were dried over $MgSO_4$ and concentrated to yield a colourless oil (180 mg, 32%) that solidified on standing. ^{1}H NMR (CD_3OD) major (*cis*) diastereomer[39] 5.81 (1H, m), 5.71 (1H, m), 4.53 (1H, br s), 3.40 (1H, m), 2.42 (1H, dt, *J*=14, 8 Hz), 1.70 (1H, m), 1.34 (9H, s). ^{13}C NMR (CD_3OD) 177.9, 158.0, 135.5, 132.9, 80.6, 57.7, 50.8, 35.8, 29.2; minor (*trans*) diastereomer 5.81 (1H, m), 5.71 (1H, m), 4.63 (1H, br m), 3.56 (1H, m), 2.42 (1H, m), 1.70 (1H, m), 1.34 (9H, s). ^{13}C NMR (CD_3OD) 177.5, 157.9, 135.1, 132.9, 80.1, 57.6, 50.8, 35.7, 28.8. Comparison of relative integrations of dispersed resonances show ratio of *cis*:-*trans*-diastereomers to be 4:1.

5.2.2. Scale up of the separation of mixture 32. 32 (1.0 g, 4.15 mmol) was suspended in 50 mM phosphate buffer, pH 7 (100 mL) and Lipase type VII (400 mg) added. The mixture was left to shake in a water bath/shaker at 26 °C for 24 h and extracted with EtOAc (3×50 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (50 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure to yield (1*S*,4*R*)-**30**, an off-white solid (220 mg, 22%). GC-MS confirmed the structure of the recovered material. To recover the *trans*-epimer, the aqueous phase was acidified with 1.2 M HCl and extracted with EtOAc (3×50 mL). The combined extracts were dried over $MgSO_4$ and concentrated to yield a white solid (270 mg, 29%) that solidified on standing. ^{1}H NMR (CD_3OD) major (*trans*) diastereomer 5.81 (1H, m), 5.71 (1H, m), 4.63 (1H, br m), 3.56 (1H, m), 2.42 (1H, m), 1.70 (1H, m), 1.34 (9H, s). ^{13}C NMR (CD_3OD) 177.5, 157.9, 135.1, 132.9, 80.1, 57.6, 50.8, 35.7, 28.8; minor (*cis*) diastereomer 5.81 (1H, m), 5.71 (1H, m), 4.53 (1H, br s), 3.40 (1H, m), 2.42 (1H, dt, *J*=14, 8 Hz), 1.70 (1H, m), 1.34 (9H, s). ^{13}C NMR (CD_3OD) 177.9, 158.0, 135.5, 132.9, 80.6, 57.7, 50.8, 35.8, 29.2. Comparison of relative integrations of dispersed resonances show ratio of *trans*:-*cis*-diastereomers to be 3:1.

5.2.3. (3α*R*,5*R*,6*R*,6α*R*)-6-Bromo-2-oxo-hexahydro-cyclopentaoxazole-5-carboxylic acid methyl ester 34. *N*-Bromosuccinimide (164 g, 0.92 mol) was added in portions to a solution of (1*S*,4*R*)-**30** (200 g, 0.83 mol) in THF (670 mL) and water (67 mL). The *N*-Bromosuccinimide was seen to completely dissolve over a 3 h period during which a mild exotherm was evident. The reaction was stirred for a further 14 h after which time it was concentrated to dryness under vacuum. The residue was redissolved in dichloromethane (1.1 L) and washed sequentially with 1 M HCl (aq.) (500 mL), saturated Na_2SO_3 (aq.) (500 mL) and brine (500 mL) before drying over $MgSO_4$. Following filtration, concentration of the organic under vacuum yielded 238 g of a light brown solid. Recrystallisation from EtOAc/heptane yielded **34** as white crystals (110 g, 50%). A further crop of crystals was obtained from the liquors though it was contaminated with a small amount of succinimide (49 g, 23%). ^{1}H NMR ($CDCl_3$) 5.79 (1H, br s,), 5.15 (1H, dd, *J*=7, 2 Hz), 4.79 (1H, m), 4.43 (1H, app dt, *J*=7, 2 Hz), 3.75 (3H, s), 3.23 (1H, app quintet, *J*=7 Hz), 2.52 (1H, dt, *J*=14, 7 Hz), 2.42 (1H, dm, *J*=14 Hz). ^{13}C NMR ($CDCl_3$) 171.2, 158.1, 87.8, 56.1, 53.3, 52.5, 51.5, 35.2. *m/z* 265, 263 (1:1, M^+), 184, 156, 140, 124, 115, 80.

5.2.4. (3*S*,4*R*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-cyclopent-1-enecarboxylic acid 35. KOH (43 g, 0.76 mol) in H_2O (150 mL) was added to a solution of **34** (36 g, 0.19 mol) in MeOH (150 mL). The mixture was heated under reflux at 90 °C for 2 days and allowed to cool. MeOH was removed under vacuum, the mixture diluted with H_2O (100 mL) and the pH of the reaction was adjusted to pH 10.5 on addition of 1 M HCl (aq.). The solution was cooled to 10 °C and a solution of Boc_2O (42 g, 0.19 mmol) in THF (60 mL) was added dropwise and the mixture allowed to warm to room temperature. The reaction was then stirred for 14 h, the layer of THF removed and the aqueous solution adjusted to pH 3 with 6 M HCl (aq). The acidic solution was extracted with EtOAc (3×200 mL) and the combined organic washings dried over $MgSO_4$. Filtration then concentration under vacuum gave **35** as a white solid (40 g, 86%). ^{1}H NMR (d_6-DMSO) 6.58 (1H, m), 6.40 (1H, d, *J*=7 Hz), 4.57 (1H, m), 4.05 (1H, m), 3.41 (1H, br s), 2.65 (1H, app dd, *J*=16, 7 Hz), 2.41 (1H, ddt, *J*=16, 6, 1 Hz), 1.46 (9H, s). ^{13}C NMR ($CDCl_3$) 168.5, 158.5, 142.8, 139.8, 80.7, 75.3, 54.2, 37.3, 29.1.

5.2.5. (3*S*,4*R*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-cyclopent-1-enecarboxylic acid methyl ester 36. Methyl chloroformate (29 mL, 0.37 mol) was added dropwise to a cooled solution (0 °C) of **35** (83 g, 0.34 mol) and triethylamine (52 mL, 0.37 mol) in MeOH (600 mL). The reaction was stirred for 1 h at 0 °C and then allowed to warm to room temperature. After stirring for 14 h it was apparent that the reaction was incomplete. After cooling to 0 °C, triethylamine (30 mL, 0.22 mol) was added then methyl chloroformate (15 mL, 0.19 mol) added dropwise. The reaction was complete after a further 2 h. The reaction mixture was concentrated under vacuum and the residue redissolved in CH_2Cl_2 (400 mL). The organic phase was washed sequentially with 1 M $KHSO_4$ (aq.) (2×200 mL), sat. $NaHCO_3$ (2×200 mL) and brine (200 mL) before drying over $MgSO_4$. Following filtration, the solvent was removed to yield an orange oil which was redissolved in MeOH (300 mL) and cooled to 5 °C. NaOMe (0.25 mL, 25 wt% solution in MeOH) was then added and the reaction mixture cooled and stirred for 4 h. The reaction was quenched with glacial AcOH, concentrated under vacuum and the residue redissolved in CH_2Cl_2 (300 mL). The organic phase was washed with sat. $NaHCO_3$ (100 mL) then brine (100 mL) and dried over $MgSO_4$. Following filtration, the solvent was removed under vacuum to yield **36** as an orange oil (76 g, 87%). ^{1}H NMR ($CDCl_3$) 6.71 (1H, m), 5.26 (1H, d, *J*=7 Hz), 4.77 (1H, br s), 4.25 (1H, br s), 3.77 (3H, s), 3.04 (1H, br s), 2.92 (1H, dd, *J*=17, 7 Hz), 2.50 (1H, m), 1.46 (9H, s). ^{13}C NMR ($CDCl_3$) 165.1, 156.1, 141.0, 137.6, 79.5, 74.7, 52.5, 51.8, 36.5, 28.3. *m/z* No M^+, 201, 183, 151, 137, 125, 106, 96, 78, 57.

5.2.6. (1*S*,3*R*,4*S*)-3-*tert*-Butoxycarbonylamino-4-hydroxy-cyclopentanecarboxylic acid methyl ester 33a. 10% Pd/C (1 g) was added under N_2 to a solution of **35** (150 g, 0.29 mol) in MeOH (300 mL) The reaction mixture was transferred to a bomb and after purging with N_2 and then H_2, a H_2 pressure of 2 bar was applied and the reaction stirred for 18 h. (Periodically, additional hydrogen was added to the reaction to re-establish the initial reaction pressure.) The pressure was released and the bomb purged with N_2. The reaction mixture was cautiously filtered through celite and then concentrated. The residue was dissolved in EtOAc (1.6 L) and *tert*-butylamine (77 mL, 0.73 mol) added dropwise. After stirring for 2.5 h, the precipitate was recovered by filtration and washed with MTBE (200 mL). This material was recrystallised from 1:3 MeOH/MTBE (700 mL) to give the *tert*-butylamine salt as a white solid (91 g). This salt was dissolved in H_2O (350 mL), and the pH adjusted to 3 with 6 M HCl. The solution was stirred for 1 h and extracted with CH_2Cl_2 (3×350 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated to give a free acid as a white solid (64 g). This was dissolved in MeOH (350 mL) and cooled to 5 °C. Et_3N (44 mL, 0.31 mol) was added followed by methyl chloroformate (25 mL, 0.31 mol) dropwise. The reaction mixture was stirred at 5 °C for 1 h, then allowed to warm to room temperature overnight. The solvent was removed in vacuo, and the residue taken up in CH_2Cl_2 (300 mL). This solution was washed with 1 M KHSO4 (2×200 mL), sat. $NaHCO_3$ (200 mL) and sat. brine (200 mL) and dried over MgSO4. Concentration of this material yielded **33a**, an orange oil (59 g, >98% d.e., 38% from **35**). ^{1}H NMR ($CDCl_3$) 5.17 (1H, br s), 4.09 (1H, m), 3.92 (1H, br s), 3.71 (3H, s), 2.91 (2H, m), 2.39 (1H, app dq, J=17 Hz), 2.12 (1H, m), 1.98 (1H, app dq, J=14 Hz), 1.74 (1H, app dq, J=17 Hz), 1.42 (9H, s). ^{13}C NMR ($CDCl_3$) 179.1, 156.2, 79.9, 73.4, 55.9, 52.8, 39.6, 36.0, 33.5, 28.8. *m/z* 241 (M^+), 202, 185, 172, 154, 140, 126, 116. $[\alpha]_D^{25}$ (*c* 1.0, MeOH) +3.3.

5.2.7. (1*S*,3*R*,4*R*)-3-*tert*-Butoxycarbonylamino-4-hydroxy-cyclopentanecarboxylic acid methyl ester 33b. Methanesulfonyl chloride (17.0 mL, 0.22 mol) was added dropwise to a solution of **33a** (43.9 g, 0.18 mol), triethylamine (50 mL, 0.36 mol) and DMAP (1.1 g, 9 mmol) in CH_2Cl_2 (500 mL) at 0 °C. After 45 min the reaction was washed with 1 M citric acid (2x200 mL), sat. $NaHCO_3$ (200 mL), H_2O (200 mL) and brine (100 mL) and then dried over $MgSO_4$. After filtration, concentration of the organic under vacuum gave the mesylate as an off-white solid (55.6 g, 96% crude), which was immediately dissolved in DMF (250 mL) and KOAc (105 g, 1.05 mol) added. The stirred mixture was heated at 60 °C for 3 days, cooled and diluted with CH_2Cl_2 (700 mL). The mixture was washed with H_2O (1 L), sat. $NaHCO_3$ (500 mL), H_2O (500 mL) and brine (300 mL) before drying over $MgSO_4$. Following filtration, concentration under vacuum gave the acetate as a brown solid which was recrystallised from MTBE and hexanes. NaOMe (0.7 mL, 25 wt% solution in MeOH) was added to a solution of the recrystallised acetate (29.2 g, 0.10 mol) in MeOH (300 mL) at 0 °C. After 7 h the reaction was quenched on addition of glacial AcOH and the solvent removed under vacuum. The residue was redissolved in CH_2Cl_2 (500 mL) and washed with H_2O (200 mL), sat. $NaHCO_3$ (200 mL), H_2O (100 mL) and brine (200 mL) before drying over $MgSO_4$. Following filtration, concentration under vacuum yielded a brown solid which was taken up in hot MTBE (80 mL) and stirred with decolourising charcoal for 30 min. After filtration, heptane was added to yield **33b** as white crystals (18.6 g, 71%). ^{1}H NMR ($CDCl_3$) 5.26 (1H, d, J=5 Hz), 4.13 (1H, m), 3.96 (1H, br s), 3.79 (1H, br s), 3.70 (3H, s), 3.07 (1H, m), 2.40 (1H, dt, J=13, 8 Hz), 2.14 (1H, m), 1.99 (1H, m), 1.71 (1H, m), 1.44 (9H, s). ^{13}C NMR ($CDCl_3$) 177.4, 156.7, 80.3, 78.6, 60.2, 52.4, 40.2, 36.5, 33.7, 28.7. *m/z* 241 (M^+), 207, 185, 154, 140, 126, 116, 100, 83, 53. $[\alpha]_D^{25}$ (*c* 1.0, MeOH) −25.9.

5.2.8. (1*R*,3*R*,4*S*)-3-*tert*-Butoxycarbonylamino-4-hydroxy-cyclopentanecarboxylic acid methyl ester 33e. ((1,2-Bis(2*R*,5*R*)-2,5-dimethylphospholano)benzene)(cyclooctadiene)rhodium(I) tetrafluoroborate (23 mg, 1 mol%) was added to a degassed solution of **36** (1 g, 3.9 mmol) in MeOH (5 mL). The reaction mixture was transferred to a bomb and after purging with N_2 and then H_2, an H_2 pressure of 5 bar was applied and the reaction stirred for 14 h. The pressure was released and the bomb purged with N_2. Concentration of the reaction mixture gave a residue which was redissolved in CH_2Cl_2 (5 mL). Addition of silica (0.5 g) with stirring removed the catalyst from the reaction and filtration and concentration of the organic gave **33e** as an off-white solid of 98% d.e. in quantitative yield. ^{1}H NMR ($CDCl_3$) 4.91 (1H, d, J=7 Hz), 4.27 (1H, m), 3.99 (1H, br s), 3.67 (3H, s), 3.10 (1H, m), 2.26 (1H, m), 2.05 (2H, m), 1.85 (1H, dt, J=13, 10 Hz), 1.43 (9H, s). ^{13}C NMR ($CDCl_3$) 176.8, 156.2, 80.0, 73.0, 55.0, 52.3, 39.5, 36.7, 32.9, 28.8. *m/z* No M^+, 202, 185, 172, 154, 140, 126, 116, 99, 87, 57. $[\alpha]_D^{25}$ (*c* 1.0, MeOH) +36.4.

5.2.9. (1*R*,3*R*,4*R*)-3-*tert*-Butoxycarbonylamino-4-hydroxy-cyclopentanecarboxylic acid methyl ester 33f. Prepared in an identical manner to **33b**. ^{1}H NMR ($CDCl_3$) 4.67 (1H, br), 4.23 (1H, br s), 4.00 (1H, m), 3.76 (1H, m), 3.70 (3H, s), 2.91(1H, m), 2.43 (1H, m), 2.34 (1H, m), 1.91 (1H, m), 1.68 (1H, m), 1.45 (9H, s). ^{13}C NMR ($CDCl_3$) 176.2, 157.3, 80.6, 79.1, 59.3, 52.5, 39.3, 36.4, 33.2, 28.7. *m/z* 241 (M^+), 202, 185, 172, 154, 140, 126, 116, 83, 57. $[\alpha]_D^{25}$ (*c* 1.0, MeOH) −9.7.

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Directed evolution of the dioxygenase complex for the synthesis of furanone flavor compounds☆

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Abstract—Herein we report a new preparation of 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one, the flavor compound strawberry furanone, based on a 'green' approach with a minimum number of steps. The first step is an enzymatic dioxygenation of *p*-xylene to form cyclohexadiene-*cis*-diol, followed by ring opening via ozonolysis, and ring closure to form the furanone. In efforts to improve the efficiency of the enzymatic step, a directed evolution approach was taken to increase the substrate specificity and selectivity of the toluene dioxygenase enzyme system.

1. Introduction

The flavor compound strawberry furanone (**1**)[1,2] is a high value fine chemical commercially available as Furaneol® from Firmenich.[3] It is found naturally in pineapples, strawberries, mangoes, as well as in roasted or cooked foods such as malt, sesame seeds, roasted beef, and soy sauce, where it is formed via the Maillard reaction.[4] Despite this natural occurrence of **1** in fruits and cooked foods, most of the commercially available flavor compound is a non-natural, synthetic product manufactured from relatively expensive 2,5-hex-3-ynediol.[5] Herein, we report a new preparation of **1** based on a chemoenzymatic approach with a minimum number of steps from inexpensive *p*-xylene using bacterial dioxygenase enzymes for enabling the strategy and the execution of the synthesis (Fig. 1). In addition, we explore the directed evolution of these bacterial dioxygenase enzymes for increased efficiency and potential for commercial applications of the synthesis of **1** and similar flavor compounds.

Microorganisms contain aryl dioxygenases that in a single step convert aromatic hydrocarbons to corresponding diene-*cis*-diols. Substituted cyclohexadiene-*cis*-diols are interesting synthons for the manufacture of a range of fine chemicals.[6] The various functionalities and stereospecific centers of these molecules can be exploited chemically, for example through oxidations, cycloadditions, ring opening and ring closure reactions, to generate precursors for synthetic applications. Economic application of microbial dioxygenases for the production of substituted cyclohexadiene-*cis*-diols has been hampered by limited volumetric productivity.

Directed evolution is a technology that allows for the diversification of enzymes into biocatalyst platforms by

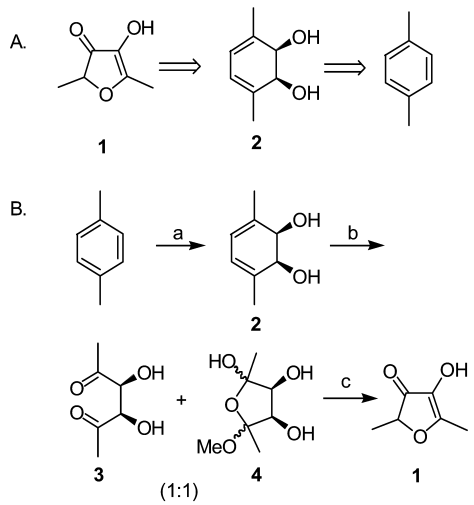

Figure 1. A novel route to strawberry furanone was envisioned in which the *p*-xylene derived *cis*-1,2-dihydroxy-3,6-dimethyl-cyclohexa-3,5-diene is converted in a short reaction sequence to the desired product. B. The strategy and execution of the synthesis. Conditions (a) *E. coli* JM109 (pTrctodNK1); (b) O_3, MeOH; then aq. $Na_2S_2O_3$, $NaHCO_3$ (84–92%); (c) *n*BuOAc, H_2O, phosphate buffer, 95 °C, 5 h (39–40%).

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recombination of homologous gene sequences that encode similar enzymes.[7] These platforms can be screened for desired biocatalyst characteristics such as substrate specificity, specific activity, enantioselectivity, regioselectivity, substrate tolerance (to allow conversions at economically relevant substrate concentrations), product tolerance (to prevent inhibition of conversion as the conversion approaches completion), and half-life time of the enzyme. The technology is applicable to single genes, encoding homomeric enzymes, multiple genes, encoding heteromeric enzymes or enzyme pathways, and even whole genomes.[8]

Aryl dioxygenases are multi-subunit, heteromeric, iron containing enzyme complexes that catalyze the incorporation of both atoms of molecular oxygen into aromatic hydrocarbons.[9] Toluene and biphenyl dioxygenase oxidize their substrates toluene and biphenyl to *cis*-1,2-dihydroxy-3-methyl-cyclohexa-3,5-diene and *cis*-1,2-dihydroxy-3-phenyl-cyclohexa-3,5-diene, respectively.[9,10] Directed evolution of toluene dioxygenase has resulted in improved substrate specificity,[11] while biphenyl dioxygenase has been evolved for remediation purposes by extending its substrate range.[12,13]

Aryl dioxygenases are encoded by four genes that constitute a gene cluster or operon. The four genes encode each of the two subunits of the oxygenase component, ferredoxin and ferredoxin reductase[14] (Fig. 2A). In all reported aryl dioxygenase enzyme improvement studies to date, only the large subunit of the oxygenase was subjected to directed evolution.[11–16] While this subunit is generally regarded as the main contributor to substrate specificity,[17–19] all components of the enzyme complex can in principle represent the rate-limiting step in substrate oxidation. In this report, we present the first directed evolution studies on a multi enzyme complex in which all components were subjected to recombination of genetic diversity. It is expected that the improved biocatalysts that are discovered in this program will enable novel chemical processes at commercial scale. One such new application is the synthesis of 3(2*H*)-furanone flavor compounds. Here we describe the application of dioxygenase to a novel route for strawberry furanone synthesis starting from *p*-xylene.

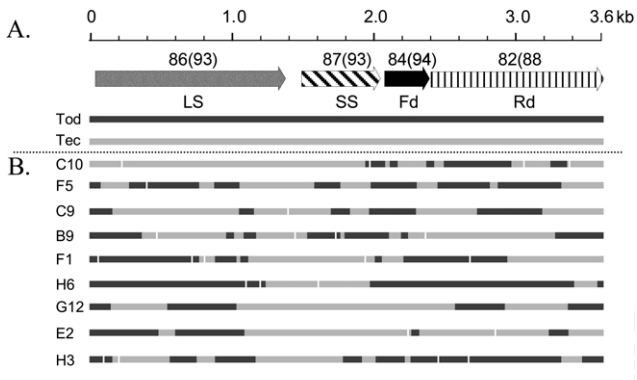

Figure 2. A. Genetic maps of the toluene (*todC1C2BA*) and tetrachlorobenzene dioxygenase (*tecA1A2A3A4*) gene clusters. The operons are approximately 3.6 kb in length and encode the oxygenase large subunit (LS, encoded by *todC1* and *tecA1*, respectively), the oxygenase small subunit (SS, *todC2* and *tecA2*), a ferredoxin (Fd, *todB* and *tecA3*) and reductase (Rd, *todA* and *tecA4*). Homology between the genes and encoded enzymes is given in percentages at the nucleotide sequence level and amino acid sequence level (in parentheses) above the gene arrows. B. Composition of the chimeric dioxygenase clusters for nine improved dioxygenase clones. Sequence derived from *todC1C2BA* is in dark grey, sequence derived from *tecA1A2A3A4* is in light grey, point mutations due to the PCR process are indicated as white lines.

2. Results and discussions

2.1. Directed evolution of aryl dioxygenase

Multigene DNA shuffling was applied to the complete toluene dioxygenase and tetrachloro-benzene dioxygenase operons to improve the biotransformation of a range of aromatics. The four gene operons encoding toluene and tetrachlorobenzene dioxygenase,[20] *todC1C2BA* and *tecA1A2A3A4*, were shuffled (Fig. 2A). The shuffled library was pre-screened according to the tier 1 assay (see Section 4) in *E. coli* DH5α for active clones using the indole oxidation assay[21] and was 70% active. Restriction enzyme digests of 24 clones from the library indicated that 23 of the randomly picked clones were chimeras of the parental genes (results not shown).

The diene-*cis*-diol resulting from *p*-xylene oxidation is of interest as a starting material for a novel synthetic route to strawberry furanone as described below. Active clones from the first tier assay were therefore, evaluated for the ability to oxidize *p*-xylene to *cis*-1,2-dihydroxy-3,6-dimethyl-3,5-cyclohexadiene. A high throughput screen was developed in which *p*-xylene toxicity to the bacterial host was circumvented by supplying the substrate in a slow-release system (Fig. 3). The amount of diol produced was compared to the amount of product produced by the parental clones. In total, 1044 different dioxygenases were screened and nine exhibited 2 to 3.5-fold higher activity when compared to the best parent.

The best nine clones were retested in multiples of eight (Table 1). The greatest improvement in activity was that of clone C10, which had a 4.4-fold higher activity. It is known that the details of a screening procedure may have an effect

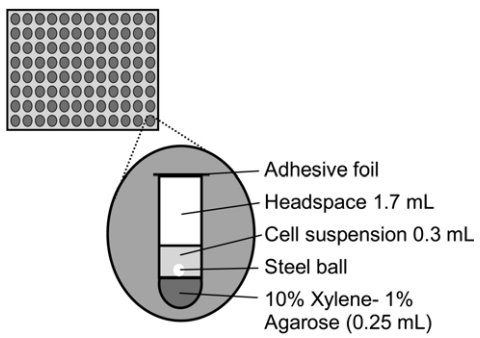

Figure 3. High throughput screen for *p*-xylene oxidation. A 96-well system was developed in which toxicity of high *p*-xylene concentrations was prevented by supplying the substrate from a slow-release formulation. Cell suspensions were added on top of the semi-solid substrate and efficient mixing was achieved by shaking with a 1 mm steel ball. Evaporation of substrate was prevented by sealing the plates with an adhesive aluminum foil. Head space over the reaction mixture was sufficient to provide oxygen for the bioconversion. Upon termination of the reaction after 1 h of incubation, the presence of diene-*cis*-diol was measured spectrophotometrically.

Table 1. Apparent activity[a] of diol formation in parental and family shuffled clones

	DH5α clones 2×YT			LS5218 clones minimal media		
	Average activity	sd[b]	Fold improvement	Average activity	sd	Fold improvement
tod	0.830	0.134	1.0	1.64	0.292	1.00
tec	0.663	0.070		1.24	0.550	
B9	1.59	0.259	1.9	1.10	0.242	(0.7)
C9	3.06	0.970	3.7	1.45	0.178	(0.9)
C10	3.67	0.894	4.4	4.12	0.683	2.5
E2	2.18	0.402	2.6	2.46	0.761	1.5
F1	3.03	0.451	3.7	3.40	0.743	2.1
F5	2.85	0.404	3.4	2.16	0.389	1.3
H3	2.99	0.902	3.6	3.59	1.13	2.2
H6	2.38	0.481	2.9	3.51	1.10	2.1
G12	2.63	0.438	3.2	2.88	0.706	1.8

[a] Apparent activity=μmol/min mL whole cells. Activities are normalized to optical density of cell suspensions.
[b] Standard deviation.

on the outcome of such screen. To test the influence of the host cell background and the growth medium on the activity of the shuffled dioxygenases, plasmid purified from the nine clones and the parental clones were transformed into *E. coli* LS5218. The transformants were tested for activity on *p*-xylene after growth in minimal media with fructose as carbon source. Improved activity was reconfirmed for seven of the nine shuffled clones, although the extent of such improvements were reduced to 1.3- to 2.5-fold. This indicates that either the host strain or the growth conditions or both have a direct impact on overall dioxygenase activity. The exact cause of this effect remains to be determined.

The same nine clones were examined for improved activity on a range of substituted benzene derivatives (Fig. 4). Depending on the substrate, among the nine clones improvements ranged from 2.7-fold (on *m*-xylene) to 16-fold on cumene. In addition to *p*-xylene, clone C10 exhibited the highest activity on *m*-xylene, 1,2,3-trimethylbenzene and naphthalene, while clone F1 showed the highest activity on cumene and toluene. DNA sequence analysis of these nine clones indicated that significant diversification had occurred (Fig. 2B). Clone C10 consisted primarily of the *tec* sequence, with significant amounts of *tod* sequence in the reductase gene only. Surprisingly, the large dioxygenase subunit in clone F1 consisted primarily of *tod* sequence where the *tod* operon was shown to have less activity on toluene than the *tec* operon. This could indicate that for oxidation of toluene, the large subunit is not necessarily the limiting factor. On this substrate, activity does not seem to be limited by the large subunit of the complex.

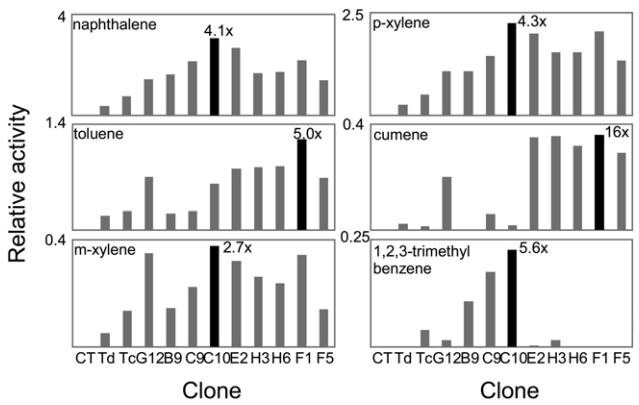

Figure 4. Substrate specificity of nine clones that were improved for oxidation of *p*-xylene. Clones were examined individually for activity on the indicated substrates. Relative activity is measured as the UV absorbance of the supernatant of cultures of each clone at the λ_{max} for each product diene-*cis*-diol. Note that the absolute activity varies per substrate. The clone with highest activity, with respect to the best parent, is indicated by the black bar with the fold improvement noted.

The availability of a wide range of highly active dioxygenase enzymes may enable the commercial application of these catalysts. One novel application would be in the manufacture of 3(2*H*)-furanone flavor compounds.

2.2. Dioxygenase enabled route to strawberry furanone

A novel route to strawberry furanone was envisioned in which the *p*-xylene derived diene-*cis*-diol is converted in a short reaction sequence to the desired product (Fig. 1A). Our chemoenzymatic approach as well as the execution of the synthesis are outlined in Figure 1B.

Biotransformation of *p*-xylene by whole cells of *E. coli* strains JM109 (pTrctodNK1) or LS5218 (pTrctodNK1) expressing toluene dioxygenase produced **2**. Cultures were grown in minimal media with fructose as the carbon source and *p*-xylene was fed as a vapor introduced into the air stream. Fed-batch cultures of 1.0 and 5.0 L volume yielded 20 and 88 g of **2**, respectively, and which was purified from cell-free culture broth by extraction. Synthesis of **2** up to 0.2 g/L had been reported for *Pseudomonas* mutants that were unable to convert *p*-xylene beyond the diol.[10]

The *meso* diol was subjected to ozonolysis in MeOH at −78 °C. When the solution turned a light blue color, it was quenched with aqueous thiosulfate/bicarbonate, and the mixture was extracted with ethylacetate. A semisolid was obtained in ~90% yield. NMR analysis of this material indicated the desired molecule as a 1:1 mixture of the diketone **3** and its diastereomeric methoxyketal–hemiketal **4**.[22] As similar mixtures were obtained when this sequence was performed on the acetonide derived from **2**, protection of the diol was not required. The crude ozonolysis products were dissolved in *n*-butylacetate and added to phosphate buffer. The mixture was heated at 95 °C for 5 h, then allowed to stand for 12 h. Furanone **1** was isolated by extraction with ethylacetate or by steam distillation. Its

physical and spectral properties were identical to those of a commercial sample. Consistent yields of ca. 40% were attained with *n*-butylacetate, even with varying cosolvents/buffers and temperature regimes. The entire sequence can be successfully performed on a 5 g scale without purification of intermediates. Further optimization at the process level will provide for a potential commercial process for this important flavor additive.

3. Conclusions

Improved dioxygenase biocatalysts were generated through multigene DNA shuffling of the entire dioxygenase gene cluster. Because such improvements were generated by recombining different fragments of the dioxygenase operons, we expect that further improvement of these catalysts is possible. Such improvements are desirable to further lower the cost of these diene-*cis*-diols. It is our estimate that with a product titer in the range of 60–80 g/L in the biotransformation, the novel route to strawberry furanone will be competitive with the current process. Analogous routes to other commercial flavor compounds such as 4-hydroxy-5-methyl-3(2*H*)-furanone and 5-ethyl-2-methyl-4-hydroxy-3(2*H*)-furanone can be developed based on toluene and *p*-ethyl-toluene, respectively and in fact, a range of new flavor compounds may be available through dioxygenase-mediated supply of diene-*cis*-diols (Fig. 5). As directed evolution of enzymes is applicable to provide efficient catalysts for any desired substrate, such applications are coming closer to reality.

4. Experimental

4.1. Biotransformation; purification of the cyclohexadiene-*cis*-diol; directed evolution

For biotransformation studies at the 1 and 5 L scale, clones containing pTrctodNK1 were preferred over pTrctecNK1. *E. coli* LS5218 (pTrctodNK1) was grown in a 2 L fermentor (New Brunswick BioFlo 2000, Edison, NJ), on E2[23] with R2 trace elements,[24] 0.2% yeast extract, 5 mM $MgSO_4$ and 0.5% fructose at 37 °C. Ampicillin was added to a final concentration of 100 μg/mL. Aeration was at 1.8 L/min and pH was maintained at 7.0 with KOH. The culture was grown to an OD_{600} of 3.5, at which time a 50% fructose, 6% ammonium chloride and 2% magnesium sulfate feed was initiated to maintain the dissolved oxygen at 30%. After the culture reached an OD_{600} 15, the temperature was reduced to 34 °C and 200 μM ferrous ammonium sulfate was added. The expression of toluene dioxygenase was induced by the addition of 250 μM of IPTG. The culture was grown for another 2 h, after which *p*-xylene was fed as vapor to the culture through the air stream at a flow rate of 3 L/min. The formation of *cis*-1,2-dihydroxy-3,6-dimethyl-hexa-3,5-diene, was monitored by measuring the absorbance of cell-free supernatant at a wavelength of 280 nm and estimating the concentration using an extinction coefficient of ε=6500 mol^{-1} cm^{-1}.[10]

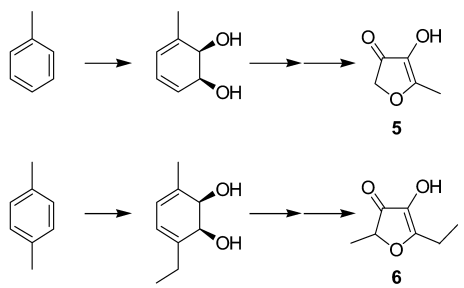

Figure 5. Analogous routes to other flavor compounds such as 4-hydroxy-5-methyl-3(2*H*)-furanone (**5**) and 5-ethyl-2-methyl-4-hydroxy-3(2*H*)-furanone (**6**) can be developed based on toluene and *p*-ethyl-toluene, respectively and in fact, a range of new flavor compounds may be available through dioxygenase-mediated supply of diene-*cis*-diols.

After the formation of the product was complete, the culture was harvested and the cells removed by centrifugation at 5,000 rpm, 20 min, at 4 °C. NaCl (280 g) was dissolved in the supernatant and the product phase extracted three times with approximately 0.6 L of ethyl acetate. The organic layers were combined and the solvent evaporated under reduced pressure to yield 20 g of nearly colorless crystals of the desired compound. The extracted samples were homogenous by TLC analysis after development in ethyl acetate giving an UV absorbance at 254 nm and staining positive with iodine vapor. The biotransformation procedure was scaled to a 5.0 L vessel (BioFlo 3000, New Brunswick, Edison, NJ) using the same medium and process conditions as described above. The 5.0 L of cell culture yielded approximately 88 g or 17.6 g/L of the desired product.

4.2. Bacterial strains and plasmid construction

P. putida F1 (ATCC 700007) was obtained from the American Type Culture Collection. The toluene dioxygenase operon *todC1C2BA* (GenBank accession number J04996) were PCR amplified using the forward primer CCATG GCTTGAAAAGTGAGAAGAC and the reverse primer TCTAGAGCGCCACCGCC TGTCACC. pSTE7 containing the tetra-chlorobenzoate dioxygenase genes from *Burkholderia* sp. PS12, was a kind gift from Dr Deitmar H. Pieper, Division of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany. The *tec* operon *tecA1A2A3A4* (GenBank accession number U78099) was PCR amplified from plasmid pSTE7 using the forward primer CCATGGCAT GAAACG TGAGGAGAC and reverse primer TCTA GAGCG CCTCCGCCCGTCACC. The PCR product was digested with *NcoI* and *XbaI*, gel purified, ligated into similarly digested expression vector pTrc99a (Amersham Pharmacia Biotech, Piscataway, NJ), and transformed into *E. coli* DH5α (Invitrogen Corp., Carlsbad, CA). Recombinant strains were maintained on LB agar plates or liquid media containing 100 μg/mL ampicillin. Strains were stored at −80 °C as 20% glycerol stocks. DNA sequences of the corresponding pTrctodNK1 and pTrctecNK1 inserts were verified by sequencing both DNA strands on an ABI 377 sequencer (Perkin–Elmer, Foster City, CA). PCR reagents were obtained from Qiagen (Valencia, CA) and restriction enzymes from New England Biolabs (Beverly, MA).

4.3. Shuffling

The two multigene operons from parent plasmids pTrctodNK1 and pTrctecNK1, were shuffled as previously

described.[25] The PCR product was digested with *NcoI* and *XbaI*, gel purified, ligated into similarly digested expression vector pTrc99a and transformed into *E. coli* DH5α. PCR product of insert DNA from randomly selected clones was digested with restriction enzyme *Hinc*II, and the restriction digest pattern compared to that of similarly digested parental PCR product to confirm that the shuffling reaction produced chimeric sequences. DNA sequences of improved shuffled clones were determined by sequencing both DNA strands on an ABI 377 sequencer (Perkin–Elmer, Foster City, CA). The sequences were compared to the parental gene sequences to confirm the formation of chimeric sequences. DNA sequences were analyzed using Sequencher version 4.0.5 (Gene Codes Corp., Ann Arbor, MI) and Vector NTI 6 (InforMax Inc., Bethesda, MD).

4.4. Screening. First tier assay

Shuffled dioxygenase libraries were initially screened for activity in vivo using the ability of the dioxygenase to transform endogenous indole to indigo.[21] Colonies containing active dioxygenase protein turn blue from the production of indigo. The shuffled libraries were transformed into *E. coli* DH5α and plated onto LB agar containing 100 μg/mL ampicillin. Plates were incubated for 20 h at 37 °C and then moved to room temperature for 24 h. The plates were checked for blue colored colonies indicating the presence of active dioxygenase. The blue color of the colonies varied from light to dark among the active shuffled clones. These active clones were picked to 96 well plates containing LB media, 100 μg/mL ampicillin, 0.2% glucose, and 20% glycerol. These master plates were incubated 20 h at 37 °C and stored at ~20 °C and used to inoculate cultures for substrate activity assays.

4.5. Second tier assay

Active shuffled clones were screened for the conversion of *p*-xylene to *cis*-1,2-dihydroxy-3,6-dimethylcyclohexa-3,5-diene in 96-well-plate format. Clones were grown in 200 μL of 2×YT media containing 100 μg/mL ampicillin and 0.2% glucose. Plate wells were inoculated with 3 μL/well of inoculum from the master plate and incubated for 20 h at 37 °C (250 rpm and 85% relative humidity (RH)). The 20-h cultures were subcultured into 2.2-mL-deep well plates containing 500 μL/well of 2×YT media containing 100 μg/mL ampicillin and 1 mM IPTG. These plates were incubated under the same conditions for 6 h. The plates were centrifuged using an Allegra 6KR centrifuge (Beckman Instruments, Fullerton, CA) (3000 rpm, 10 min, 10 °C) to pellet cells, washed once in 40 mM phosphate buffer, pH 7.0, and resuspended in 0.5 mL of 40 mM potassium phosphate buffer, pH 7.0.

The washed cell suspensions (300 μL) were transferred to a 96-deep well assay plate that contained 250 μL of solid agarose/10% *p*-xylene emulsion on the bottom of each well. The solidified agarose/substrate emulsion served as the source of volatile substrate for the biotransformation reaction. The deep well plates were sealed with Biomek Seal and Sample foil seals (Beckman Instruments, Fullerton, CA) and shaken at room temperature for 1 h. The cell suspensions were then transferred to a 96 shallow well plate, the cells pelleted (3000 rpm, 10 min, 10 °C) after which 200 μL of the supernatants were transferred to a Corning UV plate (VWR, So. Plainfield, NJ) and the absorbance of the cell-free supernatants was read at 280 and 320 nm on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA).

4.6. Analytical methods

TLC analysis of cyclohexadiene-*cis*-diols was performed on Merck silica gel 60 F_{254} plates (2.5×7.5 cm, Aldrich, Milwaukee, WI). Aliquots of *p*-xylene transformation assays were extracted three times with an equal volume of ethyl acetate, the organic layers combined and dried under nitrogen. The extracted samples were dissolved in 20 μL ethyl acetate and developed in ethyl acetate vapor giving an UV absorbance at 254 nm and staining positive with iodine vapor. The samples were compared to cyclohexadiene-*cis*-diol standard prepared from the wild type toluene dioxygenase clone.

4.7. Oxidation by ozonolysis of an unprotected diol–diene to form a diol–dione

The *cis*-1,2-dihydroxy-3,6-dimethylhexa-3,5-diene **2** was not purified prior to ozonolysis. All reagents were purchased from Fischer Scientific (Pittsburgh, PA) and used without further purification. Sodium sesquicarbonate solution A was prepared by dissolving 84 g of $NaHCO_3$ and 53 g of Na_2CO_3 in 1 L of water. The final pH of the solution was about 10–11. 5.07 g of 1,2-dihydroxy-3,6-dimethylhexa-3,5-diene **2** (vacuum dried) was dissolved in 50 mL of MeOH and ozonized at −78 °C for 3 h (until blue color persisted) (65 kV, 3 lpm). The resulting solution was transferred via insulated canula to a solution of 21 mL of 1 M $Na_2S_2O_3$ and 13 mL of 1.5 M sesquicarbonate solution A and stirred at 0 °C. The transfer took 5 min. The resulting solution quickly became peroxide negative. Stirring was continued for 30 min at 0 °C and for another 30 min at room temperature. Methanol was distilled off under reduced pressure at 40 °C, and the resulting solution (with white precipitate) was extracted 4 times with 125 mL ethyl acetate. Combined extracts were dried over anhydrous sodium sulfate and evaporated to give colorless and practically odorless oil, which after vacuum drying partially crystallized on standing (4.92 g). This material was the desired product as a 1:1 mixture of the free 2,5-diketo-3,4-dihydroxyhexane **3** and it's methoxyketal–hemiketal **4**, with combined yields in 84–92% range.

4.8. Cyclization of an unprotected diol–dione

The following condition was used after modification of the procedure published in US Patent 5,149,840,[26] which describes an optimized cyclization of rhamnose and other 6-deoxyhexoses to **1**. Crude ozonolysis product, 800 mg, was added to buffer containing: 340 mg of $NaH_2PO_4{\times}1H_2O$, 80 mg $NaHCO_3$, 0.260 mL of H_2O, 0.100 mL of 40% NaOH. The semi-solid mixture was added to *n*-butylacetate or ethylacetate and the two-phase system was, after degassing with argon, heated in a closed vial (see Table 2). The solvent was separated from the dark-reddish-brown aqueous phase, the aqueous layer was extracted with 3×5 mL EtOAc, and the organic layer and extracts were combined, dried over anhydrous $MgSO_4$ and the solvent evaporated. All

Table 2. Summary of two-phase cyclization reactions for 4-hydroxy-3[2*H*]-furanone **1** synthesis

Mass of starting material	Solvent (volume)	Buffer, amounts of $NaH_2PO_4 \cdot H_2O$ and $NaHCO_3$	Temp. (°C)	Time (h)	Weight recovered	Ratio of **1**:**3** (by NMR)
800 mg	*n*-BuOAc (3 mL)	340 mg+80 mg	95, then rt	4–16	280 mg	4:1
1.3 g	EtOAc (3 mL)	510 mg+120 mg	Reflux ~80	22	630 mg	1:1
1 g	*n*-BuOAc (5 mL)	425 mg+100 mg	95, then rt	8–15	390 mg	3.5:1
2.6 g	*n*-BuOAc (10 mL)	170 mg+40 mg	110	22	1.3 g	3:1

experiments were repeated at least 3 times. The experiments were reproducible up to 2–6 g. The material was analyzed by TLC, ^{13}C NMR, and ^{1}H NMR with 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one **1** as comparative standard.[22] Deuterochloroform was used as the solvent and D_2O exchange was also performed. The final 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one **1** product is very volatile and oxidizes readily in the presence of air, as evidenced by appearance of additional spots on subsequent TLC analysis.

4.8.1. 4-Hydroxy-3,5-dimethyl-3(2*H*)-furanone-1. ^{1}H NMR (400 MHz, $CDCl_3$): 7.6 (1H), 4.5 (dq, J=7, 1 Hz, 1H), 2.26 (d, J=1 Hz, 3H), 1.44 (d, J=7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 198.84, 174.89, 133.95, 80.02, 16.27, 13.37.

4.8.2. *cis*-1,2-Dihydroxy-3,6-dimethyl-hexa-3,5-diene-2. ^{1}H NMR (400 MHz, $CDCl_3$): 5.63 (2H), 4.06 (2H), 2.55 (2H), 1.88 (6H). ^{13}C NMR (100 MHz, $CDCl_3$): 135.14, 120.25, 72.05, 19.48. Compound **2** is unstable and dehydrates readily to 2,5-dimethylphenol.[7] This degradation product was detected in the spectra of compound **2**: ^{1}H NMR (400 MHz, $CDCl_3$): 6.97 (d, J=7.6 Hz, 1H), 6.63 (d, J=7.6 Hz, 1H), 6.59 (1H), 2.25 (3H), 2.19 (3H).

^{13}C NMR (100 MHz, $CDCl_3$): 163.8, 136.8, 130.66, 121.04, 120.68, 115.62, 20.88, 15.3.

4.8.3. 2,5-Diketo-3,4-dihydroxyhexane-3/methoxyketal–hemiketal-4 mixture: 3. ^{1}H NMR (400 MHz, $CDCl_3$): 4.38 (2H), 2.31 (6H). ^{13}C NMR (100 MHz, $CDCl_3$): 207.87, 78.53, 16.31. **4**: ^{1}H NMR (400 MHz, $CDCl_3$): 3.83 (d, J=3.5 Hz, 1H), 3.69 (d, J=3.5 Hz, 1H), 3.36 (3H), 1.4 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 105.56, 101.8, 70.89, 66.88, 48.88, 27.03, 21.46.

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One-pot chemoenzymatic synthesis of protected cyanohydrins

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Abstract—In a chemoenzymatic one-pot reaction of ethyl cyanoformate with benzaldehyde catalyzed by the hydroxynitrile lyase from *Prunus amygdalus* ethoxycarbonylated (*R*)-mandelonitrile is formed in a highly enatioselective manner. The reaction was performed both in aqueous and organic media. ^{1}H NMR investigations revealed a two-step procedure consisting of an enzyme-catalyzed addition of HCN, generated by hydrolysis of ethyl cyanoformate, to the aldehyde followed by ethoxycarbonylation of the free cyanohydrin in a second step

1. Introduction

Enantiopure cyanohydrins and their derivatives, such as α-hydroxy acids, α-hydroxy ketones and aldehydes and β-hydroxy amines, serve as important synthetic intermediates for the production of pharmaceuticals and agrochemicals.[1] These compounds can be synthesized by asymmetric addition of HCN to prochiral carbonyl compounds under the catalytic action of hydroxynitrile lyases.[2] These biocatalysts show a broad substrate acceptance including aromatic, heteroaromatic and saturated and unsaturated aliphatic aldehydes and ketones.[3]

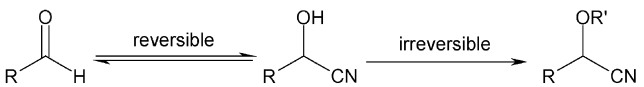
However, the reversibility of the cyanohydrin formation constitutes a major problem since the corresponding back reaction regenerates the substrate unless an excess of HCN or another cyanide donor is used. Carrying out the process in aqueous or biphasic aqueous-organic emulsion systems requires low pH values in order to stabilize the products which potentially leads to decreased biocatalyst activity. Furthermore, working at low pH suppresses the spontaneous unselective non-enzymatic HCN addition to the carbonyl substrates and products with excellent optical purity are obtained. Nevertheless, the equilibrium of the reaction often is not in favour of the desired cyanohydrins.

A potential solution of this problem would be to couple the cyanohydrin synthesis with an irreversible derivatization step in a one-pot procedure. This would furnish protected and hence more stable products. At the same time the reaction equilibrium will be shifted towards the product side resulting in higher yields (Scheme 1).

Recently, Hanefeld et al. attempted to combine two biocatalytic steps, cyanohydrin formation catalyzed by hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) and acetylation mediated by *Candida antarctica* lipase B. In this case, problems occurred due to hydrolysis of the acyl donor and subsequent deactivation of the *Hb*HNL.[4] A chemoenzymatic approach has been developed by Oda[5] and Kanerva.[6] In a dynamic kinetic resolution procedure enantiomerically enriched cyanohydrins are formed by lipase catalyzed acetylation being coupled with preliminary chemical in situ formation and racemization of the latter compounds.

In addition to cyanohydrin formation chemoenzymatic protocols for the synthesis of optically pure alcohols and amines combining chemically catalyzed racemization and lipase-catalyzed acylation or deacylation of the substrates have been developed.[7]

Recently, several groups reported that cyanoformate esters react in the presence of a chiral chemical catalyst (either cinchona alkaloids or BINOL–metal complexes) with carbonyl compounds to give the protected cyanohydrins.[8] Herein we wish to report our studies on the application of ethyl cyanoformate (**2**) as HCN donor and protecting



Scheme 1.

Keywords: cyanohydrins; hydroxynitrile lyase (*Pa*HNL); ethyl cyanoformate; chemoenzymatic synthesis.
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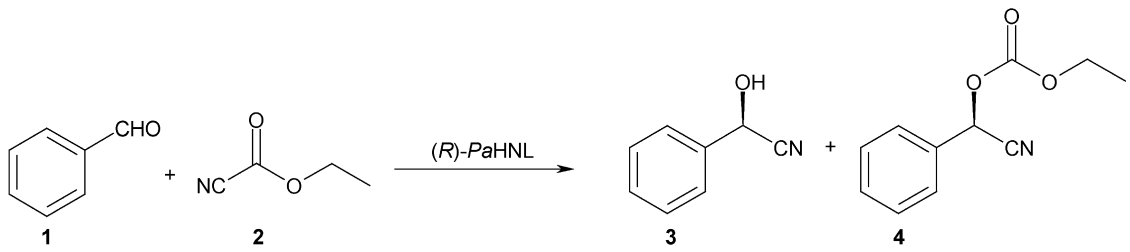

Scheme 2.

reagent in the enzymatic cyanohydrin synthesis catalyzed by the *R*-selective hydroxynitrile lyase from *Prunus amygdalus* (*Pa*HNL, from almonds). For model studies we chose benzaldehyde (**1**), the natural substrate of the enzyme, as starting material.

2. Results and discussion

Benzaldehyde (**1**) was subjected to the reaction with three equivalents of ethyl cyanoformate (**2**) in a weakly acidic aqueous solution (pH 3.3, potassium phosphate/citrate buffer) of recombinant *Pa*HNL[9] at room temperature (Scheme 2). The course of the reaction was followed by GC after extraction with *tert*-butyl methyl ether and acetylation (Ac_2O/pyridine) of a sample of the reaction mixture. After 3 h mandelonitrile (**3**) was found to be the major product whereas only a small amount of 2-ethoxycarbonyloxy-2-phenyl acetonitrile (**4**) was detected (Table 1, entry 1). Both compounds were formed with excellent enantiopurity. No other products were found. As the reaction proceeded further, **4** was formed with consumption of **3**, and only traces of **1** were left after 23 h. At this stage the ee of **3** dropped slightly probably due to slow racemization of the free cyanohydrin **3**. The yield of **4** could be increased to 66% after the addition of a further 3 equiv. of **2**.

These results and especially the formation of mandelonitrile (**3**) indicated a two step procedure which prompted us to carry out NMR studies to obtain mechanistic insights.[10] Initially the stability of **2** in D_2O was examined at 22 °C. Under aqueous conditions **2** is decomposed into HCN, CO_2 and ethanol and its half-life is approximately 4.5 h. In order to confirm the assumption of a two-step procedure the enzymatic reaction was carried out in the NMR tube using the conditions described above. Figure 1 clearly indicates that mandelonitrile (**3**) is formed prior to the acylation step furnishing **4**.

As can be seen from Figure 2, enantiomerically enriched **3** is formed by asymmetric HCN addition to benzaldehyde (**1**) catalyzed by *Pa*HNL, as in the first step HCN is generated in the preceding hydrolysis of ethyl cyanoformate (**2**). In the second step **2** acts as an activated acyl donor which leads to the conversion of **3** into **4** as the final product regenerating HCN. In a comparative experiment, the reaction of racemic **3** with **2** in an aqueous acidic solution (pH 3.3) of recombinant *Pa*HNL was examined. Apparently, since only racemic **4** was formed, only the first step in the procedure described above, namely the formation of **3**, exhibits stereoselectivity.

In order to enhance the lifetime of **2** during the reaction, further experiments were performed in organic solvents. For this purpose, the biocatalyst was immobilized on Celite® by lyophilization of a heterogeneous mixture of carrier and the buffered enzyme solution (50 mM phosphate buffer, pH 7.0). During our studies with *Pa*HNL immobilized on Celite® we found that toluene and dichloromethane are excellent solvents for carrying out cyanohydrin syntheses with this particular biocatalyst. Furthermore, studies with immobilized hydroxynitrile lyases revealed that it is necessary to have certain amounts of buffer on the carrier to achieve maximum enzyme activity.[11] For this reason either 1 or 2% (v/v) of buffer solution (50 mM phosphate buffer, pH 7.0) was added to the suspension of the immobilized enzyme in the respective solvent. All

Table 1. Yield and ee of **3** and **4** in aqueous or organic media according to the reaction in Scheme 2

Entry	Solvent	pH	(*R*)-*Pa*HNL (U/mmol)	HCN (equiv.)	**2** (equiv.)	*t* (h)	**3**[a] Yield% (%ee)	**4** Yield% (%ee)
1	H_2O	3.3	750	0	3	3	77 (>99)	8 (>99)
						23	65 (94)	33 (>99)
						44.5[b]	34 (89)	66 (95)
2	Toluene 1%	7.0	300	0	2.5	24	11 (94)	3 (94)
3	Toluene 1%	7.0	300	1	2.5	24	49 (95)	37 (98)
4	Toluene 2%	7.0	300	0	2.5	24	17 (69)	4 (58)
5	Toluene 2%	7.0	300	1	2.5	24	47 (84)	47 (99)
6	CH_2Cl_2 1%	7.0	300	0	2.5	24	5 (95)	6 (98)
7	CH_2Cl_2 1%	7.0	300	1	2.5	24	52 (97)	31 (97)
8	CH_2Cl_2 2%	7.0	300	0	2.5	24	6 (96)	4 (98)
9	CH_2Cl_2 2%	7.0	300	1	2.5	24	39 (97)	43 (98)

[a] Determined by GC after acetylation
[b] After 26 h a further 3 equiv. of **2** were added (double column).

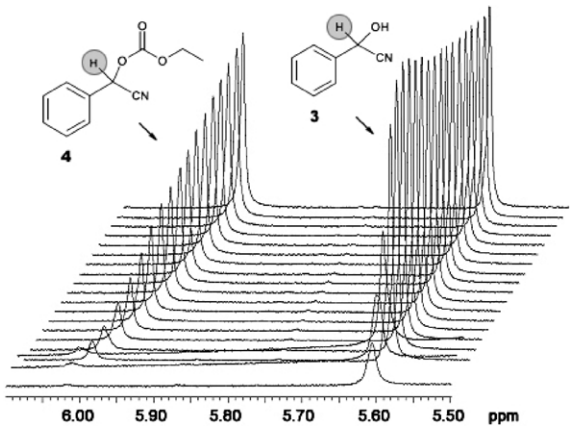

Figure 1. NMR-monitoring of the *Pa*HNL catalyzed formation of **3** and **4**. Spectra were recorded every 15 min.

transformations were performed at room temperature. The progress of the reaction was analyzed after 24 h by GC after acetylation (Ac_2O/pyridine, Table 1).

When the reaction mixture contained 1% (v/v) of buffer in toluene only 11% of **3** and 3% of **4** could be detected (Table 1, entry 2). As a plausible reason for this low product yield, the lack of free HCN owing to the enhanced stability of **2** in toluene compared to water was assumed. As a consequence the reaction was triggered by the addition of 1 equiv. of HCN at the start, yielding 49% of **3** and 37% of **4** (entry 3). Both products were formed with satisfying optical purity. This is a promising result because less than half of the catalyst was applied compared to the aqueous procedure even though enzymes are generally less active when used as immobilized formulations in organic solvents. On the other hand the solubility and stability of the reagents is increased. Adding 2% (v/v) of buffer to the system did not change the yields significantly but the optical purity of the products dropped probably due to the enhanced racemization rate of **3** (entries 4 and 5). When the reaction was carried out in dichloromethane under the same conditions as described above for toluene, the yields of **4** were slightly lower. Even when the system contained 2% (v/v) of buffer the ee values were good to excellent (entries 6–9).

In order to deepen the mechanistic understanding, the following investigations concerning the acylation step were undertaken. Reacting racemic **3** with **2** in either dry toluene or after the addition of 1% (v/v) of buffer furnished only traces of **4** after 24 h. In contrast, when the same reaction was carried out in toluene (2% buffer) in the presence of the immobilized enzyme 59% of **4** was formed after 25 h. Using Celite® as the sole additive (the enzyme solution was displaced by water prior to lyophilization) 54% of **4** was detected after 25 h. This indicates a catalytic activity of the carrier whereas the slightly increased yield of **4** in the presence of the enzyme does not seem to be significant.

In conclusion, a chemoenzymatic method for the synthesis of protected cyanohydrins in a one-pot manner was developed. This work is aiming towards an improvement of biocatalytic cyanohydrin reactions with unfavorable equilibrium conditions, whereas it is well known that benzaldehyde, our model substrate, is converted to mandelonitrile in almost quantitative yield in biphasic media (see Ref. 3a).

3. Experimental

3.1. Materials and methods

All solvents and materials were commercially available and were appropriately purified, if necessary. ^1H and ^{13}C NMR spectra were recorded either on a Varian INOVA 500 (^1H 499.82 MHz, ^{13}C 125.69 MHz) or a Varian GEMINI 200 (^1H 199.92 MHz, ^{13}C 50.25 MHz). GC analyses were performed using a Hewlett Packard 6890 instrument and either a Chirasil-DEX CB or a HP-5 column. The CHN analysis was carried out with a Perkin Elmer 2400 CHN Elemental Analyzer at the Institute of Physical Chemistry, University of Vienna.

3.2. HCN formation—caution

All reaction equipment in which cyanides were used or produced were placed in a well ventilated hood. The

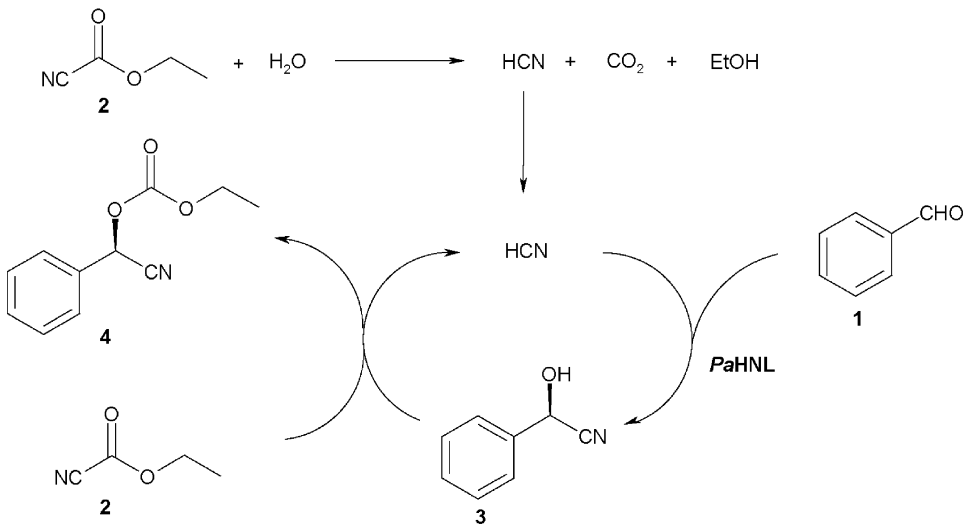

Figure 2. Tentative mechanism of the *Pa*HNL catalyzed formation of **3** and **4**.

required amount of HCN was freshly formed by dropping a saturated NaCN solution into aqueous sulfuric acid (60%) at 80 °C and trapping HCN at −12 °C in a cooling trap. For continuous warning, an electrochemical sensor for HCN detection was used. Waste solutions containing cyanides were treated with aqueous sodium hypochlorite (10%). Subsequently the pH was adjusted to 7.0 with aqueous sulfuric acid.

3.2.1. Racemic 2-acetoxy-2-phenyl acetonitrile (acetylated racemic 3). To a stirred solution of **3** (2.00 g, 15.02 mmol) and pyridine (2.90 mL, 36.04 mmol) in 15 mL dry CH_2Cl_2, acetyl chloride (1.30 mL, 18.30 mmol) was added dropwise at 0 °C under an argon atmosphere. Stirring was continued for 1 h. Subsequently, the solution was poured onto ice water. The organic phase was extracted with $NaHSO_4$ 5% and sat. $NaHCO_3$, dried (Na_2SO_4) and concentrated under reduced pressure. Purification using column chromatography (cyclohexane/EtOAc=50:1–20:1) yielded acetylated **3** (1.81 g, 69%) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ (ppm)=2.17 (s, 3H), 6.42 (s, 1H), 7.46–7.51 (m, 5H); ^{13}C NMR ($CDCl_3$): δ (ppm)=20.71, 63.09, 116.37, 128.12, 129.50, 130.66, 131.99, 169.18. The NMR data were consistent with those previously reported.[12]

3.2.2. Racemic 2-ethoxycarbonyloxy-2-phenyl acetonitrile (racemic 4). To a stirred solution of **1** (1.90 mL, 18.69 mmol) in 10 mL dry THF under an argon atmosphere were added **2** (3.70 mL, 37.45 mmol) and DABCO (210 mg, 1.87 mmol) at 0 °C. The ice bath was removed and after 1.5 h the reaction was diluted with 30 mL of ether. The single phase was washed with sat. $NaHCO_3$ (2×20 mL). The aqueous phase was re-extracted with ether (2×20 mL). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Purification using column chromatography (cyclohexane/EtOAc=15:1) yielded **4** (3.22 g, 84%) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ (ppm)=1.34 (t, J=7 Hz, 3H), 4.29 (q, J=7 Hz, 2H), 6.27 (s, 1H), 7.44–7.57 (m, 5H); ^{13}C NMR ($CDCl_3$): δ (ppm)= 14.34, 65.86, 66.60, 116.02, 128.12, 129.51, 130.87, 131.49, 153.67. Anal. calcd for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83; Found: C, 64.60; H, 5.41; N, 6.74.

3.2.3. NMR measurements. For stability studies 40 μL of **2** were dissolved in 0.5 mL of D_2O containing 1% (v/v) of DMSO-d_6. Spectra were recorded every 15 min over a period of 16.5 h at 22 °C. For the biocatalytic reaction, to 0.6 mL of a solution of recombinant *Pa*HNL (80 U/mL after dilution with D_2O and pH adjustment to 3.3 with an aqueous citric acid solution) containing 1% (v/v) of DMSO-d_6 were added 30 μL of **1** (0.30 mmol) and 90 μL of **2** (0.91 mmol). Spectra were recorded every 15 min over a period of 5 h at 22 °C. The residual water signal was suppressed by the transmitter presaturation method.

3.2.4. Reaction in water. 2.5 mL of a solution of recombinant *Pa*HNL (300 U/mL) were adjusted to pH 3.3 with an aqueous solution of citric acid and diluted with 2.5 mL of 50 mM potassium phosphate/citrate buffer (pH 3.3). **1** (106 mg, 1 mmol) and **2** (297 μL, 3 mmol) were added and the mixture was stirred at room temperature. The course of the reaction was monitored by GC after acetylation.

3.2.5. Reaction in organic solvents. 0.5 g of Celite® were swollen in 4 mL of 50 mM phosphate buffer (pH 7.0) for 2 h. After filtration, 4.2 mL of a solution of recombinant *Pa*HNL (175 U/mL) and 2 mL of phosphate buffer (pH 7.0) were added and the mixture was stirred for 10 min at room temperature. After freezing in liquid nitrogen the preparation was lyophilized. To a suspension of the dried enzyme formulation and **1** (250 μL, 2.46 mmol) in 6 mL of dry solvent (either toluene or CH_2Cl_2) were added either 60 or 120 μL of phosphate buffer [pH 7.0, 1 or 2% (v/v)] and **2** (610 μL, 6.17 mmol) and the mixture was stirred at room temperature. The course of the reaction was followed by GC after acetylation. In the case of HCN-triggering, HCN (95 μL, 2.46 mmol) was added prior to the addition of **2**.

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Remote control of enzyme selectivity: the case of stevioside and steviolbioside

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Abstract—The remote control of lipase PS site- and regioselectivity by substrate modification has been observed in the acetylation of stevioside (**1**) and steviolbioside (**2**): deglucosylation at position C-19 changed the acylation site of the sophorose moiety linked at C-13. In fact, while esterification of **1** gave mainly the corresponding 6″-*O*-acetylated derivative, acylation of **2** gave exclusively the 6′-*O*-monoester. A possible rationale has been suggested, based on the conformational behavior of the substrates in different simulated solvents.

1. Introduction

Selectivity is surely one of the key factors, if not 'the' key factor, of the growing popularity of biocatalysis among organic chemists, as testified by the hundreds of reports describing striking enzymatic performances.[1] Among them, probably the most impressive ones are the few dealing with the ability of enzymes to recognize remotely located stereocenters, as it is presently impossible to match these transformations with classical chemical catalysts. Examples of discrimination of a stereogenic center three or more bonds apart from the reaction center have been reported, describing the use of hydrolases (proteases or lipases)[2] or oxidoreductases.[2a,3] For instance, Hedenstrom and co-workers have recently disclosed the highly enantioselective *Candida rugosa*-catalyzed esterification of a series of methyldecanoic acid with 1-hexadecanol in cyclohexane: using 8-methyldecanoic acid (in which the stereogenic center is located eight bonds apart from the reactive carboxylate) as a substrate, they still measured a significant and unexpectedly high *E* value (25).[4] Remote control of enantioselectivity is the common feature of all these papers, a property that we have also exploited for the kinetic resolution of racemic *N*-Boc-piperidine-2-ethanol, a building block for the stereoselective syntheses of piperidine alkaloids.[5] On the contrary, to our knowledge, there are no reports on the remote control of enzymatic site- and regioselectivity, and this is the topic that we would like to disclose in this paper.

2. Results and discussion

Enzymatic modification of natural glycosides is one of our research interest since the late eighties,[6] as these complex and often labile molecules are ideal targets for mild and selective biotransformations. Specifically, in the past we have reported on the use of hydrolases and glycosyltransferases for the modification of flavonoid, terpene and alkaloid glycosides, and we are presently adding glycosidases to our repertoire.[7] As proposed years ago, the efficient preparation of random libraries of new derivatives (so-called 'combinatorial biocatalysis')[8] is a useful tool to improve the properties of these biologically active compounds.

Stevioside (**1**) is a sweet diterpenoid glycoside isolated from the South American plant *Stevia rebaudiana* Bertoni. The presence of three glucopyranosyl moieties makes this compound an interesting model for enzymatic transformations. Accordingly, few years ago, we submitted **1** as well as its congener steviolbioside (**2**)—easily prepared from **1** by alkaline hydrolysis—to the action of the β-1,4-galactosyltransferase from bovine colostrum (GalT).[9] Despite the presence in **1** of three possible galactosylation sites (HO-4′; HO-4″; HO-4‴), not only was this biotransformation stereo- and regio-selective, but also completely 'site-selective', producing the tetrasaccharide derivative **3** as the only product. Similarly, GalT-catalysis on **2** gave an almost quantitative conversion to the HO-4″-galactosylated compound **4**.

In order to expand the number of stevioside and steviolbioside derivatives in our hands, we investigated the regioselective acylation of these diterpenic glycosides. A

Keywords: Site selectivity; Regioselectivity; Lipase PS; Stevioside; Steviolbioside; Remote stereocenter.
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preliminary screening indicated that lipase PS was the best catalyst for this biotransformation. Accordingly, stevioside was dissolved in THF containing vinyl acetate, the enzyme was added and the suspension shaken at 45 °C for 3.5 h. An almost 70% conversion to a major product and to a minor less polar by-product was observed and both compounds were isolated by flash chromatography (51 and 4% isolated yields, respectively). At the beginning only the most abundant compound was examined and characterized as 6″-*O*-acetyl stevioside (**6**) by usual spectroscopic techniques.

The ^{1}H NMR in DMSO-d_6 (^{1}H- and ^{13}C NMR spectra of **1** and **2** were previously examined in pyridine[10] and methanol–pyridine[9]) revealed three anomeric protons at 5.24, 4.42 and 4.37 ppm, respectively. The low field signal was attributed to H-1‴, that is to the anomeric proton of the glucose moiety connected to the carboxyl function. Analysis of the extended spin connections (TOCSY) indicated that the acetate group was not located on this glucose because all proton and carbon resonances were in agreement with those of an unsubstituted saccharide. The anomeric proton at 4.42 ppm was attributed to the H-1′ of the sophorose disaccharide because of its vicinal relationship to H-2′, in turn directly linked to the low field substituted C-2′ at 82.91 ppm, and because of the long range interaction with the quaternary C-13. That the acetate group was located on the external glucose of the sophorose unit was easily deduced by the *J*-network (vicinal, long-range and spatial coupling) connecting the last anomeric proton at 4.37 ppm to the low field C-6″ methylene protons at 4.19 and 3.95 ppm via H-2″ at 3.03, H-3″ at 3.10, H-4″ at 3.19 and H-5″ at 3.30 ppm.

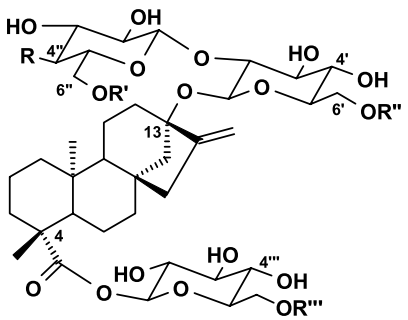
This result was coherent with the data obtained in the GalT-catalyzed reaction, as acylation took place on the external glucose of the sophorose moiety.

Enzymatic acylation of steviolbioside (**2**) was also very efficient: conversion was almost quantitative to a single product that was isolated and characterized. However, to our surprise, lipase PS directed its action to the inner glucose of the sophorose moiety of **2**, producing the monoacetate **7**. A similar result was obtained with the methyl ester **2a**, which was quantitatively converted into the monoacetate **7a**. In this case too the attribution was unequivocally supported by NMR data. The H-1′ at 4.53 ppm was connected to the low field H_a-6′ and H_b-6′ at 4.28 and 3.92 bearing the acetoxy group, whereas H-1″ at 4.36 was connected to an unsusbstitued oxymethylene at 3.59 and 3.48 ppm.

Therefore a significant effect on lipase PS site- and regioselectivity was observed by changing a remote portion of the molecule: deglucosylation at position C-19 changed the acylation site of the sophorose moiety linked at C-13.

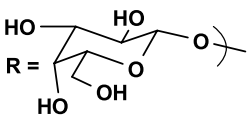
This unexpected result suggested us to examine the structure of the very minor product obtained in the acetylation of stevioside. We found that it contained two acetate groups and that one of these was on the CH_2-6‴ of the glucose attached to the carboxyl function (H-1‴ at 5.32 was connected to CH_2-6‴ at 4.23 and 4.09). In analogy with the previously described monoacetate **6**, we expected the additional acetate to be located on the external glucose of the sophorose moiety. However, in spite of our reluctance, a clear connection of the second low field methylene at 4.27 and 3.99 to H-1′ at 4.47 convinced us that the additional acyl moiety was located on CH_2-6′, and therefore to this diester the structure of 6′,6‴-di-*O*-acetyl stevioside **5** was assigned.

As the surprising structure of this very minor byproduct did not give any clue to explain the puzzling behavior of lipase PS, we decided to start a molecular modeling analysis of the conformation of the two substrates **1** and **2** in different simulation environments.[11] Our goal was to verify whether



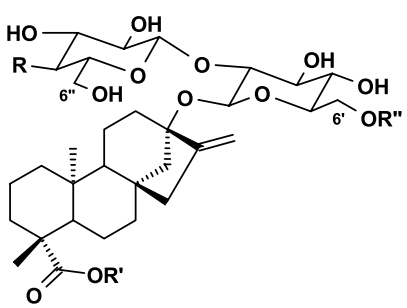
1 : R = OH ; R' = R" = R‴ = H

3 : R' = R" = R‴ = H ; R =



5 : R = OH ; R' = H ; R" = R‴ = Ac

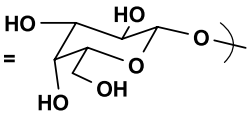
6 : R = OH ; R' = Ac ; R" = R‴ = H



2 : R = OH ; R' = R" = H

2a : R = OH ; R' = Me ; R" = H

4 : R' = R" = H ; R =



7 : R = OH ; R' = H ; R" = Ac

7a : R = OH ; R' = Me ; R" = Ac

the deglucosylation at position C-19 of **1** originated such a significant conformational variation in **2** to justify the different enzymatic selectivity experimentally observed. The solvent in these models is treated as an analytical continuum starting near the van der Waals surface of the solute. The equilibrated continuum solvent is characterized by a dielectric constant typical for the particular solvent, i.e. 78 D for water and 4.8 D for chloroform. The increased accuracy in modeling the solvent effects yields parallel increased accuracy in the conformational searches. Conformational searches on compounds **1** and **2** were performed using the Monte Carlo/Energy Minimization (MC/EM) approach implemented in MacroModel: the dihedrals (one to five at a time) corresponding to simple bonds are changed by random quantities, the new conformations obtained are scored based on the molecular mechanics energy function, and low energy conformations are stored as probable structures for the compound in solution. In this work the SUMM variant of the MC/EM algorithm was used.[11] For each compound in each different solvent, 30,000 steps of the MC/EM procedure were run. All the minima found within 50 kJ/mol of the global minimum were fully reminimized to allow a more accurate determination of the relative energies.

The global minimum structures of both **1** and **2** in chloroform (a model for a generic hydrophobic organic solvent) and in vacuo were substantially indistinguishable. Steviolbioside was found to adopt an extended conformation in water (Fig. 1, top) and a folded one in the organic solvent (Fig. 1, bottom). In water, the extended conformation allows the molecule to expose the hydroxyl groups of the sugar moieties and the COOH group to the bulk solvent, giving rise to favorable hydrogen bond type solvating interactions. On the other hand, in the organic solvent chloroform, the molecule tends to adopt a folded conformation, due to the H-bond formation between the carboxylate group at C-19 and the OH moieties on C-6″ and C-4″. This conformation causes the hydroxyl moiety on C-6′ to point out towards the solvent, and the lower steric hindrance of this group might be the reason why it suffers selective acylation when steviolbioside enters into the active site of lipase PS.

On the other hand stevioside showed a similar extended conformation in water (Fig. 2, top) and in chloroform (Fig. 2, bottom). In water the extended conformation is favored because of the high number of hydrogen bonding interactions with the bulk solvent. In chloroform, the presence of the glucopyranosyl ester moiety at C-19 prevents the formation of the stabilizing hydrogen bonds and forces the molecule to adopt again a completely extended conformation in order to minimize unfavorable steric clashes. Therefore the hydroxyl group on C-6″ is forced to point outside the molecule, thus minimizing the steric hindrance at this center and directing the acylation mainly on this group.

In conclusion, these calculations correlate well with the experimental results and suggest that the substrate conformation might be the main factor influencing and determining the recognition by lipase PS: the less sterically hindered hydroxyl group enters the active site of the catalyst more favorably, thus directing the site- and regioselective acetylation in organic solvent alternatively to the 6′-OH in **2** and to 6″-OH in **1**. Obviously, contrary to what has been proposed in the very few literature examples on simulated sugars docking inside a protease active site,[12] we were focusing on the substrate that has to be acylated by the

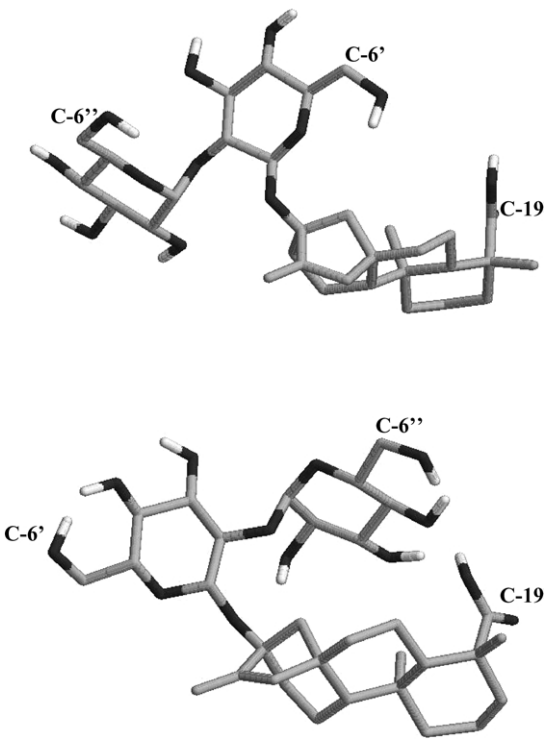


Figure 1. Conformational energy minima of steviolbioside (**2**) in water (top) and $CHCl_3$ (bottom).

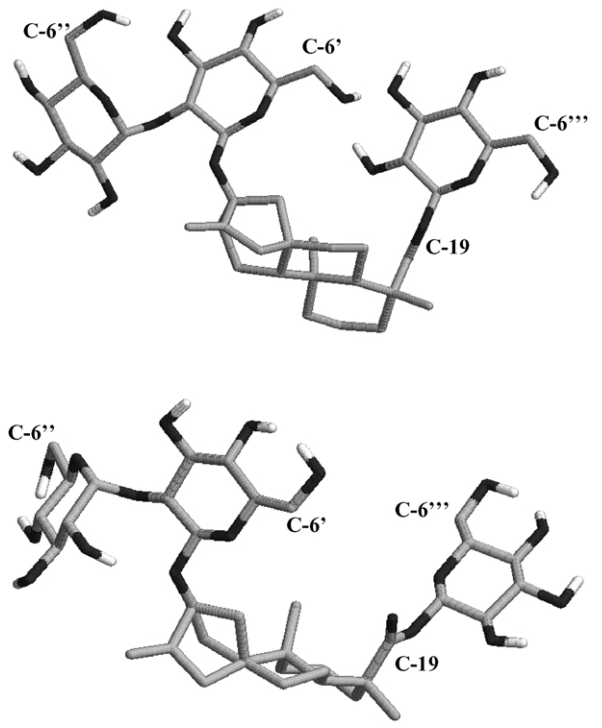


Figure 2. Conformational energy minima of stevioside (**1**) in water (top) and $CHCl_3$ (bottom).

biocatalyst and therefore the enzyme environment has not been taken into consideration. However, in the absence of a high-level ab initio calculation of the whole reaction pathway describing all the intermediates at an electronic level, useful and sound rationales to specific experimental outcomes can be more easily extrapolated using classical mechanics-based approaches.

Needless to say, the observed remote control of lipase PS selectivity by substrate modification makes stevioside and steviolbioside a peculiar case among the reported examples of biocatalyzed-site- and regioselective elaboration of natural glycosides.

3. Experimental

3.1. Materials and methods

Stevioside (**1**) and steviolbioside (**2**) were a gift from Indena, Milano, Italy. Lipase PS was adorbed on celite following a standard protocol.[13] Enzymatic transesterifications were followed by TLC on precoated silica gel 60 F_{254} plates (Merck); compounds were detected with the Komarowsky reagent.

^{1}H and ^{13}C NMR spectra at 300 MHz and 75.2 MHz were recorder in DMSO-d_6+D_2O at 80 °C. NMR spectra at 600 MHz (^{1}H homonuclear DQF-COSY, TOCSY, HMQC, HMBC, ^{1}H–^{13}C COSY and 2D-NOESY) and at 150 MHz (^{13}C) were run in DMSO-d_6 at 35 °C using standard pulse programs. FABMS spectra were recorded on a VG 70-70 EQ-HF instrument in the negative mode using Xe as a gas and 3-nitrobenzylalcohol as the matrix.

3.1.1. Stevioside (1, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid, β-D-glucopyranosyl ester]. Amorphous solid, mp 235–238 °C; $[\alpha]_D$=−37.5 (*c*=0.2, MeOH). R_f 0.25 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (300 MHz): 5.09 and 4.77 (2H, m, CH_2-17); 1.19 (3H, s, CH_3-18); 0.92 (3H, s, CH_3-20); 4.53 (1H, d, $J_{1',2'}$=7.7 Hz, H-1′); 3.30 (1H, t, $J_{2',3'}$=7.7 Hz, H-2′); 4.47 (1H, d, $J_{1',2'}$=7.7 Hz, H-1″); 3.09 (1H, t, $J_{2'',3''}$=7.7 Hz, H-2″); 5.37 (1H, d, $J_{1''',2'''}$=7.7 Hz, H-1‴); 3.21 (1H, m, H-2‴); 3.70–3.60 (3H, m, H_a-6′, H_a-6″, H_a-6‴); 3.55–3.45 (3H, m, H_b-6′, H_b-6″, H_b-6‴). ^{13}C NMR (75.2 MHz): 39.95 (C-1); 18.75 (C-2); 37.51 (C-3); 43.34 (C-4); 56.60 (C-5); 21.20 (C-6); 41.10 (C-7); 42.03 (C-8); 53.30 (C-9); 39.13 (C-10); 20.02 (C-11); 35.79 (C-12); 84.80 (C-13); 43.59 (C-14); 47.05 (C-15); 153.51 (C-16); 103.96 (C-17); 28.60 (C-18); 175.62 (C-19); 15.23 (C-20); 96.40 (C-1′); 82.58 (C-2′); 76.40[a] (C-3′); 70.47[b] (C-4′); 76.23[a] (C-5′); 61.20[c] (C-6′); 104.53 (C-1″); 75.24 (C-2″); 76.23[a] (C-3″); 69.99[b] (C-4″); 76.92[a] (C-5″); 61.00[c] (C-6″); 94.20 (C-1‴); 72.66 (C-2‴); 77.65[a] (C-3‴); 69.80[b] (C-4‴); 77.01[a] (C-5‴); 60.75[c] (C-6‴). ([a,b,c] values with the same superscript might be exchanged). FABMS: 803 (7%, $[M-H]^-$), 641 (100%,), 479 (18%,), 317 (13%,).

3.1.2. Enzymatic acetylation of stevioside (1). Stevioside (**1**) (100 mg) was dissolved 4.75 ml of anhydrous THF, vinyl acetate (250 μl) and lipase PS on celite (200 mg) were added and the suspension was shaken at 45 °C. After 3,5 h an almost 70% conversion was observed to a main product accompanied by traces of another less polar product. The suspension was filtered, the solvent evaporated and the crude residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O, from 90:15:3 to 80:15:5) to give 4 mg of 6′,6‴-di-*O*-acetyl stevioside (**5**) and 51 mg of 6″-*O*-acetyl stevioside (**6**).

Compound 6′,6‴-di-*O*-acetyl stevioside (**5**). Vitreous solid, R_f 0.35 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (600 MHz): 5.01 and 4.73 (2H, m, CH_2-17); 1.07 (3H, s, CH_3-18); 1.00 (3H, s, CH_3-20); 4.47 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.26 (1H, t, $J_{2',3'}$=8.0 Hz, H-2′); 3.38 (1H, t, $J_{3',4'}$=8.0 Hz, H-3′); 3.07 (1H, t, $J_{4',5'}$=8.0 Hz, H-4′); 3.30 (1H, ddd, $J_{5',6'a}$=2.1 Hz, $J_{5',6'b}$=6.5 Hz, H-5′); 4.27 (1H, dd, ^2J=11.9 Hz, H-6′a); 3.99 (1H, dd, H-6′b); 4.36 (1H, d, $J_{1',2'}$=7.9 Hz, H-1″); 3.02 (1H, t, $J_{2'',3''}$=7.9 Hz, H-2″); 3.15–3.20 (2H, m, H-3″ and H-4″); 3.05 (1H, m, H-5″); 3.57 (1H, dd, ^2J=12.0 Hz, $J_{5'',6''a}$=2.0 Hz, H-6″a); 3.49 (1H, dd, $J_{5'',6''b}$=6.5 Hz, H-6″b); 5.32 (1H, d, $J_{1''',2'''}$=8.1 Hz, H-1‴); 3.14–3.18 (H-2‴ and H-4‴); 3.28 (1H, dd, $J_{3''',4'''}$=8.1 Hz, H-3‴); 3.46 (1H, dd, $J_{4''',5'''}$=8.2 Hz, $J_{5''',6'''a}$=2.0 Hz; $J_{5''',6'''b}$=7.0 Hz, H-5‴); 4.23 (1H, dd, ^2J=12.1 Hz, H-6‴a); 4.09 (1H, dd, H-6‴b). ^{13}C NMR (150 MHz): 153.50 (C-16); 104.00 (C-17); 28.07 (C-18); 176.18 (C-19); 15.20 (C-20); 96.1 (C-1′); 82.2 (C-2′); 76.13[a] (C-3′); 70.31[b] (C-4′); 73.4 (C-5′); 63.8 (C-6′); 104.6 (C-1″); 75.3 (C-2″); 76.33[a] (C-3″); 70.40[b] (C-4″); 76.71[a] (C-5″); 63.8 (C-6″); 94.30 (C-1‴); 72.70 (C-2‴); 77.5[a] (C-3‴); 69.80 (C-4″); 74.4 (C-5‴); 63.3 (C-6‴). ([a,b] values with the same superscript might be exchanged). FABMS: 887 (4%, $[M-H]^-$), 725 (100%), 683 (28%,), 521 (14%), 479 (8%), 317 (23%).

Compound 6″-*O*-acetyl stevioside (**6**). Amorphous solid, mp 228–231 °C; $[\alpha]_D$=−33.5 (*c*=0.1, MeOH); R_f 0.29 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (600 MHz): 0.74 (H_{ax}-1); 1.78 (H_{eq}-1); 1.37 (H_{ax}-2); 1.78 (H_{eq}-2); 0.97 (H_{ax}-3); 2.03 (H_{eq}-3); 1.02 (H_{ax}-5); 1.70 (H_{eq}-6); 1.83 (H_{ax}-6); 1.34–1.43 (CH_2-7); 0.92 (H_{ax}-9); 1.46–1.65 (CH_2-11); 1.40 (H_{ax}-12); 1.82 (H_{eq}-12); 2.12 and 1.37 (CH_2-14); 2.05–1.95 (CH_2-15); 4.68 and 4.98 (CH_2-17); 1.11 (CH_3-18); 0.84 (CH_3-20); 4.42 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.19 (1H, dd, $J_{2',3'}$=8.2 Hz, H-2′); 3.07 (1H, dd, $J_{3',4'}$=8.3 Hz, H-3′); 3.36–3.38 (H-4′ and H-5′); 3.41 (1H, dd, ^2J=11.9 Hz; $J_{5',6'a}$=6 Hz, H-6′a); 3.66 (1H, dd, $J_{5',6'b}$=1.5 Hz, H-6′b); 4.37 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″); 3.03 (1H bdd, $J_{2'',3''}$=8.1 Hz, H-2″); 3.10 (1H, dd, $J_{3''4''}$=8.2 Hz, H-3″); 3.19 (1H, dd, $J_{4'',5''}$=8.2 Hz, H-4″); 3.30 (m, $J_{5'',6''a}$=1.5 Hz, $J_{5'',6''b}$=6.0 Hz, H-5″); 4.19 (1H, dd, ^2J=11.5 Hz, H-6″a); 3.95 (1H, dd, H-6″b); 5.24 (1H, d, $J_{1''',2'''}$=8.0 Hz, H-1‴); 3.12 (1H, dd, $J_{2''',3'''}$=8.1 Hz, H-2‴); 3.24 (1H, dd, $J_{3''',4'''}$=8.2 Hz, H-3‴); 3.14 (1H, dd, $J_{4''',5'''}$=8.2 Hz, H-4‴); 3.18 (1H, ddd, $J_{5''',6'''a}$=1.5 Hz; $J_{5''',6'''b}$=6.0 Hz, H-5‴); 3.61 (1H, dd, ^2J=12.0 Hz, H-6‴a); 3.45 (1H, dd, H-6‴b). ^{13}C NMR (150 MHz): 40.05 (C-1); 18.85 (C-2); 37.30 (C-3); 43.49 (C-4); 56.13 (C-5); 21.15 (C-6); 40.91 (C-7); 42.08 (C-8); 53.15 (C-9); 39.65 (C-10); 19.95 (C-11); 35.85 (C-12); 84.78 (C-13); 43.50 (C-14); 47.08 (C-15); 153.60 (C-16); 103.85 (C-17); 28.02 (C-18); 175.58 (C-19); 15.10 (C-20); 96.31 (C-1′); 82.91 (C-2′); 76.60[a] (C-3′); 70.51[b] (C-4′); 76.70[a] (C-5′); 61.10[c] (C-6′); 104.61 (C-1″); 75.25 (C-2″); 76.10[a] (C-3″); 69.95[b] (C-4″); 74.18 (C-5″); 63.85 (C-6″);

94.10 (C-1‴); 72.98 (C-2‴); 77.48[a] (C-3‴); 69.80[b] (C-4″); 77.15[a] (C-5‴); 60.75[c] (C-6‴); 20.55 (OCO*C*H$_3$); 169.95 (O*C*OCH$_3$) ([a,b,c]values with the same superscript might be exchanged). FABMS: 845 (8%, [M−H]$^-$), 683 (100%,), 641 (5%), 479 (18%), 317 (21%).

3.1.3. Steviolbioside (2, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid). Amorphous solid, mp 193–198 °C; $[\alpha]_D$=−32.5 (*c*=0.2, MeOH); R_f 0.22 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz): 5.13 and 4.78 (2H, m, CH$_2$-17); 1.15 (3H, s, CH$_3$-18); 0.92 (3H, s, CH$_3$-20); 4.54 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 4.47 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″). ^{13}C NMR (75.2 MHz): 39.95 (C-1); 18.91 (C-2); 37.70 (C-3); 42.89 (C-4); 56.16 (C-5); 21.68 (C-6); 41.04 (C-7); 41.85 (C-8); 53.42 (C-9); 39.13 (C-10); 19.95 (C-11); 36.36 (C-12); 85.08 (C-13); 43.75 (C-14); 47.28 (C-15); 153.11 (C-16); 104.23 (C-17); 28.69 (C-18); 178.47 (C-19); 15.31 (C-20); 96.23 (C-1′); 82.58 (C-2′); 76.42[a] (C-3′); 70.26[b] (C-4′); 76.42[a] (C-5′); 61.18[c] (C-6′); 104.48 (C-1″); 75.25 (C-2″); 76.42[a] (C-3″); 70.17[b] (C-4″); 76.87[a] (C-5″); 61.18[c] (C-6″). ([a,b,c]values with the same superscript might be exchanged). FABMS: 641 (8%, [M−H]$^-$), 479 (100%), 317 (28%).

3.1.4. Enzymatic acetylation of steviolbioside. Steviolbioside (100 mg) was dissolved in 4.75 ml of anhydrous THF, vinyl acetate (250 μl) and lipase PS on celite (250 mg) were added and the suspension was shaken at 45 °C. After 14 h an almost 90% conversion to a product was observed. After filtration and solvent evaporation the new compound was isolated by flash chromatography (eluent AcOEt–MeOH–H$_2$O, 80:15:5) to afford 75 mg of 6′-*O*-acetyl steviolbioside (**7**).

Compound 6′-*O*-acetyl steviolbioside (**7**). Amorphous solid, mp 180–183 °C; $[\alpha]_D$=−29.5 (*c*=0.1, MeOH); R_f 0.37 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (600 MHz): 0.78 (H$_{ax}$-1); 1.79 (H$_{eq}$-1); 1.37 (H$_{ax}$-2); 1.75–1.80 (H$_{eq}$-2); 0.92 (H$_{ax}$–C3); 2.02 (H$_{eq}$-3); 1.00 (H$_{ax}$-5); 1.70–1.75 (CH$_2$-6); 1.39–1.49 (CH$_2$-7); 0.92 (H$_{ax}$-9); 1.49–1.70 (CH$_2$-11); 1.48 (H$_{ax}$-12); 1.88 (H$_{eq}$-12); 1.32 (H$_{ax}$-14); 2.06 (H$_{eq}$-14); 1.98–2.04 (CH$_2$-15); 4.74 and 5.03 (CH$_2$-17); 1.10 (CH$_3$-18); 0.88 (CH$_3$-20); 4.53 (1H, d, $J_{1',2'}$=8.1 Hz, H-1′); 3.24 (1H, t, $J_{2',3'}$=8.1 Hz, H-2′); 3.41 (1H, t, $J_{3',4'}$=8.1 Hz, H-3′); 3.06 (H-4′); 3.34 (H-5′); 4.28 (1H, dd, ^2J=11.9 Hz, $J_{5',6'a}$=1.5 Hz, H-6′a); 3.92 (1H, dd, $J_{5',6'b}$=8.2 Hz, H-6′b); 4.36 (1H, d, $J_{1'',2''}$=8.1 Hz, H-1″); 2.99 (1H, t, $J_{2'',3''}$=8.1 Hz, H-2″); 3.15 (1H, t, $J_{3'',4''}$=8.1 Hz, H-2″); 3.12 (1H, t, $J_{4'',5''}$=8.1 Hz, H-4″); 3.06 (1H, m, H-5″); 3.59 (1H, dd, ^2J=11.5 Hz, $J_{5'',6''a}$=2.0 Hz, H-6″a); 3.48 (1H, dd, $J_{5'',6''b}$=4.0 Hz, H-6″b); 2.01 (C*H*$_3$COO). ^{13}C NMR (150 MHz): 40.27 (C-1); 18.91 (C-2); 37.50 (C-3); 43.03 (C-4); 55.89 (C-5); 21.59 (C-6); 40.87 (C-7); 41.53 (C-8); 53.03 (C-9); 39.70 (C-10); 19.92 (C-11); 36.49 (C-12); 85.98 (C-13); 43.37 (C-14); 46.81 (C-15); 153.02 (C-16); 104.12 (C-17); 28.69 (C-18); 178.46 (C-19); 15.40 (C-20); 96.04 (C-1′); 82.54 (C-2′); 76.10[a] (C-3′); 70.45[b] (C-4′); 73.44[a] (C-5′); 63.89[c] (C-6′); 104.55 (C-1″); 75.27 (C-2″); 76.22[a] (C-3″); 70.20[b] (C-4″); 76.91[a] (C-5″); 61.14[c] (C-6″) ([a,b,c]values with the same superscript might be exchanged). FABMS: 683 (7%, [M−H]$^-$), 641 (5%), 479 (100%), 317 (14%).

3.1.5. Steviolbioside methylester (2a, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid methyl ester). Prepared by methylation of steviolbioside with trimethysilyl- diazomethane in MeOH/THF 1:1. Amorphous solid, mp 199–202 °C; $[\alpha]_D$=−30.5 (*c*=0.15, MeOH); R_f 0.32 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz) 5.15 and 4.78 (2H, m, CH$_2$-17); 1.12 (3H, s, CH$_3$-18); 0.78 (3H, s, CH$_3$-20). ^{13}C NMR (75.2 MHz): 39.93 (C-1); 18.51 (C-2); 37.30 (C-3); 42.95 (C-4); 55.74 (C-5); 21.42 (C-6); 40.13 (C-7); 41.27 (C-8); 52.92 (C-9); 39.93 (C-10); 19.67 (C-11); 36.34 (C-12); 85.05 (C-13); 43.57 (C-14); 46.83 (C-15); 152.47 (C-16); 104.06 (C-17); 27.96 (C-18); 176.96 (C-19); 14.78 (C-20); 95.78 (C-1′); 82.05 (C-2′); 76.30[a] (C-3′); 69.98 (C-4′); 74.96[a] (C-5′); 60.79 (C-6′); 104.06 (C-1″); 75.92[a] (C-2″); 76.30[a] (C-3″); 69.98 (C-4″); 76.65[a] (C-5″); 60.79 (C-6″); 50.91 (COO*Me*) ([a] values with the same superscript might be exchanged). FABMS: 655 (13%, [M−H]$^-$), 493 (100%), 331 (23%).

3.1.6. Enzymatic acetylation of steviolbioside methyl ester (7). Steviolbioside methyl ester (**7**) (50 mg) was dissolved in 2.5 ml of anhydrous THF, vinyl acetate (80 μl) and lipase PS on celite (300 mg) were added and the suspension was shaken at 45 °C. After 11 h an almost complete conversion was observed to a single product, which, after filtration and solvent evaporation, was isolated by flash chromatography (eluent AcOEt–MeOH–H$_2$O, 80:15:5) to afford 38 mg of 6′-*O*-acetyl steviolbioside methylester (**7a**).

Compound 6′-*O*-acetyl steviolbioside methylester (**7a**). Amorphous solid, mp 185–190 °C; $[\alpha]_D$=−28.0 (*c*=0.1, MeOH); R_f 0.46 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz): 5.05 and 4.72 (CH$_2$-17); 1.10 (CH$_3$-18); 0.88 (CH$_3$-20); 4.53 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.24 (1H, dd, $J_{2',3'}$=8.1 Hz, H-2′); 3.47 (1H, dd, $J_{3',4'}$=8.2 Hz, H-3′); 3.15–3.20 (H-4′); 3.37 (1H, ddd, $J_{4',5'}$=8.2 Hz, $J_{5',6'a}$= 1.5 Hz, $J_{5',6'b}$=8.0 Hz, H-5′); 4.33 (1H, dd, ^2J=11.2 Hz, H-6′a); 4.03 (1H, dd, H-6′b); 4.48 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″); 3.00 (1H, dd, $J_{2'',3''}$=8.1 Hz, H-2″); 3.26 (1H, dd, $J_{3'',4''}$=8.2 Hz, H-3″); 3.15–3.20 (H-4″); 3.06 (1H, ddd, $J_{4'',5''}$=8.0 Hz, $J_{5'',6''a}$=4.0 Hz, $J_{5'',6''b}$=1.5 Hz, H-5″); 3.45 (1H, dd, ^2J=11.5 Hz, H-6″a); 3.68 (1H, dd, H-6″b); 2.01 (3H, s, C*H*$_3$COO); 3.62 (3H, s, COO C*H*$_3$)). ^{13}C NMR (75.2 MHz): 39.73 (C-1); 18.27 (C-2); 37.13 (C-3); 42.90 (C-4); 55.77 (C-5); 21.04 (C-6); 40.53 (C-7); 41.20 (C-8); 52.93 (C-9); 39.73 (C-10); 19.34 (C-11); 36.03 (C-12); 84.84 (C-13); 43.20 (C-14); 46.78 (C-15); 152.26 (C-16); 103.52 (C-17); 27.72 (C-18); 176.34 (C-19); 14.60 (C-20); 95.60 (C-1′); 82.65 (C-2′); 75.94[a] (C-3′); 70.07[b] (C-4′); 73.07 (C-5′); 63.36 (C-6′); 103.68 (C-1″); 74.61 (C-2″); 75.94[a] (C-3″); 70.29[b] (C-4″); 76.24[a] (C-5″); 61.12 (C-6″); 50.32 (COO*Me*); 19.93 (OCO*C*H$_3$); 169.49 (O*C*OCH$_3$) ([a,b]values with the same superscript might be exchanged). FABMS: 697 (7%, [M−H]$^-$), 655 (5%), 535 (100%), 493 (18%), 331 (13%).

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The substrate specificity of the heat-stable stereospecific amidase from *Klebsiella oxytoca*

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Abstract—The substrate specificity of the heat-stable stereospecific amidase from *Klebsiella oxytoca* was investigated. In addition to the original substrate, 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide, the amidase accepted 2-hydroxy-2-(trifluoromethyl)-butanamide and 3,3,3-trifluoro-2-amino-2-methylpropanamide as substrates. Compounds with larger side chains and compounds where the hydroxyl group was substituted with a methoxy group, or in which the CF_3 group was substituted by CCl_3, were not accepted. The biotransformation is a new synthetic route to (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid, and its related (*S*)-(−)-amide, and to (*R*)-(+)-2-hydroxy-2-(trifluoromethyl)-butanoic acid and its related (*S*)-(−)-amide.

1. Introduction

(*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid are intermediates for the synthesis of a number of potential pharmaceuticals, which include ATP sensitive potassium channel openers for the treatment of incontinence,[1] and inhibitors of pyruvate dehydrogenase kinase for the treatment of diabetes.[2] A novel heat-stable stereospecific amidase from *Klebsiella oxytoca* was selected, purified, characterised and cloned, and used to synthesise enantiomerically pure (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid, (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide.[3,4] We now report on the substrate specificity of the enzyme.

2. Results

The substrates listed in Table 1 were incubated with the heat-stable amidase from *K. oxytoca*. The enzyme used was partially purified by heat treatment of a cell-free extract from an *Escherichia coli* strain that contained the recombinant *K. oxytoca* gene.[3,4] The biotransformations were carried out at 40 °C in 100 mM potassium phosphate buffer, pH 8.0, with substrate (5.0 mg/mL) and enzyme extract (0.2 mg/mL). The reaction was followed by chiral GC analysis of the (*R*)- and (*S*)-amide substrates. The enzyme accepted substrates **2** and **7** (Table 1) and the chiral GC analyses showed that the reactions were enantiospecific. The initial rate with **2** as substrate was about a quarter of that with the original substrate **1**, and the time to complete the reaction was correspondingly longer. However, with substrate **7**, the initial rate was about 30 times faster than that with the original substrate **1**, and the time to complete the reaction was much faster (about 300 times).

To isolate the reaction products from substrates **2** and **7**, biotransformations with larger amounts of substrate were carried out (see Section 5). For substrate **2**, the biotransformation was carried out with whole cells of *E. coli* containing the recombinant *K. oxytoca* amidase, and for substrate **7**, the biotransformation was carried out with the amidase immobilised on the carrier Eupergit C, in the presence of only substrate and water. This experimental regime was to facilitate the isolation of the product (see Section 3). The reactions are shown in Scheme 1, and the detailed reaction results are shown in Table 2. The amide **8** was obtained with a yield of 34.4% and an ee value of >98%. The corresponding acid **9** was obtained with a yield of 39.1% and an ee value of 85.2%. The amide **10** was obtained with a yield of about 50% and an ee value of >99%, whereas the corresponding acid **11** was obtained with a yield of 42% and an ee value of 95.4%.

3. Discussion

There is considerable interest in the synthesis of chiral

Keywords: Biotransformation; Amidase; (*R*)-(+)-3,3,3-Trifluoro-2-amino-2-methylpropionic acid; (*S*)-(−)-3,3,3-Trifluoro-2-amino-2-methylpropanamide; (*R*)-(+)-2-Hydroxy-2-(trifluoromethyl)-butanoic acid; (*R*)-(+)-2-Hydroxy-2-(trifluoromethyl)-butanamide.
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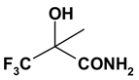
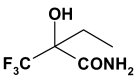
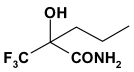
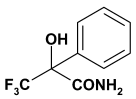
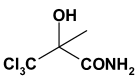
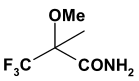
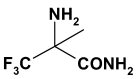
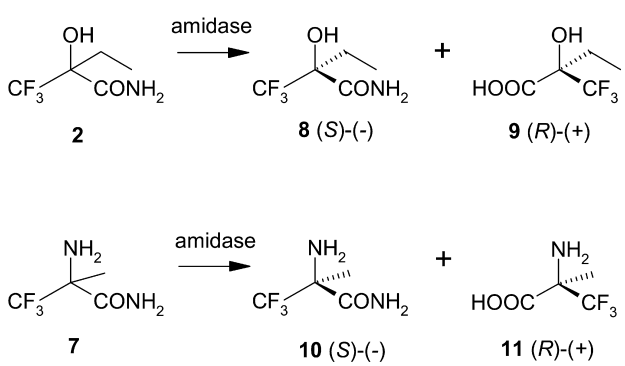
Table 1. Substrate specificity of the amidase

Substrate	Name	Structure	Initial rate (μmol/min/mg protein) (relative)	Time to complete conversion
1	3,3,3-Trifluoro-2-hydroxy-2-methylpropionamide		1.9 (1)	~5 h
2	2-Hydroxy-2-(trifluoromethyl)-butanamide		0.52 (0.27)	>80 h
3	2-Hydroxy-2-(trifluoromethyl)-pentanamide		0	
4	3,3,3-Trifluoro-2-hydroxy-2-phenyl-propionamide		0	
5	3,3,3-Trichloro-2-hydroxy-2-methylpropionamide		0	
6	3,3,3-Trifluoro-2-methoxy-2-methylpropionamide		0	
7	3,3,3-Trifluoro-2-amino-2-methylpropanamide		54.0 (28.41)	1.0 min

trifluoro-substituted acids as pharmaceutical intermediates[1,2] and fluorinated amino acids have been synthesised for use as mechanism based inhibitors of amino acid decarboxylases and transaminases.[5,6] Trifluoro-substituted acids have been synthesised chemically[1,7,8] and we[3,4,9] and another group[10] have used enzymatic methods for their synthesis. The fluorinated amino acid **11** (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid (3,3,3-trifluoro-2-methyl-alanine) has been synthesised chemically,[5] and also by enzymatic resolution.[6]

Various derivatives of the original enzyme substrate with substitutions at each of the three positions other than the amide group were synthesised, and used to test the substrate specificity of the amidase. Cell-free extracts were used for these tests, to ensure that the permeability of the *E. coli* cell wall to the various substrates would not influence the results. When larger groups substituted the methyl group on the substrate, the enzyme was active with the ethyl compound (at 27% of the rate shown with **1** as substrate), but there was no measurable activity with the propyl and phenyl derivatives. It follows that the larger the substrate the more difficulty it has to fit in the active site. Substituting the CF_3 group with CCl_3 also resulted in no activity, again probably due to the larger size of the CCl_3 group and after a time the pH 8 buffer solution slowly began to hydrolyse the CCl_3 group. The influence of the hydroxyl group on the substrate was investigated by two further derivatives. Masking the hydroxyl group with a methoxy group resulted in no activity. Substituting the hydroxy group with an amino group resulted in a 28-fold increase in the rate of amide hydrolysis over substrate **1**. The amidase active site probably requires a direct or indirect proton interaction to function, either in binding or in a more direct chemical role.

The amides **8** and **10** rotated polarised light to the left (−) and the acids **9** and **11** rotated to the right (+). The absolute configurations of **9** and **11** have been reported as (*R*)-(+).[1,5,10]



2 **8** (*S*)-(-) **9** (*R*)-(+)

7 **10** (*S*)-(-) **11** (*R*)-(+)

Scheme 1. Amidase catalysed resolutions of **2** and **7**.

Table 2. Amidase catalysed resolutions of **2** and **7**

#	Yield[a] (%)	Optical rotation	^{1}H NMR (DMSO)	^{13}C NMR (DMSO)	Elem. anal. or LC–MS	Mp (°C)	ee (%)
8	34.4	$[\alpha]_D=-13.2$ $c=3.75$ (MeOH)	7.60 (br s, 1H, $CONH_2$), 7.45 (br s, 1H, $CONH_2$), 6.46 (s, 1H, OH), 2.00 (m, 1H, CH_2), 1.70 (m, 1H, CH_2), 0.87 (t, 3H, J=6.7, CH_3)	169.3 (s, $CONH_2$), 124.9 (q, J_{CF}=287 Hz, CF_3), 77.2 (q, J_{CCF}=26.2, quaternary), 24.7 (t, CH_2), 6.7 (q, CH_3)	Calcd (found) for $C_5H_8F_3NO_2$ C 35.1 (35.4), H 4.7 (4.5), N 8.2 (8.1)	57–61	>98
9	39.1	$[\alpha]_D=+9.75$ c=3.31 (MeOH)[b]	1.92 (m, 1H, CH_2), 1.73 (m, 1H, CH_2), 0.88 (t, 3H, J=7.0 Hz, CH_3)	169.7 (s, COOH), 124.6 (q, J_{CF}=287 Hz, CF_3), 77.4 (q, J_{CCF}=27 Hz, quaternary), 25.6 (t, CH_2), 7.0 (q, CH_3)	Calcd (found) for $C_5H_7F_3O_3$ C 34.9 (35.2), H 4.1 (4.1)	107–111	85.2
10	53[c]	$Na_D=-5.4$ $c=0.6$ (water)	7.50 (s, br, 1H, $CONH_2$), 7.44 (s, br, 1H, $CONH_2$), 2.46 (s, br, 2H, NH_2), 1.35 (s, 3H, CH_3)	171.3 (s, $CONH_2$), 126.3 (sq, J_{CF}=285 Hz, CF_3), 60.2 (sq, quaternary C, J_{CCF}=26 Hz), 20.1 (qq, J_{CCCF}=2 Hz, CH_3)	Mass=156 Mass found=m/z 157 ($[M+1]^+$)	98–101[d]	>99[e]
11	42.0	$Na_D=+8.4$ c=0.8 (water)	5.60 (br, COOH, NH_2), 1.35 (s, 3H, CH_3)	170.4 (s, COOH), 126.9 (sq, J_{CCF}=284 Hz, CF_3), 60.1 (sq, quaternary C, J_{CCF}=25 Hz), 21.0 (q, CH_3)	Mass=157 Mass found=m/z 158 ($[M+1]^+$)		95.4[e]

[a] The theoretical maximum yield is 50%.
[b] Literature[10] (60% ee) $[\alpha]_D=+7.3°$ (c=5.33, MeOH).
[c] A yield of 53% is not theoretically possible. The higher figure is probably due to experimental error (trace moisture in the amino acid).
[d] This value was obtained from a separate larger biotransformation containing cell extracts. The sample passed elemental analysis, but the GC shows a trace impurity that may be (*S*)-**1**. $[\alpha]_D$ (MeOH, c=1.01) of this sample was −13.2 (23 °C). Anal. Calcd for $C_4H_7F_3N_2O$: C 30.8, H 4.5, N 17.9. Found C 31.1, H 4.7, N 17.7.
[e] Biotransformation with cell-free extract.

It follows, therefore, that the acids **9** and **11** that we isolated have the (*R*) configuration, and the amides **8** and **10** the (*S*) configuration.

Isolation of the product **11** from the reaction mixture was initially a problem. Although **10** could be easily extracted into ethyl acetate under basic conditions, **11** could not be isolated due to the presence of many buffer salts, free proteins and other cellular components. Extraction of amino acids such as **11** also requires precise knowledge of their iso-electric point in the mixture, and this point could not be determined. These problems were solved by using a reaction that contained only the substrate **7**, immobilised enzyme and water. The enzyme could easily be removed by filtration from the reaction after it was complete, **10** was extracted into ethyl acetate and **11** remained essentially alone in the water to be collected by evaporation of the water.

4. Conclusion

The amidase shows a marked preference for hydrolysis of only the (*R*) configured amide group, but it will accept only a limited range of substrates. However, the substrates that it did accept resulted in products that were unknown when this work was carried out (**8**, **9** and **10**) or have considerable synthetic interest (**11**). The biotransformation with the amidase therefore serves as a new synthetic route to these compounds.[11] Since this work was carried out, **9** has also been synthesised by enzymatic ester resolution,[10] and we were able to assign the configuration of the **9** that we synthesised based on the results in that paper.

5. Experimental

5.1. Analytical methods—general

The methods for NMR (Varian UNITY-400), chiral GC of the substrates to monitor the reactions, ammonia determination to monitor the reactions and chiral GC of the acid products have already been described.[3] For ^{1}H NMR the frequency was 400, 376 MHz for ^{19}F NMR and 100 MHz for ^{13}C NMR. For the ^{1}H and ^{13}C NMR the values reported are in ppm downfield from a reference of TMS, for ^{19}F NMR the values are reported upfield of the reference compound ($CFCl_3$). FTIR (Nicolet 20 SXB) was determined on KBr pellets. Only a sample of the wavenumbers of the major strong peaks is presented as a reference. Optical rotations were measured with a Perkin–Elmer PE 241 Polarimeter and LC–MS was carried out with a Finnigan LCQ Deca. Except where otherwise reported, MS refers to GC–MS.

5.2. Synthesis of racemic substrates for amide biotransformation

5.2.1. 3,3,3-Trifluoro-2-hydroxy-2-methylpropionamide 1. The title compound was synthesised from ethyl 4,4,4,-trifluoroacetoacetate by standard chemical methods[7,12] in three steps with an overall yield of 59.4%. Appearance: white powder. ^{1}H NMR (DMSO): 7.55 ppm (br s, 1H, NH_2); 7.53 ppm (br s, 1H, NH_2); 6.75 ppm (s, 1H, OH); 1.43 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 170.7 ppm (s, $CONH_2$); 124.9 ppm (q, CF_3, J_{CF}=286.9 Hz); 74.1 ppm (q, quaternary C, J_{CCF}=27.5 Hz); 19.8 ppm (q, CH_3, J_{CCCF}=1.6 Hz). ^{19}F NMR (DMSO): −77.9 ppm (s, CF_3). Mp 143.6–145.0 °C (3× recrys. from AcOEt/Hex). IR: 3452 (s, OH); 3327 (s); 3271 (s); 1695 (sh); 1680 (s, $CONH_2$). Anal. Calcd for $C_4H_6F_3NO_2$: C 30.6, H 3.9, N 8.9. Found C 30.5, H 3.7, N 8.6.

5.2.2. 2-Hydroxy-2-(trifluoromethyl)-butanamide 2. 2-Hydroxy-2-(trifluoromethyl)-butanenitrile (Fluorochem) 8 g was added slowly dropwise to conc. H_2SO_4 (15.3 g) the mixture was heated to 115 °C for 15 min, cooled to 8 °C and 21.8 g of water added. Diethyl ether (50 mL) was then added, and the organic phase was washed with water (25.0 mL), saturated aqueous $NaHCO_3$ (25.0 mL) and again with water (25.0 mL). The diethyl ether phase was dried over Na_2SO_4, filtered and evaporated. The resulting oil was treated with n-hexane, and the resulting crystals (4.27 g) were collected by filtration. Appearance: white powder. ^{1}H NMR ($CDCl_3$): 6.21 ppm (br s, 2H, NH_2); 4.09 ppm (s, OH); 2.01–1.91 ppm (m, 2H,CH_2); 0.98 ppm (t, 3H, J=7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$): 169.7 ppm (s, $CONH_2$); 124.2 ppm (q, CF_3, J_{CF}=284.9 Hz); 77.6 ppm (q, quaternary C, J_{CCF}=27.9 Hz); 25.8 ppm (t), 6.5 (q) (CH_2CH_3). ^{19}F NMR ($CDCl_3$): −79.1 ppm (s, CF_3). Mp 75.8–78.0 °C (cryst. from toluene). IR: 3472 (s, OH); 3286 (s); 1695 (s, $CONH_2$); 1593 (s). MS: *m/z* (rel. intensity) 172 (M+1, 1), 143 (10), 142 (17), 127 (4), 109 (6), 108 (100), 93 (20), 79 (6), 69 (7), 67 (6), 57 (30), 45 (18), 44 (57).

5.2.3. 2-Hydroxy-2-(trifluoromethyl)-pentanamide 3. 1,1,1-Trifluoropent-2-one was synthesised[13] and converted to the corresponding cyanohydrin in a similar way to that previously described.[4] The cyanohydrin was then converted to the amide (7.80 g, yield 42.7%) by reaction with H_2SO_4 similarly to the substrate above. Appearance: white powder. ^{1}H NMR ($CDCl_3$): 6.29 ppm (br s, 1H, NH_2); 6.24 ppm (br s, 1H, NH_2); 4.15 ppm (s, 1H, OH); 1.95–1.82 ppm (m, 2H); 1.61–1.48 ppm (m, 1H); 1.34–1.21 (m, 1H); 0.97 (t, 3H, J=7.4, CH_3). ^{13}C NMR (DMSO): 170.0 ppm (s, $CONH_2$); 124.1 ppm (q, CF_3, J_{CF}=285.7 Hz); 77.3 ppm (q, quaternary C, J_{CCF}=28.6 Hz); 34.7 ppm (t), 15.7 (t), 13.9 (q). ^{19}F NMR ($CDCl_3$): −79.2 ppm (s, CF_3). Mp 77.0–79.0 °C. IR: 3512 (s, OH); 3486 (s); 3400 (s); 3381 (s); 3253 (s); 1706 (s, $CONH_2$); 1687 (s); 1667 (s). MS: *m/z* (rel. intensity) 186 (M+1, 2), 148 (6), 143 (M+1−C_3H_7 or $CONH_2$, 26), 122 (64), 103 (7), 94 (56), 71 (24), 59 (11), 44 (100).

5.2.4. 3,3,3-Trifluoro-2-hydroxy-2-phenyl-propionamide 4. The title compound was prepared as described previously[14] and converted to the amide (7.89 g) by reaction with H_2SO_4 similarly to the substrate above. Appearance: pale orange powder. ^{1}H NMR (DMSO): 7.74–7.37 ppm (m, 7H, arom CH and NH_2); 3.35 ppm (s, 1H, OH). ^{13}C NMR (DMSO): 169.5 ppm (s, $CONH_2$); 124.1 ppm (q, CF_3, J_{CF}=286.5 Hz); 77.5 ppm (q, quaternary C, J_{CCF}=27.0 Hz); 135.1 ppm (s), 128.7 (d), 128.0 (d), 126.3 (d). ^{19}F NMR (DMSO): −73.4 ppm (s, CF_3). Mp 96.4–97.1 °C. IR: 3506 (s); 3390 (s); 3199 (s); 1700 (s); 1568 (s). Anal. Calcd for $C_9H_8F_3NO_2$ C 49.3, H 3.7, N 6.4. Found C 49.5, H 3.9, N 6.3.

5.2.5. 3,3,3-Trichloro-2-hydroxy-2-methylpropionamide 5. The title compound was synthesised from 1,1,1-trichloroacetone via the cyanohydrin (69% yield) and by reaction with H_2SO_4 (66% yield) similarly to the substrates above. Appearance: white powder. ^{1}H NMR (DMSO): 7.48 ppm (br d, 2H, $CONH_2$); 1.64 ppm (s, CH_3). ^{13}C NMR (DMSO): 170.8 ppm (s, $CONH_2$); 105.3 ppm (s, CCl_3); 82.5 ppm (s, quaternary C); 22.0 ppm (q, CH_3). Mp 172.5.0–175.2 °C (from toluene/hexane, lit.[15] 174–176 °C). IR: 3490 (s, OH); 3378 (s); 3196 (s); 3381 (s); 1689 (s, $CONH_2$); 1572 (s). MS *m/z* (rel. intensity): 208, 206 (M+1 Cl-isotopes, 0.6 and 0.4) 179 (2), 165 (6), 163 (16), 161 (17), 128 (62), 126 (100), 91 (13), 88 (20), 62 (15), 43 (73). Anal. Calcd for $C_4H_6Cl_3NO_2$ C 23.3, H 2.9, N 6.8. Found C 23.0, H 3.1, N 7.1.

5.2.6. 3,3,3-Trifluoro-2-methoxy-2-methylpropionamide 6. The title compound was synthesised by methylation of 3,3,3-trifluoro-2-hydroxy-2-methylpropionamide with dimethylsulfate resulting in a 38% yield of the methyl ether. Appearance: white powder. ^{1}H NMR (DMSO): 7.65 ppm (br s, 2H, $CONH_2$); 3.35 ppm (s, 3H,=OCH_3); 1.50 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 168.5 ppm (s, $CONH_2$); 124.6 ppm (q, CF_3, J_{CF}=286.5 Hz); 80.0 ppm (q, quaternary C, J_{CCF}=27.0 Hz); 14.8 ppm (q, CH_3). Mp 82.3–86.8 °C (from Tol/Hex).

5.2.7. 3,3,3-Trifluoro-2-amino-2-methylpropanamide 7. The title compound was synthesised via low yielding (3% overall yield), but reliable, literature method[16] from trifluoroacetone. Appearance: white powder. ^{1}H NMR (DMSO): 7.50 ppm (br s, 1H, $CONH_2$); 7.43 ppm (br s, 1H $CONH_2$); 2.43 ppm (s, 2H, NH_2); 1.35 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 171.3 ppm (s, $CONH_2$); 126.3 ppm (q, CF_3, J_{CF}=285.7 Hz); 60.2 ppm (q, quaternary C, J_{CCF}=26.3 Hz); 20.1 ppm (q, CH_3, J_{CCCF}=2.0 Hz). ^{19}F NMR (DMSO): −76.6 ppm (s, CF_3). Mp 81.3–84.8 °C (sublimation followed by EtOAc recrys, lit.[16] 84–85 °C). IR: 3452 (s, OH); 3327 (s); 3271 (s); 1695 (sh); 1680 (s, $CONH_2$). MS: *m/z* (rel. intensity) 157 (M+1, 1), 156 (M+, 1), 113 (4), 112 (100, M+−$CONH_2$), 94 (3), 93 (5), 92 (18), 69 (2), 62 (5), 42 (31).

5.3. Biotransformations

Biotransformations to test the substrate specificity of the enzyme were carried out with a cell-free extract from cells of *E. coli* XL-1 Blue MRF/pPRS7. Cells were cultivated in Nutrient Yeast Broth with ampicillin (100 μg/mL) to an OD_{650} of 3.6. They were then washed in 100 mM phosphate buffer, pH 8.0, and resuspended in the same buffer to an OD_{650} of 190. The cells were then broken open by 3 passes through a French press, after which the cell extract was heated to 75 °C for 5 min, and cell debris and precipitated protein removed by centrifugation at 20,000 *g*. The clear supernatant had a protein concentration of 9.75 mg/mL and was used in the biotransformations. To test the various substrates the conditions were: racemic substrate, 5.0 mg/mL; enzyme extract, 0.2 mg/mL; 100 mM potassium phosphate, pH 8.0; temperature, 40 °C. The reactions were followed by GC analysis and ammonia determination.[3] The same GC method was used for all the substrates. For substrates **4** and **5**, the enantiomers were not separated by the GC method, but it was still possible to determine if there was conversion of substrate by monitoring the size of the amide peak or by ammonia determination.

Biotransformations with **2** and **7** as substrates to produce product for isolation were carried out as follows. For substrate **2** cells of *E. coli* XL-1 Blue MRF/pPRS7 were cultivated in Nutrient Yeast Broth with ampicillin (100 μg/mL) to an OD_{650} of 4.7. They were then washed in 100 mM phosphate buffer, pH 8.0, and re-suspended in the same buffer to an OD_{650} of 140. The biotransformation conditions were: racemic substrate, 5.0 mg/mL; cells, OD_{650}=10; 100 mM potassium phosphate, pH 8.0; temperature, 37 °C; volume 1.0 L; stirring at 120 rpm. For substrate **7** the biotransformation conditions were: racemic substrate, 5.0 mg/mL; amidase from *E. coli* XL-1 Blue MRF/pPRS7, immobilised on Eupergit C, 3.0 g (118 mg protein extract immobilised/g Eupergit C); solvent, water; temperature, 20 °C; volume 200 mL; stirring at 120 rpm. For both substrates **2** and **7** the larger scale biotransformations were monitored by chiral GC analysis of the substrates, and the reactions were stopped when all of one of the two amide enantiomers had been converted to product.

5.3.1. (*S*)-(−)-2-Hydroxy-2-(trifluoromethyl)-butanamide 8 and (*R*)-(+)-2-hydroxy-2-(trifluoromethyl)-butanoic acid 9. Solution from the biotransformation (902 mL) was adjusted to pH 10.0 with NaOH, and then extracted 3 times with ethyl acetate (600 mL). The pH of the aqueous phase was re-adjusted to 10.0 between each extraction. The three portions of ethyl acetate were combined, dried over Na_2SO_4, and the ethyl acetate removed under reduced pressure at 40 °C. The resulting orange oil was dissolved in hexane and placed at −18 °C overnight. The product suspension was filtered, washed with cold hexane and dried. The product was then recrystallised from hot toluene and dried to give 1.72 g of **8** as an off-white solid.

The aqueous phase was adjusted to pH 1.0 with HCl and extracted 2 times with ethyl acetate. The combined ethyl acetate fractions were dried over Na_2SO_4, and the ethyl acetate removed under reduced pressure at 40 °C. Toluene (15 mL) was added to the residue and the mixture dried to give 2.18 g of brown solid. The product was then twice recrystallised from hot toluene and dried to give 1.97 g of **9** as an off-white solid (mp 104–112 °C). For characterisations of **8** and **9** see Table 2. Additional FT-IR data: compound **8**: 3471 (s, OH), 3295 (br s), 1696 (s, $CONH_2$), 1593 (s), 1277 (s), 1166 (s); compound **9**: 3422 (s, OH), 3035 (br s), 1750.4 (s, COOH), 1320 (s), 1268 (s), 1229 (s), 1200 (s), 1185 (s), 1161 (s).

5.3.2. (*S*)-(−)-3,3,3-Trifluoro-2-amino-2-methylpropanamide 10 and (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid (3,3,3-trifluoro-2-methyl-alanine) 11. Removing the immobilised enzyme by filtration stopped the biotransformation. The aqueous reaction mixture was extracted 3 times with ethyl acetate. The combined ethyl acetate fractions were evaporated to give 0.53 g of **10** as a white powder. The aqueous phase was also evaporated to give 0.42 g of **11**, also as a white powder.

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A dynamic combinatorial screen for novel imine reductase activity

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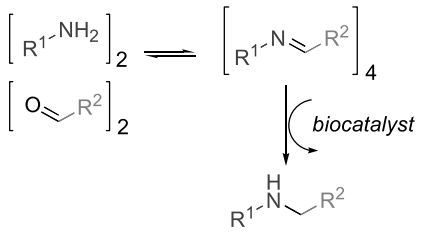
Abstract—New imine reductase activity has been discovered in the anaerobic bacterium *Acetobacterium woodii* by screening a dynamic combinatorial library of virtual imine substrates, using a biphasic water–tetradecane solvent system. Benzylidine aniline and butylidine aniline were reduced to the corresponding amines by caffeate-induced cells, whereas uninduced cells reduced butylidine aniline only. The reductions were detected despite side reactions that consumed some of the starting materials. The new screen can now be extended to discover synthetically useful imine reductases and enzymes that catalyse reactions for which biocatalytic equivalents of the chemical reactions have not yet been discovered.

1. Introduction

Combinatorial chemistry is a well-established tool for drug discovery. In particular, dynamic combinatorial approaches provide a simplified, rapid and versatile approach to discover new molecules that interact selectively with biological targets.[1] In drug discovery, a virtual library of candidate molecules is constructed by reversible self-assembly from basic component molecules. The active library constituents (e.g., selective enzyme inhibitors) are 'expressed' by binding to the biological target (e.g., the enzyme). The active constituents are 'locked in' so that they can be identified. Since the inhibitor is not modified by the enzyme, this is achieved by a chemical transformation or a change in reaction conditions. This quenches the reversible process and converts the active constituent to a stable chemical product[1,2] or stabilizes it sufficiently for it to be retrieved and characterized.[1]

Here, we report the first application of a dynamic combinatorial library to screen whole cell biocatalysts for a novel class of biotransformation, the reduction of imines to secondary amines. Virtual libraries of imines can be self-assembled from a mixture of component amines and aldehydes, and this has already been used to screen for enzyme inhibitors.[2,3] The key conceptual—and practically significant—advance is to use the enzyme substrate selectivity to perturb the equilibrium for self-assembly of the active library constituent and then to use the biocatalytic reaction to lock in the expressed constituent, so that the preferred imine is reduced to the corresponding secondary amine (Scheme 1).



Scheme 1.

A dynamic combinatorial screen for biocatalytic imine reduction would be extremely useful. Asymmetric reduction of ketimines, or related C=N containing compounds (hydroxylamines, hydrazones and oxime ethers), is an important target since this approach should provide a versatile strategy to synthesize α-chiral amines.[4]

Although chemical imine reductions are improving all the time,[5,6] biocatalysis may provide a route to enantiopure amines that are not yet accessible by chemical catalysis. However, imine reduction is one of a number of reaction classes for which there are no known enzymes that catalyse synthetically useful reactions,[7] but where there are no theoretical reasons why such enzymes should not exist.

Many laboratories have attempted to isolate useful imine reductases but with no success. A major problem is that imines tend to hydrolyse very readily in aqueous reaction systems, making it difficult to screen whole cell biocatalysts for imine hydrogenation. Furthermore, substrate instability means that living organisms are not exposed to imines in

Keywords: Virtual library; Imine reductase; Anaerobic biocatalysts.
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natural ecosystems, except as transient intermediates of metabolic pathways.[7a–d] Therefore, we cannot expect specialized enzymes to have evolved for imine metabolism. On the other hand, there may be enzymes that catalyse imine reduction fortuitously. This still presents problems, because we cannot predict their natural biological function or their substrate preference. This makes it extremely difficult to design suitable screens for imine reductases.

Dynamic combinatorial screens would be extremely effective in solving these problems. Thus, the target enzymes can 'choose' their preferred substrates from the virtual library and a very wide range of substrates can be screened simultaneously. This is ideal for detection of biocatalysts with unknown substrate preferences. Furthermore, the screen actually exploits the hydrolytic equilibrium, since the substrate selectivity of the enzyme forces the equilibrium towards generation of the preferred substrate by continuously converting it to the reduced product. Therefore, substrate instability is no longer a problem.

We developed the dynamic combinatorial screen using the strictly anaerobic bacterium, *Acetobacterium woodii*. We chose to use an anaerobe for screening for several reasons. Aerobic microorganisms and facultative anaerobes are more likely to hydrolyse the C=N bond than reduce it, because this produces aldehydes and amines which can be oxidized very easily.[14] In contrast, hydrogenation produces secondary amines, which are much more resistant to oxidative enzymatic attack. Secondary amines are either metabolized by oxidation to the imine[8] (which would be a futile cycle for imine oxidation) or by reductive cleavage to a primary amine, which only occurs in obligate anaerobes,[9] and is, therefore irrelevant to aerobic metabolism. Therefore, hydrogenation of imines is unlikely to occur in oxidative metabolic processes, and the search for imine reductases should be directed towards the reductive sector of metabolism.

Hydrogenation of organics is very favourable when the reaction serves as the electron-accepting process for growth of anaerobic bacteria.[10] This is a type of anaerobic respiration, where hydrogenation of the organic is analogous to the reduction of oxygen to water in aerobic respiration. The reactions are single step, occur at high rates and the hydrogenated products accumulate without further metabolism. Most importantly, the enzymes often have broad substrate ranges.[10] Therefore, if imine hydrogenation is to occur at all, it is most likely to occur as a fortuitous reduction catalysed by an enzyme involved in anaerobic respiration.

We used *A. woodii* in this study because this organism can reduce C=C double bonds as a respiratory process.[11] Our reason for this choice is that there is good chemical analogy between reduction of alkenes (of conjugated systems) and imines and we hoped that a C=C reductase might accept imines as fortuitous substrates. We wish to report the successful use of a combinatorial dynamic screen to identify a new imine reductase activity in whole cells of *A. woodii*.

2. Results and discussion

This work targeted the reduction of aldimines as a model for establishing the principle of this screening approach and the identification of new biocatalytic C=N reductions. The aldmines to be screened were chosen to include combination of alkyl–alkyl, alkyl–aryl and aryl–aryl components (from the carbonyl and amine components, respectively). Although, these would not yield chiral products, the use of aldehydes provides a very robust test of dynamic combinatorial screening for a number of reasons.

1. Substrate and product toxicity is a frequent complication in whole cell biotransformations and may hinder detection of target reactions in a screen. Aldehydes are more toxic than the corresponding ketones that would be needed for self-assembly of prochiral ketimines, since aldehydes react very readily with cellular amines (e.g., proteins, etc.). Therefore, use of the aldehydes provides a good test of the robustness of the approach.

2. Whole cells contain a multiplicity of enzymes, which might act on the starting materials or the components of the virtual library, and thus reduce the availability of potential substrates and cause the formation of side products. Aldehydes are more susceptible to biocatalytic side reactions than ketones, and thus provide a better indication of any problems that might be caused by competition between the target biotransformation and side reactions.

We produced *A. woodii* cells for use as the biocatalyst using growth conditions and methods for cell harvesting developed previously.[12] We used hydrogen as the electron donor for hydrogenation of the virtual library, since H_2 can be oxidized efficiently by the cells to yield the reduced cofactors needed for the biotransformation.[12b] The biocatalyst was grown in the presence and absence of the inducer, caffeate, to produce cell preparations containing and lacking the enzyme needed for C=C reduction,[12b,c] respectively. This would allow us to test whether or not the C=C reductase is responsible for any C=N reductions detected.

First of all, we confirmed that the biocatalyst had behaved as expected. Thus, the growth rate was 12% faster and the final

Table 1. Growth[a] and caffeate reduction[b] by *A. woodii* in the presence and absence of caffeate

Growth conditions	Growth rate (h^{-1})	Final biomass concentration ($g\,l^{-1}$)	Caffeate reduction rate (mmol h^{-1} kg dry $weight^{-1}$)
No additions	0.157	2.3	0
Caffeate	0.176	4.18	1615

[a] *A. woodii* was grown with or without caffeate (1 mM) and the growth rate and final biomass concentration were determined. The cells were then harvested from each culture and tested for reduction of caffeate (2.5 mM).
[b] Using harvested cells.

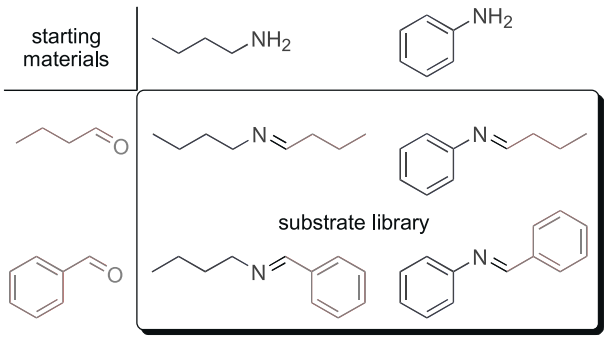

Figure 1. Virtual library of aldimines tested as substrates for *A. woodii*.

biomass concentration was 75% higher in the cultures grown with caffeate than in cultures grown in the absence of caffeate (Table 1). Similarly, the harvested cells could only reduce caffeate after growth in the presence of caffeate, confirming that reduction of C═C double bonds was inducible.[12b,c] It should be noted that we tested for caffeate reduction in the presence of tetradecane (phase ratio 0.2), since we planned to use a 2-liquid phase reaction system to deliver the virtual library, and we wanted to confirm that the cells were not affected by the solvent. In fact, caffeate reduction occurred at a slightly higher rate than reported previously with H_2 as electron donor in the absence of tetradecane,[12b] possibly because H_2 is more soluble in tetradecane than water.

The same preparations of harvested cells (induced and uninduced) were then tested for reduction of a virtual library of aldimines with the constituents shown in Figure 1. The constituents were derived from a mixture of aniline, benzaldehyde, butanal and butylamine. The two-liquid phase reaction system was used to ensure that all starting materials, virtual library constituents and any reaction products were in solution phase, and, thus, in contact with the biocatalyst.

Two reduced products, *N*-butylaniline and phenylbenzylamine, were formed in small quantities in reaction mixtures containing cells which had been grown with caffeate. Cells grown without caffeate produced only *N*-butylaniline (Table 2), but the concentration of *N*-butylaniline was much higher than with the cells grown in the presence of caffeate. Neither product was formed in reaction mixtures containing boiled cells or without any cells. This shows that reduction of the virtual library constituents was biocatalytic rather than chemical.

Only one constituent of the virtual library, benzylidine aniline, could be detected in any reaction mixtures. Thus, relatively large quantities of benzylidine aniline were present in control reaction mixtures containing boiled cells or without cells (Table 2). When active biocatalyst grown without caffeate was present, the concentration of benzylidine aniline was much lower, whilst this library component could not be detected at all in reaction mixtures containing cells that had been grown in the presence of caffeate. This trend reflects the formation of phenylbenzylamine, and further indicates that the cells were converting benzylidine aniline from the virtual library to the product.

Although *N*-butylaniline was produced by the biocatalyst, the starting material, butylidine aniline, was not detected in any reaction mixture. Butylidine aniline is less stable in water than benzylidine aniline,[13] and this may explain our inability to detect this library constituent. This confirms that the 'actual' substrate does not need to be present at detectable levels in the reaction mixture and that the presence of the 'virtual' substrate is sufficient to detect the biocatalytic activity.

Of the starting materials, only aniline could be detected in all of the reaction mixtures (results not shown). Benzaldehyde was detected in reaction mixtures containing boiled cells or no cells, but not with active cells. This suggested that the cells had consumed this substrate completely. Butanal and butylamine were not detected in any reaction mixtures, presumably because they could not be resolved from the tetradecane used as the solvent in the two-liquid phase system.

Table 2. Reduction of a virtual library by *A. woodii*[a]

Conditions for growth of biocatalyst	Biocatalyst preparation and no. of replicates	Library components, peak area×10^{-6}		Products, peak area×10^{-6}				Side products, peak area×10^{-6}			
		Benzylidine aniline		*N*-Butylaniline		Phenylbenzyl amine		Benzyl alcohol		Benzoic acid	
		C14	H_2O	C14	H_2O	C14	H_2O	C14	H_2O	C14	H_2O
With caffeate	Active (3)	0	0	0	0.065	0	0.0113	17.6	308	0	208
	Boiled (1)	835	0	0	0	0	0	1.7	86	0	44
	None (1)	729	0	0	0	0	0	0	31.3	0	13
No additions	Active (3)	6.03	8.13	0.267	0.307	0	0	0	391	0	71
	Boiled (1)	387	0	0	0	0	0	0	0	0	153
	None (1)	280	0	0	0	0	0	0	0	0	71

[a] *A. woodii* was grown with or without caffeate (1 mM), harvested and tested for reduction of a virtual library in a 2-liquid phase reaction system containing tetradecane and water (active). These were the same cell preparations as those used for the experiments shown in Table 1. Control reaction mixtures containing boiled cells (boiled) or no cells (none) were tested under identical conditions. The data are averages for the number of replicate reaction mixtures for each biocatalyst preparation shown in parentheses. The tetradecane (C14) and aqueous (H_2O) phases were analysed for library components, reaction products and side products. Data for each component detected are given for each phase as peak areas×10^{-6}.

Side products were also formed. Thus, benzaldehyde was converted to benzyl alcohol and benzoic acid in approximately equal quantities by biocatalyst that had been grown with caffeate (Table 2). In contrast, cells grown without caffeate produced mainly benzyl alcohol. Small quantities of benzyl alcohol and benzoic acid were also formed in control reaction mixtures containing boiled caffeate-grown cells, but only benzoic acid was formed in the presence of boiled cells grown without caffeate. This suggests that the side reactions were partly non-enzymatic and that some benzyl alcohol formation was caused by chemicals associated with caffeate-grown cells. In control reaction mixtures without cells, one reaction mixture contained smaller quantities of both benzyl alcohol and benzoic acid, whilst one contained only benzoic acid, again confirming that the reactions were partly non-enzymatic.

3. Conclusions

Reduction of a virtual library of aldimines was achieved using whole cells of *A. woodii*. Two constituents of the library (benzylidine aniline and butylidine aniline) were expressed in the presence of cells that were induced for caffeate reduction. In contrast, only butylidine aniline was expressed in the presence of uninduced cells. This suggests that *A. woodii* may contain two imine reductase activities, one of which (the benzylidine aniline reductase) is coinduced or identical with the caffeate reductase, and one (the butylidine aniline reductase) that is produced constitutively. The quantities of product were very low in each case, so it is possible that screening a larger virtual library would reveal better substrates. Once these are identified, further work will be needed to characterize these novel imine reductases.

In this study, product formation was detected using a whole cell biocatalyst. This demonstrates that the physical barrier of the bacterial cell membrane does not hinder the generation and biotransformation of a virtual library. Even though toxic substrates were used and the cells catalysed side reactions, low concentrations of product could still be detected. The most remarkable aspect is that the induced cells produced phenylbenzylamine even though one of the precursors, benzaldehyde, was also converted to benzyl alcohol and benzoic acid. This demonstrates that biocatalytic lock in of the reaction products perturbs the equilibrium sufficiently to direct the flow of precursors to the target biotransformation even in the presence of side reactions.

In this preliminary study, we have demonstrated that it is feasible to use dynamic combinatorial screens to identify new biocatalytic activities in whole cells, even with toxic substrates and in the presence of side reactions. It will now become possible to screen a wider range of potential biocatalysts with larger virtual libraries of prochiral ketimines so that synthetically useful biocatalysts can be identified. There is also scope to apply dynamic combinatorial screens to other reaction classes and thus obtain biocatalysts for a range of reactions that are not feasible at present.

4. Experimental

4.1. Microorganisms and growth

A. woodii DSM 1030 was maintained and grown in 'Balch' medium as described previously[14] at 30 °C in an anaerobic cabinet (Don Whitley). The medium was made anaerobic as follows. The basal medium solution containing NH_4Cl (0.1 g l^{-1}), $MgSO_4.7H_2O$ (0.1 g l^{-1}) K_2HPO_4 (0.4 g l^{-1}), KH_2PO_4 (0.4 g l^{-1}), Cysteine HCl (0.5 g l^{-1}), yeast extract (2.0 g l^{-1}), resazurin solution (1.0 ml l^{-1}; 0.1 g l^{-1}), $CaCO_3$ (for agar plates only; 5 g l^{-1}) was boiled and then cooled in an ice bath whilst gassing with N_2. The anaerobic solution was transferred by N_2 pressure to a Buchner flask (containing agar powder if required; 20 g l^{-1}) fitted with a football bladder on the side arm, which had been evacuated and flushed with N_2. The flask was sealed and autoclaved at 121 °C for 15 min. The football bladder acted as a buffer for pressure changes during autoclaving. The flask was cooled for approximately 40 min in a water bath at 60 °C, and transferred to the anaerobic cabinet. All other medium components were made anaerobic by dispensing into universal bottles, sealing with Suba seals and sparging with O_2-free nitrogen for 20 min via syringe needles inserted through the Suba seals. Wolin B trace elements, sodium sulfide, selenite-tungstate and caffeate solutions were then autoclaved at 121 °C for 15 min and transferred to the anaerobic cabinet when cool, whilst Wolin B vitamins, $NaHCO_3$ solution and fructose solution (100 g l^{-1}) were transferred to the anaerobic cabinet and filter sterilized (Acrodisc 32, pore size 0.2 μm).

The complete medium was prepared by adding deoxygenated, sterilised solutions to the basal medium in the following sequence, with thorough mixing after addition of each solution: Wolin B vitamin solution (10 ml l^{-1}), Wolin B trace elements (10 ml l^{-1}), $NaHCO_3$ solution (20 ml l^{-1}), $NaS.9H_2O$ (10 ml l^{-1}), selenite–tungstate solution (2 ml l^{-1}), fructose solution (as required). The medium was then transferred to sterile culture vessels in the anaerobic cabinet.

Stock cultures were maintained on agar plates and subcultured every 2 weeks and were grown with fructose (2.5 g l^{-1}) and $CaCO_3$. All other cultures were grown with fructose at 5 g l^{-1}, without agar and $CaCO_3$. Sodium caffeate (1 mM) was added when required and was prepared by dissolving caffeic acid in 10% of the final volume in deoxygenated NaOH (1 M) in the anaerobic cabinet. This was diluted to 60% of the final volume with deoxygenated H_2O, adjusted to pH 7.0 using deoxygenated HCl (1 M) and then made to the desired volume by adding deoxygenated H_2O to prepare a 0.15 M solution. The NaOH, water and HCl had been deoxygenated by sparging with N_2 for 20 min. The solution was filter sterilised, kept under anaerobic conditions and used within 2 d.

Precultures (15 ml) were inoculated with a single colony from a stock culture and grown for 48 h. Inocula for biocatalyst production (15 ml) were inoculated from the precultures (10% v/v) and incubated for 48 h. Two cultures were then used to inoculate 400 ml medium in Duran bottles capped with foam stoppers. The cultures were mixed on a

magnetic stirrer at 100 rpm. The empty Duran bottles had been autoclaved aerobically. Air was removed using the air lock of the anaerobic cabinet, before moving the bottles into the anaerobic cabinet and dispensing the medium into the bottles.

4.2. Cell harvesting

Caffeate-containing cultures were harvested after caffeate in the medium had been completely reduced. Cultures grown without caffeate had been inoculated at the same time as the caffeate-containing cultures and were also harvested at the same time. The cultures were transferred to airtight, screw-capped bottles (COWIE PTFE Technology, UK). The bottles were sealed, removed from the anaerobic cabinet and centrifuged at 5000 rpm for 30 min in a Thermo IEC Centra-3M Centrifuge (USA). The supernatants were discarded and the cells washed twice using deoxygenated 20 mM potassium phosphate buffer (pH 7.0). The buffer was deoxygenated by the same method as described for the basal medium. The cells were then resuspended in the buffer at approximately 10% of original culture volume and used as the biocatalyst.

4.3. Preparation of reaction mixtures

Virtual libraries were prepared from butylamine, aniline, butanal and benzaldehyde which were stored under N_2. Stock solutions (0.1 M) were dissolved in deoxygenated distilled water (butylamine, aniline, butanal) or tetradecane (benzaldehyde) in a fume cupboard under N_2 flow and then transferred to the anaerobic cabinet to prepare the reaction mixtures. The solvents were deoxygenated by sparging with N_2 for 20 min.

The overall volume of the reaction mixtures was 6.25 ml in 20 ml bottles, and all substrates were added to 2.5 mM relative to the overall volume. The aqueous phase (5 ml) contained harvested cell suspension (1 ml) mixed with the butylamine, aniline, and butanal solutions in potassium phosphate buffer (pH 7.0). The overall buffer concentration was adjusted to 20 mM by adding a 40 mM solution to compensate for the volume of aqueous substrate solutions added and then making the final aqueous phase volume to 5 ml with a 20 mM solution. The organic phase (1.25 ml) contained benzaldehyde solution mixed with anaerobic tetradecane. Thus, the phase ratio was 0.2.

The reaction bottles were sealed with suba seals, which were secured with self-lock cable ties. Reactions were started by adding hydrogen as the electron donor. A slight vacuum was created by removing the headspace gas (approximately 13.75 ml) into a 50 ml syringe, and then a slight over-pressure was created by injecting H_2 (50 ml). The reaction mixtures were stirred using a magnetic stirrer bar at 300 rpm for 24 h. Samples of the reaction mixture were placed in a narrow neck test tube and the phases allowed to separate. Samples from the tetradecane phase and the aqueous phase were transferred into Eppendorf tubes and centrifuged at 13,000 rpm for 5 min using a microcentrifuge (MICRO 20, CE, Germany) to remove the cells from the samples and remove residual water from the tetradecane phase. The supernatants were transferred into GC autosampler glass vials and stored at $-20\,^{\circ}C$ for subsequent analysis by GC–MS.

When caffeate was used as the substrate, the same reaction mixture was used except that only caffeate (2.5 mM) was added as the sole substrate. Samples (0.2 ml) were taken at intervals to measure rates of caffeate reduction. Samples were made aerobic and put on ice to stop the reaction, then centrifuged at 13,000 rpm for 3 min. The aqueous phase was withdrawn and diluted 40 times for determination of residual caffeate.

4.4. Analytical methods

Cell growth was estimated by measuring the OD at 660 nm after an appropriate serial dilution of samples of cultures. Growth rates (μ) were calculated from plots of ln OD (x) versus time (t), using the equation $\ln x_t = \mu t + \ln x_0$. Biocatalyst concentrations were determined by converting OD_{660} to dry weight using a calibration curve. This was prepared using a harvested cell preparation, which had been washed and resuspended in distilled water in place of phosphate buffer and then diluted to give a range of OD values. Dry weights were determined by centrifuging the diluted samples, washing the pellets into preweighed glass vessels using distilled water and drying at 105 °C overnight. An OD value of 1 was equivalent to a dry weight of $0.382\ g\ l^{-1}$.

Reaction products from dynamic combinatorial screens were identified by GCMS analysis using a Hewlett–Packard 5890A series 1 gas chromatograph coupled to a VG Tritech TS250E mass spectrometer. Ultra-high purity helium (99.9995%) was used as the carrier gas at 120 kP head pressure. The samples from the tetradecane phase were analysed directly by on-column injection of samples (0.25 μl) at 140 °C onto a Durabond DBwax capillary column (30 m×0.25 mm, 0.25 μm phase thickness, Jones Chromatography). Sample components were resolved using a temperature programme comprising isothermal chromatography at 140 °C for 2 min, followed by a linear gradient of $15\ ^{\circ}C\ min^{-1}$ to produce a final temperature of 250 °C, which was maintained for 5 min.

The aqueous phase samples were also analysed without extraction, by on-column injection of samples (0.5 μl) at 120 °C onto a Durabond DB1701 capillary column (30 m×0.25 mm, 0.25 μm phase thickness, Jones Chromatography). Sample components were resolved using a temperature programme comprising isothermal chromatography at 120 °C for 2 min, followed by a linear gradient of $15\ ^{\circ}C\ min^{-1}$ to produce a final temperature of 265 °C which was maintained for 5 min.

Mass spectra were acquired using positive ion electron impact ionization at 70 eV with the source temperature at 240 °C. Mass spectra were scanned from 450 to 45 mass units over 1 s and the resolution was 300. Analyses were conducted at the maximum sensitivity of the instrument. Compounds were identified by comparing mass spectra with authentic spectra from the National Institute of Standards (NIST) database, or by comparing the spectra and retention times with authentic standards when these were available.

Caffeate concentrations were determined by measuring absorbance at 312 nm using a UV spectrophotometer (S&M CECIL 1020, UK) and calculated according to a reference curve of absorbance against caffeate concentration. Reduction of caffeate was determined by measuring the loss of absorbance at 312 nm due to hydrogenation of the C=C double bond.

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An efficient enzymatic preparation of rhinovirus protease inhibitor intermediates

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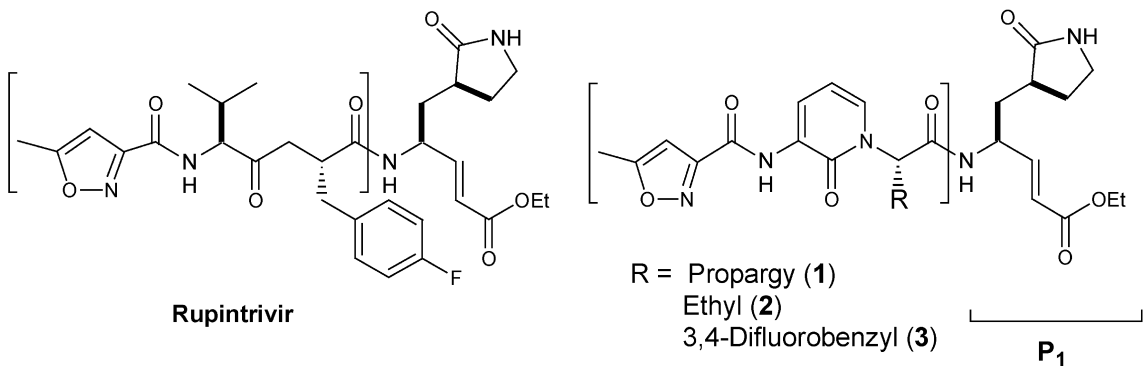
Abstract—The development of an efficient route for the preparation of (2*S*)-2-[3-{[(5-methylisoxazol-3-yl)carbonyl]amino}-2-oxopyridin-1(2*H*)-yl]pent-4-ynoic acid (**4**), a key intermediate in the synthesis of a human rhinovirus (HRV) protease inhibitor, is presented. In the presence of 40% acetonitrile, the alkaline protease from *Bacillus lentus* can catalyze the kinetic resolution of racemic ester **7** to afford (*S*)-acid **4** in 49% chemical yield/per cycle with 98% ee and >98% HPLC purity. The (*R*)-ester can then be readily recycled via a DBU catalyzed epimerization. The enzymatic preparation described here is superior to the existing chemical resolution route, exhibiting lower costs as well as higher yields, enantioselectivity, and substrate loads. In addition, this protease displays broad substrate specificity toward this class of compounds and can be easily extended to the preparation of other tripeptide mimetics of rhinovirus protease inhibitors.

1. Introduction

Currently, no effective therapy exists to directly treat the common cold. Since the condition is mainly caused by rhinovirus infections, the use of inhibitors that target the 3C protease, a protein required for viral replication, has been reported. The design and development of substrate-derived tripeptidyl Michael acceptor-containing human rhinovirus protease inhibitors have been extensively investigated.[1] A series of promising compounds including Rupintrivir™ and other related molecules (**1**–**3**, Scheme 1) have emerged as the lead candidates for this ailment entering human clinical trials. All of these molecules share the same right wing piece (P_1).[2] One of the most difficult issues in the synthesis of these compounds is to install the desired chiral center in the tripeptide mimetics [bracketed] with high optical purity.[3]

In this paper, we report an efficient and cost-effective enzymatic preparation of a key intermediate **4** towards the synthesis of compound **1** (Scheme 2). The method relies on enzymatic kinetic resolution of a racemic ester precursor using a protease isolated from a *Bacillus* species strain also known as *Bacillus lentus*.[4] The enzyme is a very inexpensive and commercially available serine protease,



Scheme 1.

Keywords: Rhinovirus protease inhibitor; Kinetic resolution; Enzymatic hydrolysis; Process development; Solvent engineering; *Bacillus lentus* protease; Substrate recycling.
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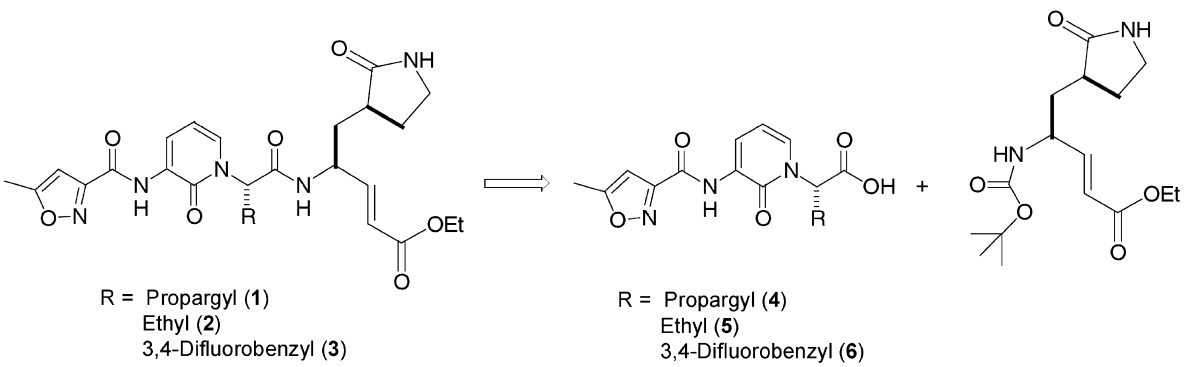


Scheme 2.

which is used in the detergent industry to remove protein-based stains and in the textile industry for wool finishing. However, in view of its ease of availability and low cost, the enzyme appears to be underused as a preparative catalyst. The enzymatic resolution strategy presented displays broad substrate range and could be easily extended to the preparation of tripeptide mimetics **5** and **6** needed for the synthesis of other rhinovirus protease inhibitors **2** and **3**.

Herein, we describe the screening performed in order to identify the enzyme, as well as process optimization carried out to improve the yield, enantioselectivity and substrate load. The dramatic solvent effect revealed during optimization of enzyme selectivity as well as the details on the development of a repetitive batch process to recycle unreacted ester with wrong stereochemistry, will be described. Finally, enzymatic and chemical processes will be compared.

2. Results and discussion

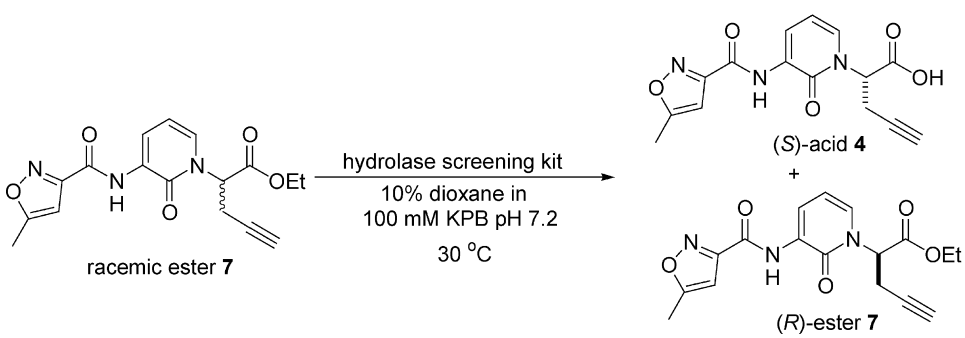
The development of a process for the resolution of racemic ester **7**[5] (Scheme 3) will be used to illustrate the strategy. The process development involved three main steps: (1) screen for an enzyme from an internal collection containing most commercially available lipases, proteases and esterases, (2) perform reaction optimization primarily to improve enantioselectivity of the enzyme hits found and subsequently to determine the optimal pH, temperature and substrate loads, and (3) development of a procedure for the regeneration of racemic ester from enriched unreacted ester (recovered after one kinetic resolution cycle) in order to perform a repetitive batch process with yields greater than 50%.

2.1. Enzyme screening

The most suitable catalyst for the kinetic resolution of racemic ester **7** was identified by using an unbiased screening of commercially available hydrolases. A general high-throughput enzyme screening method was used to screen the enzymes.[6] After initial screening using 10% dioxane, several hydrolases were identified as hits for the enzymatic hydrolysis of the racemic substrate **7**. The active enzymes found were pig liver esterase, subtilisin carlsberg, *Candida antarctica* lipase-A, *Candida antarctica* lipase-B, *Aspergillus oryzae* lipase, *Aspergillus* species protease, *Bacillus lentus* protease, *Bacillus subtilis* protease, *Aspergillus oryzae* protease, *Aspergillus melleus* protease and the acylase from *Aspergillus* species. Most of them showed good reactivities but unfortunately poor enantioselectivities ($E<5$). *Candida antarctica* lipase-B carried out the reaction with very good enantioselectivity (>98% ee at 50% conversion), however the reaction was very slow with only 0.5% w/v substrate loads being possible. Although process optimization was attempted initially with this enzyme, no major improvements in terms of reactivity were obtained afterwards. The substrate loads and the cost of the enzyme could not compete with the existing chemical process, where the racemic acid from ester **7** upon chemical hydrolysis was resolved using chiral norephedrine (see discussions below).

2.2. Optimization of enantioselectivity through the use of organic co-solvents

The use of solvent engineering to improve the enantioselectivity of enzymes has been extensively well documented.[6,7] A comprehensive study was thus initiated to



Scheme 3.

Table 1. Effect of dioxane contents on reactivity and enantioselectivity of proteases[a] (for the hydrolysis of substrate **7**)

Enzyme	[Dioxane] (%)	ee (%)	Conversion (%)	*E*
Bacillus lichenformis protease (subtilisin Carlsberg)	5	5	49	1.2
	35	48	45	4.1
	40	60	43	6.2
	45	66	41	7.6
	50	68	22	6.3
Aspergillus species protease	5	4	45	1.2
	35	70	12	6.2
	40	66	5.6	5.1
	45	72	2.4	6.3
	50	60	2	4.1
Bacillus lentus protease	5	30	43	2.3
	35	97	49	>200
	40	98	48	>200
	45	98	43	144
	50	98	28	143

[a] Reactions were conducted at pH 7.2, 5 mg/mL substrate **7**, 10% v/v enzymes, 30 °C and 5–50% solvent content. The reactions were allowed to proceed for 1 h and then quenched and analyzed by HPLC.

study the effects of solvent content on the enantioselectivity of the enzyme hits, which were reacted under various concentrations of 1,4-dioxane at constant pH 7.2. Both solvents and solvent contents showed intriguing effect on the enantioselectivity of the catalyzed hydrolysis of ester **7** by the three very inexpensive protease hits, which displayed relatively good reactivity (Tables 1 and 2). Subtilisin carlsberg, *Aspergillus* species protease and *Bacillus lentus* protease exhibited an enhanced *E* value as the solvent contents in the reaction mixture was increased. Moreover, *Bacillus lentus* protease (BLP) exhibited a dramatic solvent content effect, having an *E* value of >200 at 40% solvent content compared to an *E* value of only 2.3 at 5% 1,4-dioxane. As stated previously, this solvent effect has been found to be quite general for a variety of potential processes,[6] further emphasizing the need for a routine implementation of a thorough solvent study in every enzyme screening. Once an appropriate solvent content was identified, other solvents were screened at that content in order to identify potentially more reactive and environmentally friendly solvents while retain high enantioselectivity of the enzyme. As can be seen from Table 2, although the enzyme was still very enantioselective in most solvents at 40% solvent content, the reactivity differed significantly, with some reactions not proceeding at all notably in protic solvents (glycerol, MeOH, EtOH) and nonpolar solvents (methylene chloride, THF, toluene and hexanes). Acetone and acetonitrile were used in further optimization studies.

Table 2. Effect of solvents on reactivity and enantioselectivity of BLP[a] (for the hydrolysis of substrate **7**)

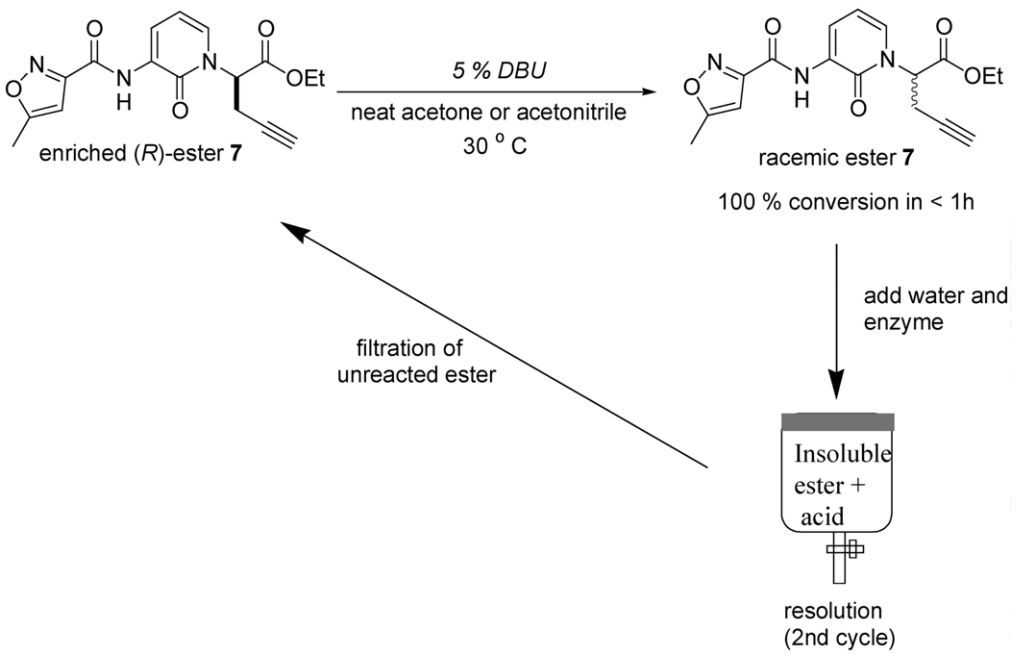
Solvent	Conversion (%)	ee (%)
50% Glycerol	<5	>99
Methanol	<5	>99
Ethanol	<5	>99
Isopropanol	29.6	99.2
Acetonitrile	41.8	98.8
Methyl-*t*-butyl ether	27	98.7
Methylene chloride	No reaction	—
Tetrahydrofuran	No reaction	—
1,4-Dioxane	48	98.8
Acetone	46	97.7
Toluene	<5	>99
Hexane	<5	>99

[a] Reactions were conducted at pH 7.2, 5 mg/mL substrate **7**, 10 mg/mL of enzyme, 30 °C, and 40% solvent content. The reactions were allowed to proceed for 1 h and then quenched and analyzed by HPLC.

2.3. Other optimization studies

Further optimization studies were performed in order to determine parameters such as maximum substrate load, optimum pH and temperature. Substrate load as high as 10% (100 mg/mL) required approximately 24 h reaction time. It was observed that proper stirring is very important since most of the substrate remained out of solution after saturation at 30 g/L concentration. The fact that the reaction was performed in a heterogeneous system was in fact advantageous since removal of the leftover ester only involved a simple filtration once the kinetic resolution was complete. Under these conditions, a batch process was chosen for simplicity. BLP is an enzyme that remains active in a broad pH range (6–11) with greater activity at higher pH. The background chemical hydrolysis was significant (>1%) at pH values above 8.5. Therefore a pH of 8.25 was optimum in terms of having maximum activity in the absence of deleterious background hydrolysis leading to low ee's. The enzyme-catalyzed reaction was studied at temperatures ranging from 20–50 °C. Temperatures above 37 °C favored background hydrolysis at the optimum pH mentioned above. Since the reaction was fast enough, there was no need to increase temperature above 30 °C. The amount of enzyme to be used was also studied by varying the amount of enzyme from 0.1–50% v/v. It was found that 10% enzyme content was the optimum protein concentration with minimal complication in the workup.

The enzyme catalyzed kinetic resolution to afford (*S*)-**4** was thus optimized to reach 49% chemical yield/cycle, >98% HPLC purity and 98% ee. BLP catalyzes the selective hydrolysis of the (*S*)-ester of the racemic mixture of **7** in the presence of 40% acetonitrile as co-solvent. After the reaction is over, the remaining ester (*R*)-**7** is filtered from the reaction mixture and the acid is recovered from the aqueous mother liquor after acidification to pH 3.5 without further purification.



Scheme 4.

2.4. Repetitive batch process

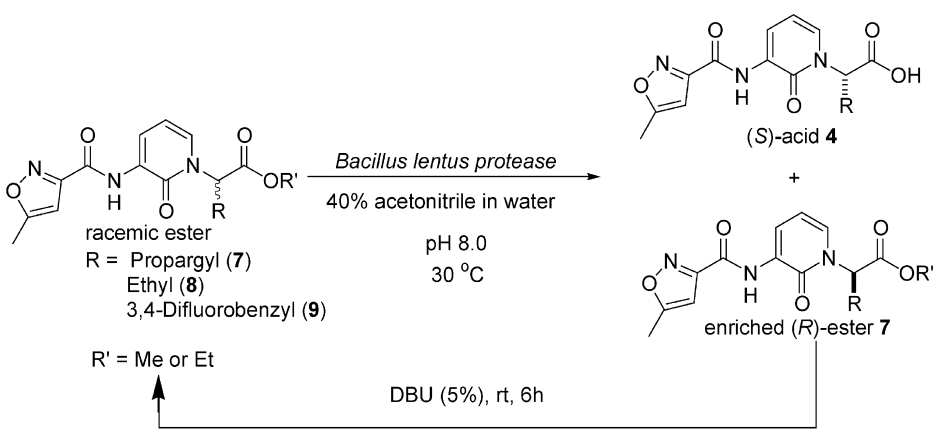
Due to the limitations associated with performing a kinetic resolution, a method for the recovery or in situ conversion of the remaining ester (*R*)-**7** was explored. A dynamic resolution process was attempted, however it was not possible to find conditions in which racemization took place efficiently under the optimum conditions catalyzed by BLP. However, the wrong enantiomer could be easily racemized under non-aqueous conditions (Scheme 4), thus allowing a repetitive batch approach for the preparation of (*S*)-**4**. The ester was epimerized by a catalytic amount of DBU (5 mol%) in 100% acetone or acetonitrile. The second resolution cycle continues after the addition of enzyme and water to the mixture under optimized conditions. The small amount of DBU did not produce any observable effect on the activity of the enzyme in the second cycle.

2.5. Scope of the method

Considering the attractiveness of the above process to resolve **7** using BLP, the kinetic resolution of **8** and **9** to prepare **5** and **6** was investigated under similar conditions optimized for substrate **7** (Scheme 5). Both methyl and ethyl esters were studied as suitable substrates. The remarkable solvent effect on enantioselectivity was also observed at concentrations above 30%. Both compounds were good substrates for the enzyme with enantiopurities of the products **5** and **6** being >97% after half of the racemic substrate was hydrolyzed. The base catalyzed racemization procedure outlined above for (*R*)-**7** could also be applied to substrates **8** and **9**, thus allowing a repetitive batch process as well. The enzyme accommodates both methyl and ethyl esters and displays very similar reactivities and enantioselectivities.

3. Summary

In summary, an efficient preparation of intermediate **4** has been described using *Bacillus lentus* protease (BLP). As seen from Table 3, this enzymatic preparation is superior to



Scheme 5.

Table 3. Comparison of chemical and enzymatic processes for the production of (*S*)-**4**

	Chemical	Enzymatic
Substrate	*rac*-**4**	*rac*-**7**
Method	Diastereomeric salt formation (norephedrin)	BLP catalyzed hydrolysis
Substrate load (g/L)	100	100
Yield/cycle (%)	33	49
ee of acid (%)	96	98
Catalyst load	84 g/L	100 ml/L
Catalyst price (per kg)	$315	<$50
Recyclability	Complicated: CDI-base treatment	Simple: filtration-DBU treatment

the chemical resolution approach. First of all, the enzyme BLP is significantly cheaper than chemical resolving agent norephedrine. In addition, the enzymatic process has much higher yields (49% vs 33% per resolution cycle). The high yielding makes the enzymatic route far more attractive considering it is the penultimate step of the overall synthesis of the drug candidate. Furthermore, removal of the leftover ester involved simple filtration once the kinetic resolution was complete, and efficient racemization allowed for the development of a repetitive batch process. In contrast, the chemical recycling is much more complicated involving activation of the undesired acid followed by epimerization. In the BLP catalyzed process described herein, >4000 mol of ester are converted per mole of enzyme per batch. The enzyme has high stability under a wide range of pH, temperature, and high organic solvent contents (40%). Remarkably, this bacterial enzyme also displayed broad substrate specificity and could be extended to the preparation of chiral precursors **5** and **6** needed for the synthesis of rhinovirus protease inhibitors **2** and **3**. While BLP shows similar reactivity toward both methyl and ethyl esters ignoring the difference among propargyl (**4**), ethyl (**5**) and difluorobenzyl (**6**) substituents, it differentiates each enantiomer stereochemically with kinetic perfection. The preparation of these types of densely functionalized optically active compounds with high molecular weight is a difficult task that still presents a major challenge in organic synthesis in the pharmaceutical industry. Through the use of a very robust hydrolytic enzyme such as BLP, a challenging chemical process has been resolved in a matter of weeks resulting in an efficient and environmentally acceptable route with great savings.

4. Experimental

4.1. Materials

The majority of enzymes utilized in the preparation of screening kits were obtained from various enzyme suppliers including Amano (Nagoya, Japan), Roche (Basel, Switzerland), Novo Nordisk (Bagsvaerd, Denmark), Altus Biologics Inc (Cambridge, MA), Biocatalytics (Pasadena, CA), Toyobo (Osaka, Japan), Sigma and Fluka (see Ref. 5 for details on preparation of screening plates, including specific enzyme sources for each enzyme, as well as a detailed description of the screening methodology). HPLC analysis of the screened samples was performed on an Agilent 220 HPLC auto sampler. Reactions were performed in an Eppendorf thermomixer-R (VWR). Solvents utilized during optimization were obtained from EM Science (Gibbstown, NJ) and were of the highest purity available. Chiral HPLC columns used in analysis were obtained from Chiral Technologies (Exton, PA) and Phenomenex (Torrance, CA). Commercially available *Bacillus lentus* protease was purchased from Altus Biologics (Boston, MA) as Altus 53 in crude form. A semi-purified form of the enzyme can also be obtained from Sigma as Savinase. This preparation showed no significant difference in the resolution, both in terms of reactivity and enantioselectivity when compared to Altus 53 preparations. HPLC methods: chiral method using detector wavelength: 254 nm; Chiralcel OJ-R, 3μ, C18, 4.6×100 mm; flow rate 0.5 mL/min; injection volume: 10 μL; mobile phases: (A) 25 mM NaH_2PO_4 pH 2.0; (B) Acetonitrile; isocratic: 40% B for 31 min, 3 min post run. Every HPLC sample was made by taking 5×200 μL from the reaction slurry, then combined and diluted with 4 ml of acetonitrile. 100 μL of that solution were further diluted with 400 μL of acetonitrile and injected in the HPLC.

4.2. Procedure for enzyme screening

The resolution of ester intermediate **7** was carried out as following. A 96-well plate screening kit prepared in house[6] was thawed for 5 min. 80 μL of potassium phosphate buffer (0.1 M, pH 7.2) was then dispensed into the wells using a multi-channel pipette. 10 μL of the substrate stock solution (50 mg of **7**/mL dioxane) was then added to each well via a multichannel pipette, and the 96 reactions were incubated at 30 °C and 750 rpm. The reactions were sampled after 1 and 16 h by the transfer of 25 μL of the reaction mixture into a new 96-well plate, which was then quenched by the addition of 150 μL of acetonitrile. The 96-well plate was then centrifuged, and the organic supernatant transferred from each well into another 96-well plate. Sampled reactions were then sealed using a penetrable mat cover and transferred to an HPLC system for analysis. The same plate was used to analyze the samples for both reactivity and enantioselectivity using alternating columns on the HPLC simultaneously.

4.2.1. Procedure for the preparation of compound 4. To a 150 mL jacketed flask equipped with a pH electrode, an overhead stirrer and a base addition line, was added the racemic ester **7** (10 g, 29.12 mmol, 1.00 equiv.) and acetone (40 mL). A 718 Stat Titrino-Metrohm pH titrator (Brinkman instruments, Inc.) was used to control the pH of the reaction. The resulting slurry was stirred at 30 °C for 5 min. A mixture of *B. lentus* protease (10 mL from commercial Altus 53 solution) and distilled water (50 mL) was added to the reaction flask. The suspension was then stirred at the

same temperature for 24 h maintaining the pH of the reaction constant at 8.2 using 1 M NaOH. Both the conversion and ee's of the reaction was monitored by RP-HPLC following the product, and stopped after 50% starting material was consumed (approximately 24 h under these conditions). The heterogeneous mixture was filtered and the remaining ester was recovered as white crystals, washed once with distilled water and dried under vacuum to afford 4.81 g of (*R*)-ester (48% yield, >98% ee). The remaining aqueous solution was extracted once with 50 mL of ethyl acetate to remove any traces of starting material, acidified to pH 3.5 with 1 M HCl, and extracted three times with 50 mL of ethyl acetate. The acid fractions were pooled, dried with sodium sulfate and concentrated in vacuum. The crude solid was washed once with hot water, filtered and dried overnight. Compound **4** was obtained as white crystals (4.68 g, 98% ee, 49% yield, >98% UV purity). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.44 (s, 1H), 8.31 (dd, J=1.67, 7.38 Hz, 1H), 7.54 (dd, J=1.72, 7.02 Hz, 1H), 6.72 (d, J=0.91 Hz, 1H), 6.42 (t, J=7.17 Hz, 1H), 5.28 (dd, J=4.55, 10.68 Hz, 1H), 3.19 (ddd, J=2.65, 10.72, 17.46 Hz, 1H), 2.97 (ddd, J=2.70, 4.55, 17.47 Hz, 1H), 2.84 (t, J=2.59 Hz, 1H), 2.50 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.77, 169.17, 158.83, 157.15, 156.84, 132.88, 127.64, 123.54, 105.67, 101.60, 80.03, 73.67, 60.81, 19.37, 12.17. Mp 175–182 °C; IR (KBr) ν_{max}/cm^{-1}: 3301, 1752, 1645, 1542, 1459, 1221, 1188; HRMS (CI) *m/z*: 316.0940 (316.0933 calculated for $C_{15}H_{14}N_30_5$).

4.2.2. Data for compound 5. Compound **5** was obtained as white crystals from the resolution of racemic ester **8** with 97% ee, 48% yield, >98% UV purity. ^{1}H NMR (700 MHz, $CDCl_3$): δ 9.58 (s, 1H), 8.49 (dd, J=1.7, 7.5 Hz, 1H), 7.11 (dd, J=1.9, 7.2 Hz, 1H), 6.82 (s-broad, 1H), 6.49 (s, 1H), 6.38 (t, J=7.4 Hz, 1H), 5.38 (dd, J=5.1, 10.1 Hz, 1H), 2.5 (s, 3H), 2.34 (m, 1H), 2.05 (m, 1H), 0.93 (t, J=7.7 Hz, 3H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 172.7, 171.8, 158.7, 157.9, 157.7, 129.2, 128.6, 123.8, 107.3, 101.4, 61.5, 23.7, 12.5, 10.6. Mp 158–160 °C; IR (KBr) ν_{max}/cm^{-1}: 3345, 1744, 1699, 1643, 1534, 11459, 1202; HRMS (CI) *m/z*: 305.2940 (305.2933 calculated for $C_{14}H_{15}N_30_5$).

4.2.3. Data for compound 6. Compound **6** was obtained as white crystals from the resolution of racemic ester **9** with 97% ee, 45% yield, >98% purity. ^{1}H NMR (700 MHz, $CDCl_3$): δ 9.50 (s, 1H), 8.45 (dd, J=1.5, 7.5 Hz, 1H), 7.63 (s-broad, 1H), 7.00 (m, 1H), 6.92 (m, 1H), 6.82 (dd, J=7.2, 1.5 Hz, 1H), 6.77 (m, 1H), 6.49 (s, 1H), 6.24 (t, J=7.2 Hz, 1H), 5.28 (dd, J=4.9, 10.2 Hz, 1H), 3.55 (dd, J=14.8, 5.0 Hz, 1H), 3.41 (dd, J=14.8, 10.5 Hz, 1H), 2.50 (s, 3H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 171.9, 171.2, 158.2, 157.9, 157.3, 132.9, 130.4, 128.7, 125.2, 124.3, 107.4, 101.4, 63.7, 34.8, 12.5. Mp 152–155 °C; IR (KBr) ν_{max}/cm^{-1}: 3347, 1718, 1650, 1594, 1535, 1460, 1286, 1213; HRMS (CI) *m/z*: 403.0940 (403.1033 calculated for $C_{19}H_{15}F_2N_30_5$).

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Mechanistic studies on the enzymatic transesterification of polyesters

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Abstract—The effect of solvents on the enzymatic transesterification of aliphatic polyesters has been investigated. It has been shown that the hydrophobicity and the polarity of the solvent have little effect on the reaction, however, the molecular weight of the product depends on the solubility of the product in the medium. Above a certain molecular weight, when the product is no longer soluble in the medium, transesterification does not occur. Deuterium NMR studies have shown that transesterification tends to take place at the ends of the polymer chain rather than at random along the polymer chain.

1. Introduction

In recent years the enzymatic synthesis of polyesters has aroused considerable interest because of the possibility of producing novel polyesters with unusual properties. The early work was done using activated acids and or diols, such as divinyl adipate and halogenated alcohols in non-aqueous solvents,[1] the resulting polyesters were oligomers[2] of relatively low molecular weight of approximately 2000 Da. It was shown subsequently that using the enzyme *Candida antarctica* lipase B supported on acrylic beads (*Novozyme* 435™) it was possible to synthesise much higher molecular weight polyesters in the absence of solvent.[3] A number of workers have also studied the enzymatic catalysis of polyesters by the transesterification of both activated and unactivated diesters with diols[4] and by the ring opening transesterification of lactones.[5]

When the polyesters prepared by enzymatic synthesis in the absence of solvent were studied it was found that they had significantly different properties compared to those prepared by the conventional high temperature process. It was found that not only was it possible to obtain higher molecular weight polymers but they had a lower dispersity, that is a narrower molecular weight distribution than the conventional polyesters with the result that products made from these materials have superior physical properties.[6] These results were explained by the apparent absence of a transesterification reaction during the synthesis.[2] It was subsequently shown that if the reaction was carried out in the presence of toluene then not only was the dispersity higher but that the molecular weight obtainable was reduced. The conclusion being that transesterification occurs in the presence of toluene as solvent causing scission of the growing polymer. This conclusion was confirmed when the synthon, 1,6-hexanedioic acid di(4-hydroxybutyl) ester (BAB) was shown not to react with itself in the absence of solvent, whereas in toluene the enzyme catalysed the transesterification and a number of higher molecular weight oligomers were formed.[7]

Lipase catalysed transesterification reactions in organic media with monoesters are well known and have been used to separate racemic mixtures of alcohols and carboxylic acids or to select a specific ester or alcohol group within a molecule as substrate.[8] Therefore, the original question of why the enzyme appears to catalyse the transesterification of polyesters in some circumstances and not in others becomes more interesting, because one would expect it to catalyse transesterification in all circumstances. It had been considered initially that the enzyme might adopt a different configuration in solvent thereby affecting the reaction and the resulting products. However, it has been shown, using synchrotron CD spectroscopy that no major changes in the structure of the protein take place when used in solvents such as hexane and toluene.[9] The possibility that solvent molecules were being absorbed onto the hydrophobic regions in or around the active site was considered, as even one or two bulky solvent molecules such as toluene absorbed in a critical area could easily affect the rate at which the substrate could diffuse into the active site of the enzyme.

In all our earlier work, the acid-diol esterification reaction

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and any transesterification reaction between ester groups, leading to the formation of the polyester would be taking place simultaneously, with the final composition containing the products of both. Therefore, in order to study the transesterification reaction, it was necessary to develop a method whereby the transesterification reaction could be studied independently of the esterification reaction. If the reaction was being affected by solvent absorbed at the active site, then the effects of the size, shape and physical properties of the solvent on the transesterification reaction were of interest.

2. Results

2.1. Transesterification reactions

The hydroxyl group of the added diol involved in the transesterification reaction causes scission of the high molecular weight polyester, the extent of transesterification being proportional to the reduction in the average molecular weight of the polyester. The results obtained are shown in Table 1.

Table 1. Effect of solvent on the transesterification of polyester

Solvent	M_w	M_n	Dispersity
PTMEG 650	36,000	32,000	1.1
1,4-butanediol	31,000	26,000	1.2
Toluene	5800	2900	2.0
Dioxane	1400	750	1.8

It appears from these results that the amount of transesterification is independent of hydroxyl concentration. The greatest breakdown of the high molecular weight polyester occurred with toluene and dioxane and not with 1,4-butanediol or polytetramethylene ether glycol of molecular weight 650 (PTMEG 650), which have much higher concentrations of hydroxyl groups. This observation appears to contradict the conclusion of Kumar and Gross,[10] who proposed that there is slower transesterification in higher molecular weight polycaprolactones because the higher the molecular weight, the fewer terminal hydroxyls being present to take part in the transesterification reaction.

It had been found by Harffey[11] that the addition of as little as 6% w/w of toluene to the reaction medium gave the same result as when the reaction was carried out in toluene as the reaction solvent. The possibility that toluene was being absorbed from the medium onto the hydrophobic areas of the protein, thereby affecting the mechanism of the reaction, was considered. Therefore, the effect of low concentrations of toluene in 1,4-butanediol on the degree of transesterification was determined. The results are shown in Table 2.

Table 2. Effect of toluene concentration on transesterification

Principal solvent	[Toluene] (mM)	M_w	M_n	Dispersity
1,4-BD	0.11 (0.001%)	26,000	14,000	1.9
1,4-BD	1.1 (0.01%)	28,000	19,000	1.5
1,4-BD	67 (0.625%)	27,000	17,000	1.6
1,4-BD	200 (1.87%)	28,000	18,000	1.6

The differences between these results are not considered to be significant. It appears that small additions of solvent do not affect the degree of transesterification. It is unlikely, therefore, that absorption of solvent into the hydrophobic areas of the enzyme takes place, as it would be expected that the hydrophobic attraction of the lipase would extract toluene from such a polar medium as 1,4-butanediol, even at these low concentrations.

The effect of the shape of the solvent molecule was then investigated. The standard transesterification experiment was carried out with a number of solvents of different shapes and hydrophobicity. The results are as shown in Table 3 below.

Table 3. Effect of solvent configuration on transesterification

Solvent	M_w	M_n	Dispersity	$C \log P$
Toluene control	5800	2900	2.0	2.7
n-Butylbenzene	5200	2800	1.9	4.0
iso-Butylbenzene	5900	3000	2.0	4.0
tert-Butylbenzene	5900	2900	2.0	4.1
4-Chlorotoluene	5900	2900	2.0	3.3
Hexane	18,000	8600	2.0	3.8

The GPC profiles for all the experiments listed in Table 3 were essentially the same for all the aromatic solvents.

It is evident from these results that the degree of transesterification as measured by chain scission is not affected by the geometry of the solvent molecule. The differences seen in the experiments with the aromatic solvents are not considered significant; the only significant difference being between the aromatic solvents and the aliphatic hexane. It does not appear, however, that the hydrophobicity as measured by $C \log P$ is the cause of the difference; as there is significant transesterification at a $C \log P$ of -0.4 in dioxane and also at $C \log P$ of 4.1 in *tert*-butylbenzene.

The transesterification reaction was repeated using solvents that were substantially more polar in order to determine if the reaction is affected by the polarity of the solvent. Propylene carbonate, triethylene glycol methyl ether and tetraethylene glycol methyl ether were chosen as the added solvent. In all cases extensive transesterification took place and the molecular weight fell to around 3000 Da as measured by GPC. There did not appear to be any significant difference in the amount of transesterification in relation to the polarity of the solvent.

The only two media in which transesterification does not occur are 1,4-butanediol and polytetramethylene ether glycol both of these are very poor solvents for the high molecular weight polyester. It would appear that the transesterification reaction takes place in any solvent in which the higher molecular weight polyester is soluble.

2.2. NMR studies

In order to elucidate the mechanism, the transesterification reaction was studied using deuterium NMR. The insertion of deuterated 1,4-butanediol into the polyester being followed

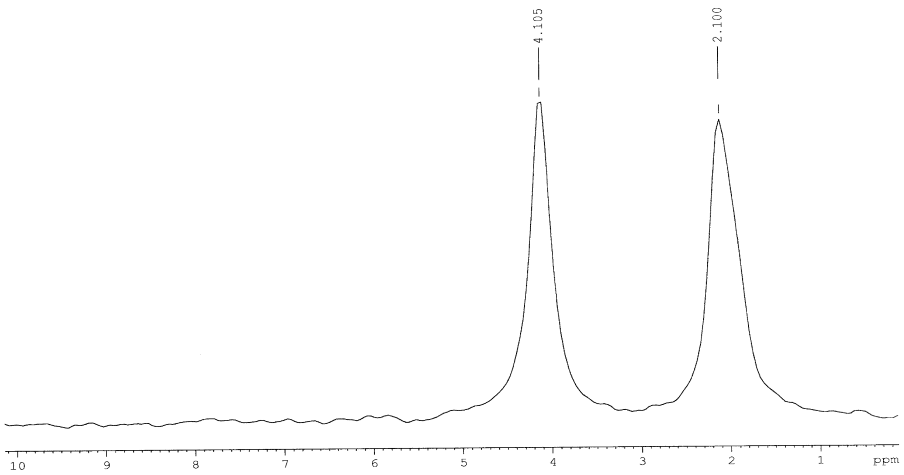

Figure 1. ^{2}H NMR spectrum of deuterated 1,4-butanediol.

by observing the downfield shift due to the presence of adjacent ester carbonyl groups.

A 1% solution of d^{8}-1,4-butanediol in 1,4-butanediol was prepared and the ^{2}H NMR spectrum obtained (Fig. 1).

The two peaks of the deuterated diol were at 4.11 ppm, corresponding to the 1,1′- and 4,4′-deuteriums and 2.14 ppm, corresponding to the 2,2′- and 3,3′-deuteriums. The signal-to-noise ratio was 150:1, therefore we were confident that using this method we would be able to see whether transesterification had taken place, by looking for the insertion of the deuterated 1,4-butanediol into the polyester.

In order to maximise the visibility of the deuterated diol in the polyester, the concentration of the deuterated diol was increased to 10%. Samples were taken at 2, 5 and 21 h and the ^{2}H NMR spectra obtained, no difference could be seen in any of the spectra, thus indicating that in the 1,4-butanediol medium no observable transesterification had taken place. After 24 h a very small peak at 4.68 ppm was starting to show, indicating that a small amount of deuterated diol had been incorporated into the polyester. After 48 h a somewhat larger peak was observed at 4.68 ppm, which indicated that transesterification does take place in 1,4-butanediol, but that it is very slow.

This experiment was repeated after adding 6 ml of toluene and stirring at 60 °C a ^{2}H NMR spectrum with excellent signal-to-noise ratio (2000 scans) was obtained. After 24 h, there was a small but distinct peak at 4.68 ppm due to the increased chemical shift when one end of the diol is incorporated into an ester group and the peak at 2.12 ppm was starting to split, with a pronounced shoulder at 2.21 ppm, as a result of the d^{8}-1,4-butanediol now forming a significant part of the ester groups. This indicated that the d^{8}-1,4-butanediol had been incorporated into the polyester and that transesterification had occurred (see Fig. 2A).

After 48 h it could be seen that both of the deuterium resonances had split and the new peaks had moved downfield (see Fig. 2B). The $OC^{2}H_{2}$ peak had moved in its entirety to 4.73 ppm, leaving only a small peak at 4.18 ppm. The $C^{2}H_{2}-C^{2}H_{2}$ peak had also moved downfield to 2.33 ppm, which proved that there had been significant incorporation of the deuterated diol into the polyester.

The initial experiments without added toluene were repeated using a polyhexane adipate polyester of 2000 Da. The sample taken at 24 h showed that the $HO-C^{2}H_{2}$ peak at 4.11 ppm had split equally with the $COO-C^{2}H_{2}$ peak at 4.68 ppm. Similarly, the diol $C^{2}H_{2}-C^{2}H_{2}$ peak at 2.21 ppm had also split equally with the polyester $C^{2}H_{2}-C^{2}H_{2}$ peak at 2.12 ppm. This showed that after 24 h, significant chain scission caused by transesterification with the added diol had taken place and after 48 h even more transesterification was found.

The polyester with the lowest and most uniform molecular weight is the simple oligomer 1,6-hexanedioic acid di(4-hydroxybutyl) ester (BAB). In order to see if this was also susceptible to transesterification in the absence of solvent, a sample of BAB was prepared starting from 6-carboxy-11-hydroxy-7-oxaundecanoic acid (AB) synthesised by the method of Harffey.[11] AB was reacted with a two-fold molar excess of 1,4-butanediol using *Novozyme* 435 as catalyst. After 24 h ^{1}H NMR spectroscopy showed that 100% conversion to BAB had taken place. The enzyme and solvent were removed and 10% deuterated diol and *Novozyme* 435 were added and the mixture heated at 60 °C for 24 h. ^{2}H NMR spectroscopy on the purified sample showed a very small, deuterated ester peak at 4.68 ppm, this indicated that some but not very much transesterification had occurred.

Samples from the experiments above were analysed by GPC to see if the effect of the transesterification could be determined. Most interestingly, the GPC of the high molecular weight polyester transesterified with deuterated 1,4-butanediol in the absence of toluene showed that the peak molecular weight, M_{w} 36,000, had declined very little but a number of low molecular weight oligomers had appeared (Fig. 3), whereas the 2000 M_{w} polyester had reduced to 980 M_{w} in the same time.

This result shows that the scission is not taking place at random along the polyester backbone, but rather that it takes

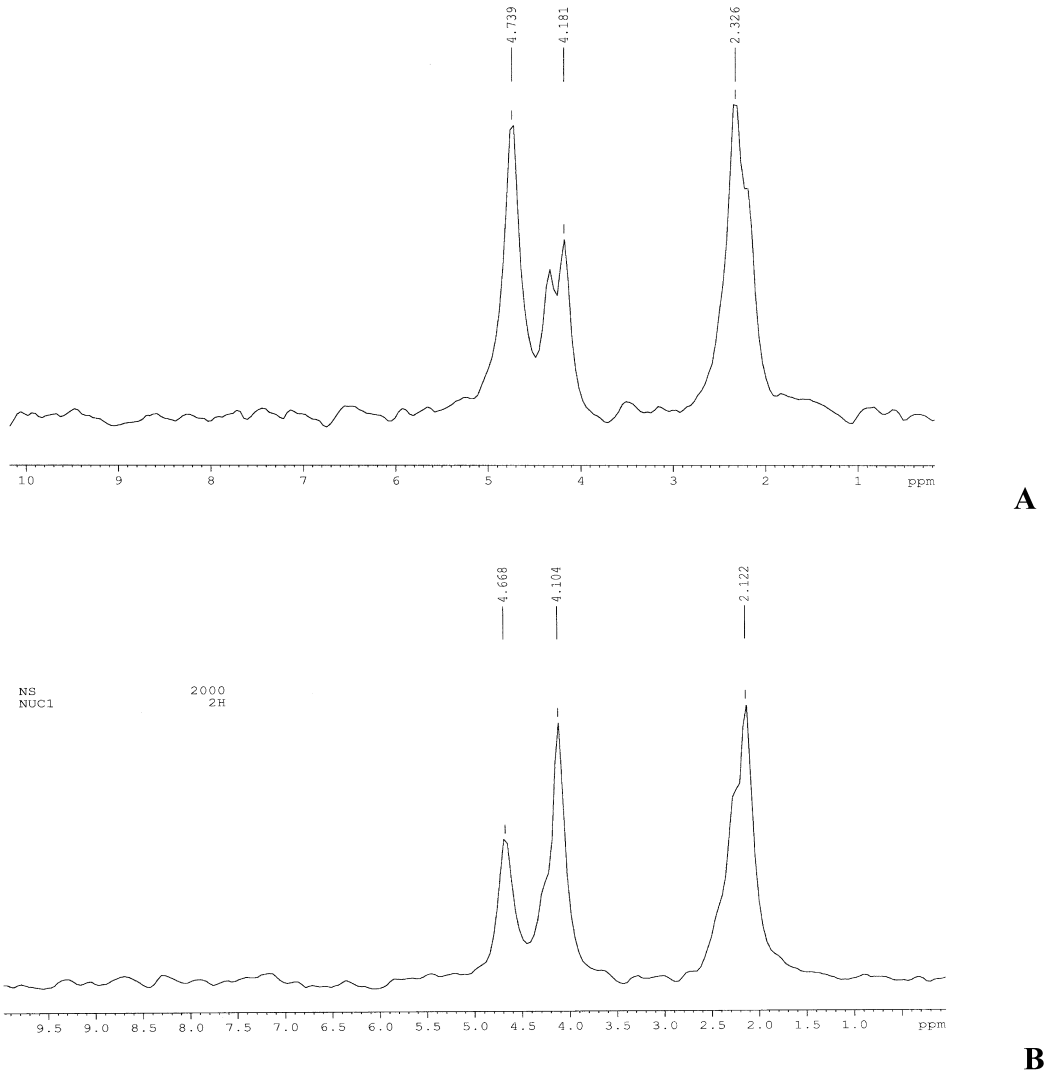

Figure 2. ^{2}H NMR spectrum of polyester+$^{2}H_8$-1,4-butanediol+toluene A after 24 h and B after 48 h.

place at the ester groups near to the end of the polymer chain. This is unlikely to be due to any property of the enzyme because each ester group and its environs are identical and any could fit into the pocket of the enzyme. It is more likely to be a property of the polyester. It is possible that it forms a tight coil in these media and it is only the ends of the chain that are available to the enzyme.

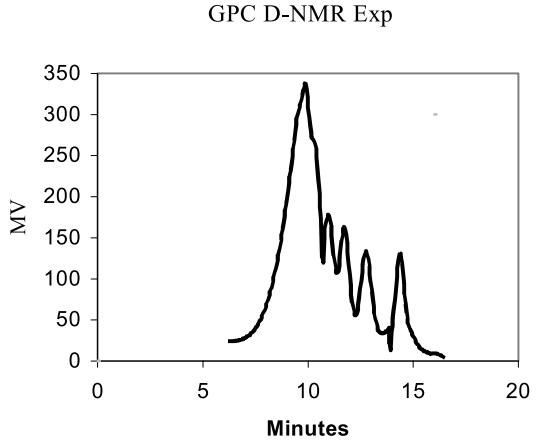

Figure 3. High molecular weight polyester after transesterification with deuterated 1,4-butanediol.

It appears from these results, that the rate of transesterification reaction, measured by the amount of insertion of $^{2}H_8$-1,4-butanediol into the polyester, whilst occurring in 1,4-butanediol is very much faster in toluene. The amount of insertion into the high molecular weight polyester and the 2000 molecular weight polyester appear to be approximately the same, however, the results of the transesterification are quite different. In the case of the high molecular weight polyester there is nominal reduction in the molecular weight as the chain scission only occurs at the ends of the molecule. In the 2000 Da polyester the chain scission occurs at random along the chain with the result that there is a significant reduction in molecular weight.

The transesterification of the oligomer BAB appears to be quite rapid, however, BAB cannot polymerise in the presence of excess diol, therefore the deuterated diol can only react at one of its two hydroxyls and this is reflected in the relative size of the peak shifted downfield.

The conclusion from these experiments seemed to be that

the reason for the lack of transesterification in the absence of solvent is the insolubility of the high molecular weight polyester in 1,4-butanediol or PTMEG 650. It is proposed that in the absence of solvent the growing polymer chain is held in the proximity of the enzyme active site by hydrogen bonding to the surface of the enzyme, a mechanism known in the synthesis of biopolymers such as RNA.[12] It is only when the polymer is in solution that the visceral ester groups are available to the enzyme for transesterification to take place. It might be that as the polyester reaches a critical molecular weight it starts to drop out of solution in the diol, thus limiting the transesterification reaction.

Modelling using Sybyl 6.0 and Sculpt 3.0 produced a very similar result, in both cases the growing polyester partially wrapped around the surface of the enzyme, bound by multiple hydrogen bonds to the surface of the enzyme. The non-bound part of the polymer chain adopted a tightly coiled configuration held together by intramolecular hydrogen bonds. If this is an accurate simulation then in a medium in which the polyester is not soluble it is only at or near to the ends of the molecule that the ester groups are available for transesterification. This would explain the fact that in the absence of solvent much higher molecular weight products are obtained, there being no transesterification to cause scission of the growing polymer.

3. Conclusions

Transesterification reactions catalysed by *Novozyme* 435 have been carried out between high molecular weight polyester and the diols 1,4-butanediol, 1,6-hexanediol and PTMEG 650 in the presence of a number of different solvents. It appears that the transesterification reaction depends predominantly on the solubility of the polyester in the medium and that excess hydroxyl content does not influence the transesterification if the polyester is not soluble in the diol. The hydrophobicity, polarity and shape of the solvent molecules do not influence the nature of the transesterification reaction. It appears that unless the polyester is soluble in the medium the growth of the polymer is not limited by the transesterification reaction and high molecular weight polyester is formed. The limited amount of transesterification occurs preferentially at the ends of the polymer rather than at random along the chain.

4. Experimental

4.1. Synthesis of polyhexane adipate polyester

R_1-[$OCH_2(CH_2)_4CH_2OCO(CH_2)_4COO]_n$-$R_2$

A high molecular weight polyhexane adipate polyester was synthesised using *Novozyme* 435 as the catalyst. A 1:1 molar ratio of 1,6-hexanediol and adipic acid was stirred at 60 °C for 1 h, 0.5% w/w *Novozyme* 435 added and a vacuum of 100 mb applied for 24 h. The vacuum was increased to 10 mb and the stirring and heating continued for a further 24 h at which point the vacuum was increased to 3 mb for a further 24 h. When the polyester had reached a molecular weight of 36,000 as measured by GPC the reaction was stopped and the acid number and hydroxyl number determined by titration. The bound enzyme was filtered off and the residual enzyme deactivated by heating the polyester at 200 °C for 15 min. This polyester had a M_n of 17,500, a M_w of 37,000 and dispersity 1.9. The acid number was measured as 2 mg KOH g^{-1} and the hydroxyl number was 13 mg KOH g^{-1}. This polyester sample became the standard for all our subsequent transesterification experiments. The molecular weights for this and all subsequent reaction products were determined by using a Waters HPLC with a model 510 pump, model 410 refractive index detector and the Waters 717 autosampler. The column was packed with Polymer Labs. 1000 Å polystyrene copolymer packing and the eluent used was THF stabilised with 250 ppm of butylated hydroxytoluene (BHT) at a flow rate of 1 ml min^{-1}. The sample concentration for all experiments was 0.5% w/vol with an injection volume of 40 μl. The data were analysed using the Millennium 32 GPC software. The GPC trace for the polyhexane adipate polyester is shown in Figure 4.

4.2. Procedure for transesterification

In order to study the effects of different solvents on the transesterification reaction a standard transesterification reaction was developed.

2 g of high molecular weight polyester plus 8 ml of solvent, 0.1 g of 1,6-hexanediol and 0.1 g of *Novozyme* 435 were added to a stirred cell reactor and heated at 60 °C for 24 h. The reaction was then stopped by filtering off the bound enzyme and cooling rapidly to 20 °C. In order to simulate the conditions that had existed in earlier syntheses, the transesterification was carried out in the presence of the solvents toluene and dioxane in the presence of 1,4-butanediol and polytetramethylene ether glycol (PTMEG) 650 to simulate the excess monomer and oligomer that is present in the early stages of the solvent free polymerisation. *n*-butylbenzene, *iso*-butylbenzene, *tert*-butylbenzene, 4-chlorotoluene, hexane, propylene carbonate, triethyleneglycol methyl ether and tetraethyleneglycol methyl ether were used to investigate the effect of hydrophobicity, polarity and size of the solvent molecule on the transesterification

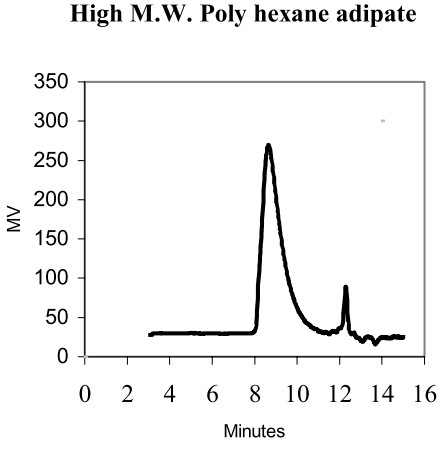

Figure 4. GPC of the high molecular weight polyhexane adipate used as substrate for transesterification.

reaction. All materials were purchased from Sigma-Aldrich and were standard reagent grade purity.

4.3. NMR studies

The toluene and 1,4-butanediol experiments were repeated using 1,1′,2,2′,3,3′,4,4′-octadeutero-1,4-butanediol in place of the 1,6-hexanediol, as the transesterification agent. A 10% solution of the d^8-1,4-butanediol in 1,4-butanediol was added to 5 g of the polyester, and 0.1 g of *Novozyme* 435. The mixture was stirred in a cell reactor at 60 °C and sampled at 24 and 48 h. Filtering off the *Novozyme* stopped the reaction and the residual 1,4-butanediol was stripped off using a Kugelrohr evaporator prior to observing the NMR spectra.

4.4. Molecular modelling

The molecular modelling was done using both Sculpt 3.0[13] and Sybyl 6.0[14] on a SGI Octane UNIX work station. The enzyme was modelled using the pdb file 1tca. A 2000 Da polybutane adipate polyester was constructed and docked manually to the Ser 105 residue of the active site. The Sculpt model was energy minimised using MM3 with both van der Waals and electrostatic interactions. The Kollman all Atom forcefield was used for the Sybyl model, which was then energy minimised using the Powell method within the Sybyl program.

Acknowledgements

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Generation of a dynamic combinatorial library using sialic acid aldolase and in situ screening against wheat germ agglutinin

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Abstract—This paper describes the generation of a dynamic combinatorial library of sialic acid analogues using sialic acid aldolase. Addition of wheat germ agglutinin to the equilibrating libraries results in selective amplification of one or more members.

1. Introduction

1.1. Dynamic combinatorial chemistry—the concept

Dynamic combinatorial chemistry (DCC) is a rapidly emerging field which offers a possible alternative to the approach of traditional combinatorial chemistry (CC).[1] Whereas CC involves the use of irreversible reactions to efficiently generate static libraries of related compounds, DCC relies upon the use of reversible reactions to generate dynamic mixtures. Selective binding of one member of the dynamic combinatorial library (DCL) to a molecular trap, such as the binding site of a protein, is expected to cause a shift in equilibrium to favour the formation of that member (Fig. 1). Comparison of the equilibrium product distribution of the 'perturbed' library with that of a control DCL will therefore indicate which members of the library are interacting with the trap.

The DCC approach offers in situ screening of combinatorial libraries, without the need for separation or deconvolution steps. The members of a DCL need not be produced with equal efficiency, since even members formed in trace amounts may still be amplified sufficiently for identification. In the extreme case, where amplification results in the collapse of the library to a single species, the method may be considered a tool for preparative synthesis.

Although attractive, the DCC approach presents significant experimental difficulties which have hindered its development. In particular, the choice of reversible reaction suitable for DCC is severely limited:

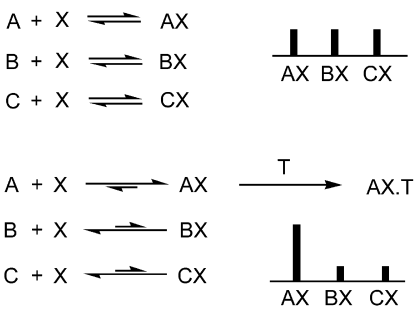

Figure 1. The DCC concept; reversible reactions performed with a limiting amount of **X** generate a mixture of compounds **AX**, **BX**, **CX**. Binding of **AX** to molecular trap **T** causes perturbation of the equilibria involving **A** and **X**, giving overall amplification of **AX** at the expense of the other library members.

- The reaction must accept a broad range of reacting components, in order to maximise the diversity of the library formed.
- It must proceed reversibly and efficiently under conditions which are appropriate to the recognition stage, and do not lead to a loss of binding activity in the trap. For drug discovery, in which the trap is usually envisaged as a protein or other biomolecule, this requirement translates to the use of near-physiological aqueous conditions.
- Once the system has reached equilibrium it must be possible to stop the reaction, converting the dynamic library to a static library for analysis.

Very few reversible reactions meet the above requirements, and consequently only a limited number of successful DCC experiments (involving small libraries) have been reported.

Keywords: Dynamic combinatorial chemistry; Aldolase; Sialic acid; Wheat germ agglutinin.
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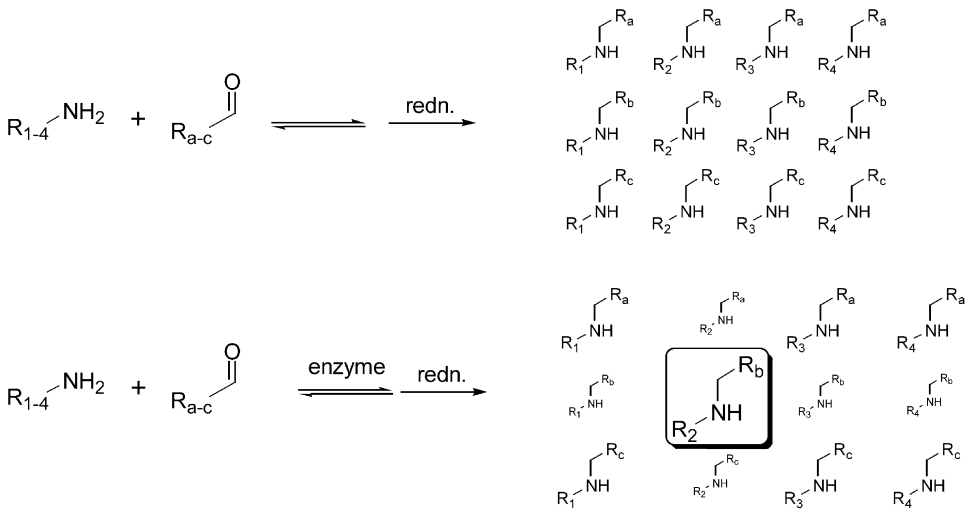


Scheme 1. Lehn's DCC experiment. When a (4×3) DCL of imines was prepared in the presence of an enzyme with binding affinity for one member, the product distribution was changed; the amine derived from the binding imine was amplified at the expense of other members derived from common components.

The discovery (or re-discovery) of reaction systems which are well-suited to DCC is therefore of prime importance to the future development of the method.

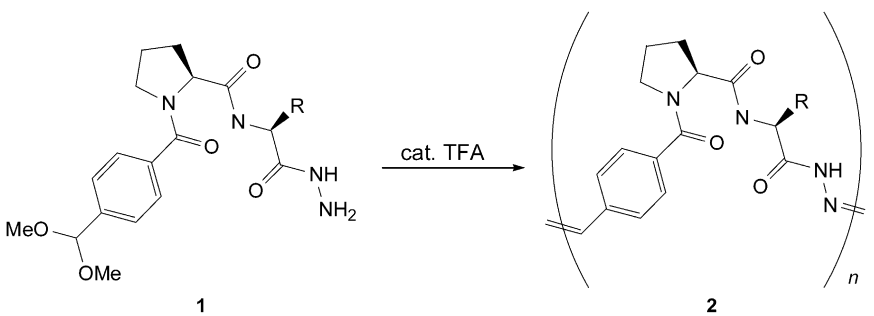
1.2. Examples of DCC

The DCC concept was clearly stated for the first time in 1996,[2] and shortly thereafter the first successful examples were reported. In 1997, Venton et al. reported the use of a protease (thermolysin) to generate a DCL, analysed by HPLC and sequencing, of at least fifteen short oligopeptides from an initial mixture of one dipeptide and one tripeptide.[3] A monoclonal antibody known to bind to one member of the DCL was used as the thermodynamic trap, and a modest amplification of the binding member (statistical analysis was necessary to verify that the variations recorded were significant) was observed. The use of enzyme catalysis enabled the generation of a DCL based on a transamidation reaction under physiological conditions; simple ultrafiltration removed the enzyme and reduced the DCL to a static library of robust compounds suitable for analysis.

A more impressive demonstration of DCC was provided by Lehn, also in 1997. Lehn employed imine formation as the reversible process, allowing the use of mild conditions in the generation of the DCL;[4] since the products were labile the library was 'frozen' by slow cyanoborohydride reduction, and the resulting mixture of amines analysed by HPLC. When a (4×3) library of twelve imines was prepared, both in the presence and in the absence of a stoichiometric amount of the enzyme carbonic anhydrase II (CA), clear qualitative differences were observed between the respective HPLC traces (Scheme 1). These differences indicated amplification of a strongly-binding imine (structurally similar to known inhibitors of CA) at the expense of the other library members sharing common amine or aldehyde components.

Several other examples of DCLs based on the formation and exchange of C=N bonds have been reported.[5,6] Systems based on transimination of hydrazones generated from amino acid-derived building blocks **1** have also been developed (Scheme 2),[7] in which TFA was employed both to deprotect the masked aldehyde function of the monomer, and to catalyse the formation of hydrazone-linked oligomers **2**. After generation of the DCL, the solution was adjusted to neutral pH, to give a static library of stable compounds for analysis. Again, the pseudopeptides produced in these experiments were screened against charged guest species. In one example, generation of the DCL in the presence of a stoichiometric amount of lithium iodide caused the collapse of the entire library to a single trimeric species, which was isolated in essentially quantitative yield.[7c] This efficient synthesis of a novel receptor for Li^+ from a diverse DCL provides a striking demonstration of the potential of (1→*n*) libraries.

Disulfide exchange also allows for the generation of DCLs



1 **2**

Scheme 2. In the presence of TFA, building blocks **1** were deprotected, and DCLs of hydrazone-linked oligomers **2** were generated.

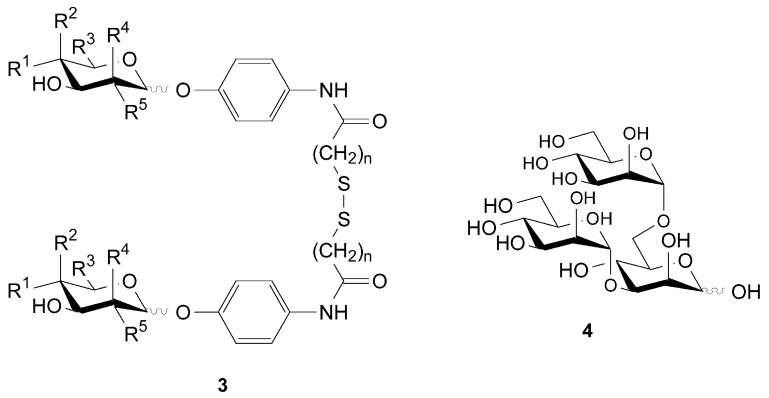


Figure 2. Disulfide exchange between 'bola-disacharides' **3** formed the basis for Lehn's DCL of carbohydrate mimetics. The library was screened against ConA, the preferred ligand for which is the trisaccharide **4**.

under mild conditions. Lehn investigated disulfide exchange as the basis for the generation of a DCL of 'bola-disaccharides' **3** (Fig. 2).[8] In the presence of dithiothreitol rapid disulfide exchange between a set of homodimers generated a DCL of up to twenty-one members, which was frozen by adjusting the solution to pH 4. This library was screened against Concanavalin A (ConA), a well-studied plant lectin whose preferred ligand is the mannose trisaccharide **4**.[9] Immobilised ConA was used to 'fish out' the binding species, which were eluted and analysed separately. The mannose homodimer was represented most strongly in the subset library of binding members; heterodimers containing one mannose residue were also bound. When the lectin was present throughout the scrambling process, some amplification of the mannose homodimer was observed.

1.3. Enzyme catalysis in DCC

As the above examples illustrate, the attractive features of DCC have been demonstrated in a number of successful experiments. Nevertheless, the development of DCC as a tool for drug discovery continues to be hindered by the requirement that, for this purpose, DCLs must be generated under physiological conditions.

We believe that enzyme catalysed reactions are ideally suited to the generation of DCLs for the following reasons:

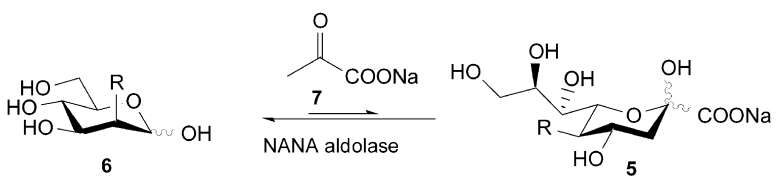
- They are characteristically reversible under aqueous, physiological conditions.
- The products of an enzyme catalysed reaction are usually stable compounds; simple removal or inactivation of the enzyme stops the reaction, reducing the dynamic mixture to a static library which may be analysed directly, without the need for a derivatisation step to freeze the product distribution.
- Many enzymes with broad specificity (required for library diversity) are already commercially available, and the application of modern techniques in directed evolution may be expected to increase their number.

Apart from the early work by Venton,[3] the use of enzyme catalysis has been largely overlooked in the approaches to DCC published to date. In the current study the generation of DCLs of carbohydrate-related compounds, and their screening against commercially available lectins, was investigated. The results of this work are presented in Section 2.[10]

2. Results and discussion

Initially, the enzyme-catalysed generation of a simple ($m\times n$) DCL, to be screened against a protein with known binding affinity, was undertaken. *N*-Acetylneuraminic acid aldolase (NANA aldolase, EC 4.1.3.3) was chosen as a suitable enzyme for this purpose. NANA aldolase catalyses the cleavage of *N*-acetyl neuraminic acid (sialic acid, **5a**) to *N*-acetyl mannosamine (ManNAc, **6a**) and pyruvate **7** (Scheme 3).[11a]

In the presence of excess pyruvate the equilibrium may be driven in the direction of aldol product formation. For this 'forward' reaction the enzyme requires sodium pyruvate as the nucleophilic equivalent, but will accept a range of reducing sugars as the electrophilic component. This synthetic activity has been exploited in the enzymatic



6a R = NHAc
6b R = OH

5a R = NHAc
5b R = OH

Scheme 3. NANA aldolase catalyses the cleavage of sialic acid **5a** to ManNAc **6a** and pyruvate **7**; in the presence of excess pyruvate, aldol products **5a** and **5b** may be generated from the respective substrates **6a** and **6b**.

preparation of a number of sialic acid analogues.[11] It was envisaged that a (1×2) DCL (the smallest possible) of aldol products **5a** and **5b** (ketodeoxynonulosonic acid, KDN) would be produced through the action of NANA aldolase on a mixture of the corresponding substrates **6a** and **6b** (D-mannose).

Wheat germ aglutinin (WGA), a well-studied and readily available plant lectin,[12,13] was chosen as the molecular trap. WGA is known to specifically bind sialic acid with modest (mM) affinity, where the diequatorial C-4 hydroxy and C-5 acetamido groups of sialic acid form the primary recognition motif. Thus, amplification of **5a** over **5b** was expected when the DCL was generated in the presence of WGA.

2.1. Library generation

Generation of the two-component DCL proved straightforward; a mixture containing equimolar amounts of the two substrates **6a** and **6b**, with 2 equiv. of sodium pyruvate, was incubated overnight in the presence of NANA aldolase. After thermal inactivation of the enzyme and filtration, the mixture was analysed by high-field ^{1}H NMR. A 1:1 mixture of the aldol products, resulting from 40% conversion from the respective substrates, was indicated, confirming that the enzyme accepted both substrates with similar affinities.

In order to generate the 'perturbed' library, the incubation was to be performed in the presence of a stoichiometric amount of WGA binding sites, based on the observed equilibrium concentration of **5a**. At neutral pH WGA exists as a dimer of four-domain monomers, total molecular weight 43 kDa, with four independent binding sites. The reported affinity constants for sialic acid binding to WGA are based on whole-cell agglutination experiments, which must be interpreted with caution, but these indicate 50% saturation of WGA in 5 mM sialic acid. The incubations were therefore scaled up to produce ca. 1 μmol of each aldol product (at ca. 1–5 mM) at equilibrium. At this scale ^{1}H NMR was no longer practical, and all analyses were performed by ion-exchange HPLC. An HPLC trace of a control incubation is shown below (Fig. 3); the aldol product peaks are clearly resolved and have roughly equal areas.

Longer incubations produced the same product distribution, indicating that the reaction had reached equilibrium overnight. However, the composition of the diluted aliquots did change on standing; in these solutions, a slow drift toward the substrates was observed, indicating that some residual enzyme activity remained after heat treatment. When the denaturation step was made more stringent (5 min at 95 °C), some decomposition of the aldol products was observed. The use of molecular weight cut-off filters to remove the enzyme by filtration was also investigated, but there were concerns that, in the presence of WGA, some lectin-bound sialic acid might be retained on the filter. The most direct solution to these problems was adopted; aliquots of the incubation mixtures were diluted and immediately injected for HPLC analysis. Although somewhat crude, this procedure did prove successful in 'freezing' the DCL without decomposition, and ensuring quantitative recovery of bound species for analysis. Using this method, and provided that aliquots of the incubation mixtures were much diluted before injection, consistent and linear response factors were observed in the peaks assigned to the aldol products.

By reference to stock solutions made up from commercial samples, a ca. 15% conversion from substrate was estimated for each product. A solution was made up with the estimated equilibrium composition; this solution produced an HPLC trace very similar to those observed for 'real' incubation mixtures, and on incubation with NANA aldolase the composition changed only in that it moved closer to the observed equilibrium mixture.

That the enzyme does indeed catalyse both aldol formation and cleavage on the timescale employed was demonstrated by re-equilibrating a mixture of **5a** and **6b** in the presence of NANA aldolase; after incubation overnight the mixture contained, in addition to the initial components, **5b** and **7** which could only arise through a retro-aldol cleavage of **5a** followed by aldol formation of **6b**.

Having confirmed that NANA aldolase was able to accept both **6a** and **6b** as substrates with similar affinity, that roughly equimolar mixtures of the corresponding aldol products could be readily generated and analysed, and that the mixtures produced were truly dynamic, incubations in the presence of WGA were attempted. Since the incubations contained no internal standards, and hence could not be normalised to account for variations in aliquot volumes, equilibrium product distributions were expressed as fractions of the total area of the peaks assigned to aldol products (% Total Aldol Product Area, %TAPA). Relative amplification was then determined by comparing %TAPAs determined in the presence and absence of WGA.

Overnight incubations performed in the presence of WGA

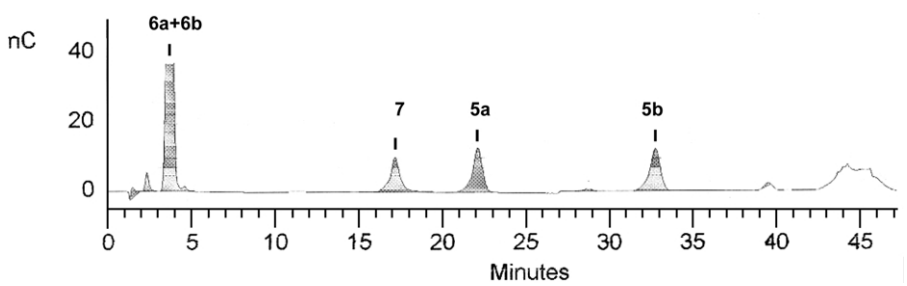


Figure 3. HPLC trace for the (1×2) control DCL. Substrates **6a** and **6b** co-elute, but the aldol products **5a** and **5b** are well-resolved and give reproducible relative areas. This product distribution was present after 24 h incubation, and remained unchanged for at least the following 6 days.

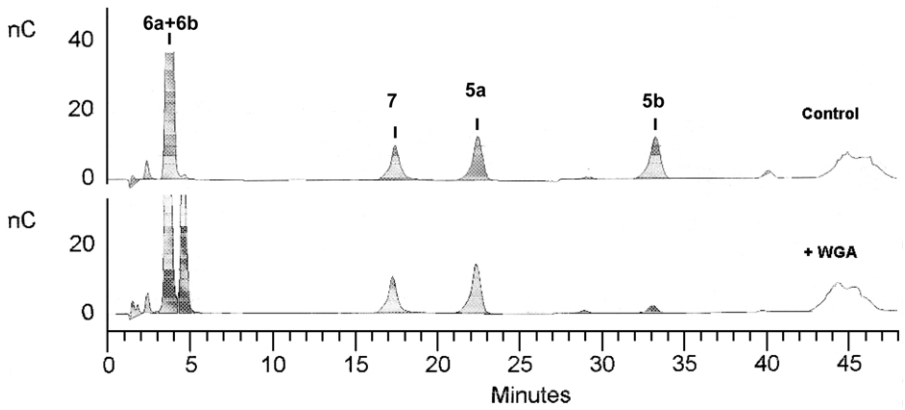


Figure 4. Comparison of control and 'perturbed' (1×2) libraries after 7 days incubation. In the presence of WGA, the production of nonbinding member **5b** has been almost completely suppressed in favour of WGA-binding member **5a**.

showed reproducible but small (ca. 10%) amplification of **5a** over **5b**, but the extent of amplification increased with increasing reaction time (Fig. 4). The system appears initially to approach a similar distribution to that observed in control incubations, but thereafter slowly to deviate toward a new equilibrium position. After 7 days incubation the peak assigned to **5a** had risen from 49 to 85% TAPA, a 73% amplification, while the peak assigned to **5b** had dropped from 51 to 15% TAPA, a 71% suppression.

In order to prove that the observed changes were not caused by a selective aldolase activity associated with the lectin preparation, a mixture approximating the equilibrium distribution of the control incubation was prepared from commercial samples, and incubated in the presence of WGA (but not NANA aldolase). No change from the initial distribution was observed, demonstrating that WGA affects the DCL by acting as a thermodynamic trap, and not as a selective catalyst for KDN degradation.

Microcalorimetry experiments were undertaken to provide independent confirmation of the observed WGA binding affinities (Fig. 5); although **5a** binding to WGA is weak, a binding affinity of 172 M^{-1}, corresponding to 50% binding at 5.8 mmol L^{-1}, was determined, while no detectable binding affinity was observed for **5b**.

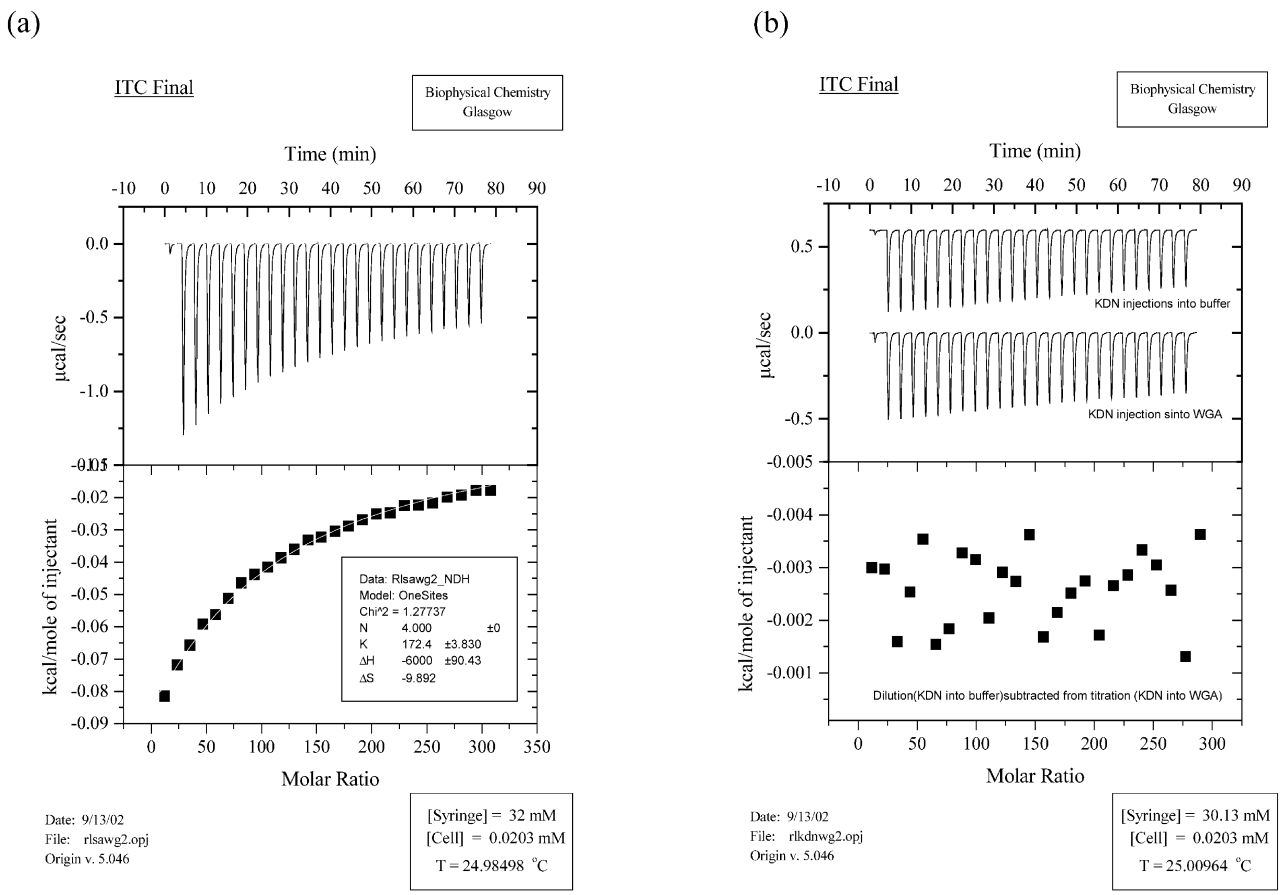
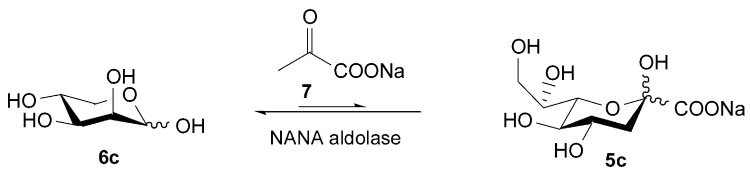


Figure 5. Isothermal titration curves for the binding of WGA to (a) **5a** and (b) **5b**.



Scheme 4. KDO **5c**, the third library member, is produced from D-lyxose **6c**.

2.2. Three components

D-Lyxose **6c** was chosen as a third substrate for the generation of a (1×3) DCL (Scheme 4). Despite having the same C2–C4 configuration as **6b**, **6c** has been shown to be a relatively poor substrate for NANA aldolase.[11a] The corresponding ketodeoxyoctulosonic acid **5c** (KDO) is the eight carbon analogue of **5b**, and was thus not expected to bind to WGA.

Incubations performed with the three substrates **6a**–**c** gave 1:1:2 mixtures (in terms of peak area) of the respective aldol products **5a**–**c**. **5c** is not available commercially, so a quantitative analysis of the mixture was not attempted, but it was assumed that the response factor for this compound was unlikely to differ greatly from those of **5a** and **5b**. Conversion from **6c** to **5c** was thus estimated to be in the range 15–30%. When the incubation was performed in the presence of WGA, a similar pattern of amplification of **5a** (53%) with suppression of **5b** (72%) was observed. The peak assigned to KDO was not suppressed, but showed a small relative amplification (10%).

This observation that **5b** and **5c** were not suppressed to the same extent might be due to WGA having a weak binding affinity for **5c**, such that the latter species may also experience some measure of relative amplification during incubation in the presence of the lectin. In effect, the extent of relative amplification observed in the above experiment might suggest a ranking order of the three components based on the relative affinities of WGA for each of them. In this case, the two-component DCC experiment involving only **5b** and **5c** should demonstrate some degree of amplification of **5c**, while that involving only **5a** and **5c** should show amplification of **5a** over **5c**. When these two-component experiments were performed, the results were not conclusive; the predicted amplifications were observed, but with much smaller magnitude than those involving the **5a**–**5b** pair. If **5c** has appreciable binding affinity for WGA there should be significant amplification of **5c** over **5b** (which has no binding affinity), whereas if **5c** has only very small binding affinity for WGA then the amplification of **5a** acid over **5c** should be significant (Table 1).

An alternative explanation for the observed results is that the equilibrium amount of **5c** is not sensitive to **5a** binding to WGA. When **5a** binds to WGA the resulting changes in product distribution are mediated by NANA aldolase. If the rate of **5c**–**6c** interchange is slow compared to the rate of **5b**–**6b** interchange, then the latter couple might be expected to bear the brunt of re-equilibration.

Table 1. Summary of the results from two-component libraries after 7 days incubation

Components	Control %TAPA		Perturbed %TAPA		% Amplification	
5a+**5b**	49	51	85	15	+73	−71
5a+**5c**	36	64	40	60	+11	−6
5b+**5c**	19	81	15	85	−21	+5

Timecourses for the three-component DCLs were measured in order to investigate the relative rates of formation of the library members. The graph below (Fig. 6) shows the development of the control library in terms of absolute peak areas for each aldol product (a) and in terms of %TAPA (b). The data clearly indicate that **5a** is formed most rapidly, while **5c** is generated relatively slowly. After 24 h incubation the system is at equilibrium, and the distribution is then stable for at least the next 6 days.

In the presence of WGA, the formation of the DCL proceeds in the same manner, with slow generation of **5c** (Fig. 7). The product distribution does not stabilise after 24 h, but drifts in the direction of increased **5a** and decreased **5b**, due to WGA binding of the former. A plot of relative amplification over time for each component (Fig. 7c), shows these changes more clearly. The slight (10%) amplification observed for **5c** may not be significant considering the variations in %TAPA observed for this component over the first two hours incubation. Similarly, a small kinetic effect (amplification of **5a** in the first two hours of incubation) is recorded, but may not be significant.

2.3. Four components

In order to further investigate the behaviour of poor enzyme substrates in the DCL, a (1×4) library was investigated. The fourth substrate chosen was D-galactose **6d**, which is known to be a poor substrate for NANA aldolase.[11a] Further, the nonulosonic acid produced (Scheme 5, designated somewhat glibly as 7-*epi*-KDN **5d**) differs from **5b** only in the configuration at C-7, and thus is not expected to possess any greater binding affinity for WGA than does the latter. Thus, if in general poor enzyme substrates produce false positives in the presence of better substrates, then both **5c** and **5d** should show no suppression in the four-component DCL. If the observed behaviour of **5c** is due to a real binding affinity, the four-component DCL should show suppression of both **5b** and **5d**.

The HPLC traces for the control and perturbed four-component DCLs after 7 days incubation are shown below (Fig. 8). Although the peaks due to **5b** and **5d** were not fully resolved with the elution protocol used, and hence accurate peak areas were not available for these components individually, it can be seen that in the perturbed library **5b** has been greatly suppressed, while **5d** has remained relatively unchanged. This result is supported by the results from the two-component DCL formed from **5a** and **5d**

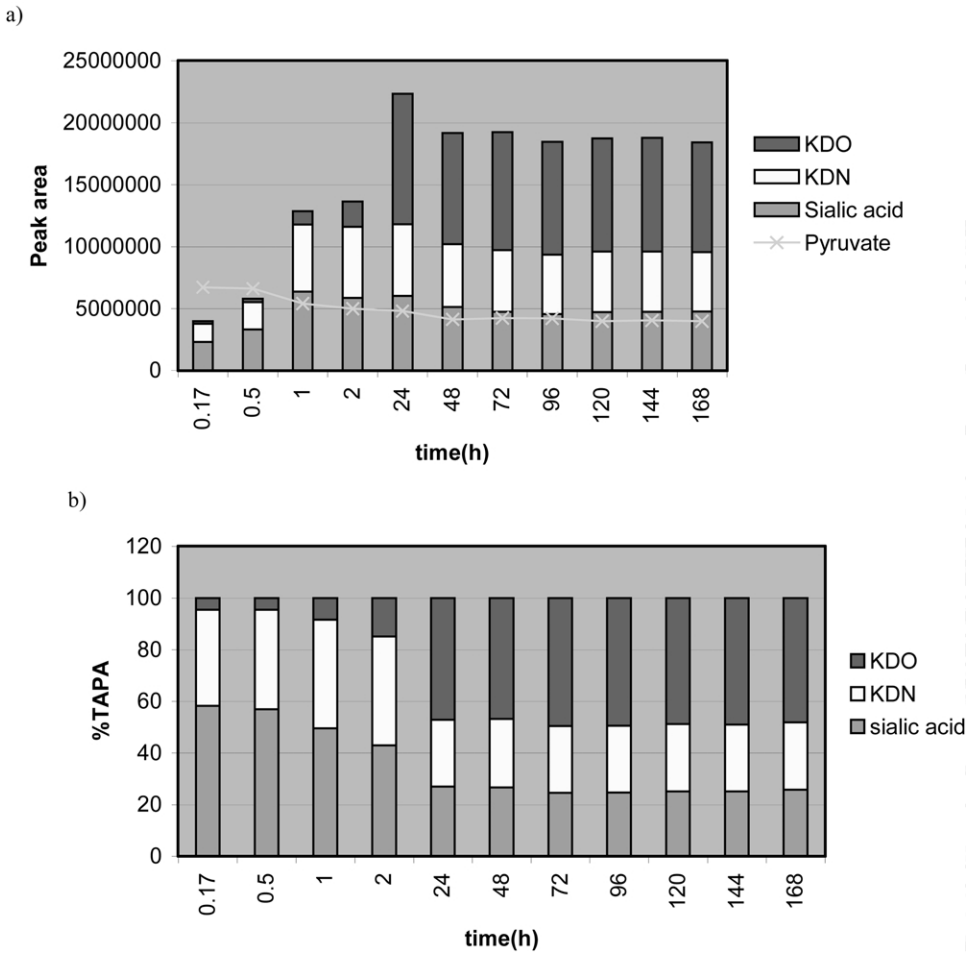


Figure 6. Timecourse for three-component control DCL, measured by (a) absolute peak areas and (b) %TAPA.

(Table 2), which again shows at best only slight amplification of **5a** over **5d**.

The experiments described above constitute the first clear demonstration of the application of enzyme catalysis to DCC. The use of an aldolase allowed for the straightforward preparation of a DCL through stereoselective carbon–carbon bond formation; the members of the library produced were complex molecules of biological interest which are difficult to prepare chemically. It is significant for the development of DCC in general that even the very weak binding affinity of WGA for sialic acid was still sufficient to produce a marked change in the equilibrium product distribution of the DCL; traps with greater affinity should allow for successful detection at lower concentrations.

The major limitation of enzyme catalysis in DCC was also highlighted in the example studied; the preparation of libraries of useful size requires an enzyme with broad specificity. In this example, only two components of the four-component DCL were good substrates for NANA aldolase, and only these two responded to the presence of WGA as a thermodynamic trap. It is important to note that the data still clearly indicates sialic acid **5a** as the best binder to WGA, but it would be of interest to repeat the aldolase/WGA experiments with a different aldolase for which **5a** was the poorer substrate. In such a system, sialic acid binding to WGA would still occur, and sialic acid amplification (and the subsequent suppression of other components) would be expected to follow, though at a lower rate. Provided that significant amplification may still be observed when the binding member is a poor substrate, the absence of suppression among the poorer response from some with binding affinity may still produce significant amplification In general, the problem of high enzyme specificity might be addressed by performing independent DCC experiments with enzymes of different specificity. Ultimately it is anticipated that the application of modern techniques in directed evolution will increase the number of available enzymes with truly broad specificity; there is evidence that increased activity through directed evolution is often associated with a loss of specificity.[14]

3. Experimental

HPLC analyses were performed on a Dionex DX-500 Chromatography system consisting of a LC30 Chromatography Module, GP40 Gradient Pump, ED40 Electrochemical Detector (gold electrode and amperometry cell), AS3500 Autosampler (stainless steel needle), controlled from a workstation running PeakNet v4.30. A CarboPac PA-100 column with guard column was used, flow rate 1 mL min^{-1}. Solvents used were 100 mM sodium hydroxide, 100 mM sodium hydroxide/1 M sodium acetate, water.

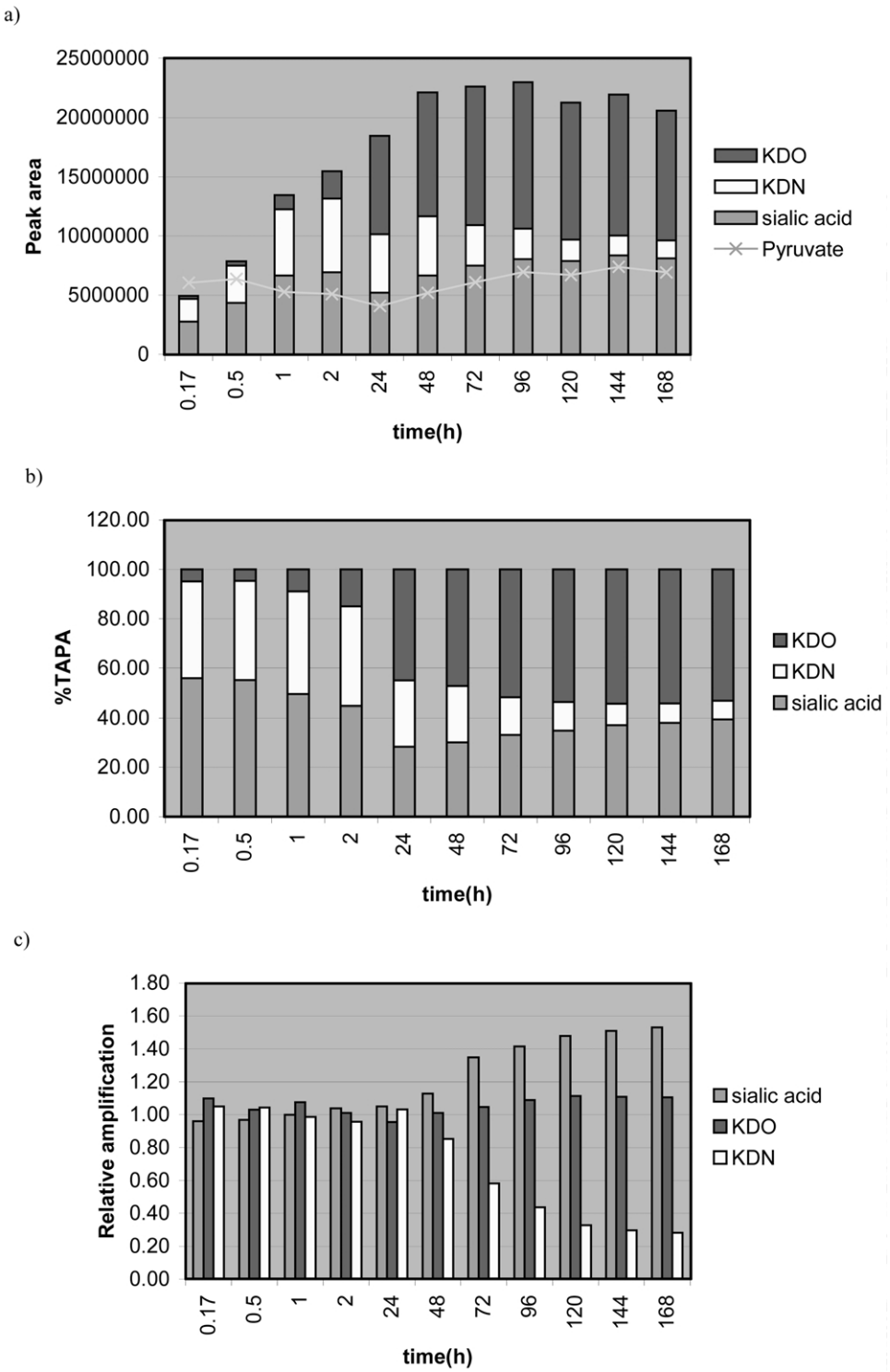
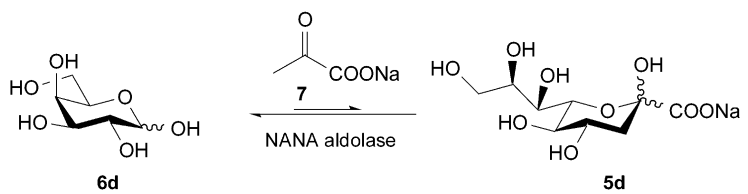


Figure 7. Timecourse for the three-component peturbed DCL, measured in (a) absolute peak areas, (b) %TAPA, and (c) relative amplifications over the control DCL.



Scheme 5. 7-*epi*-KDN **5d**, the fourth library member, is produced from D-galactose **6d**.

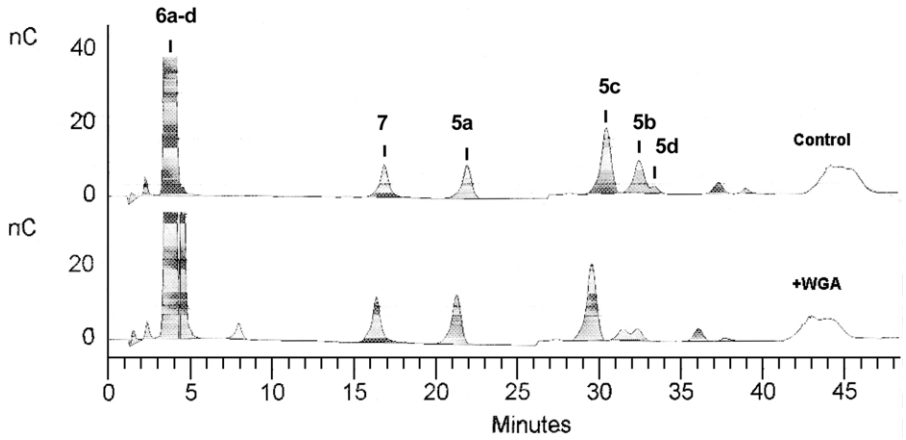


Figure 8. Comparison of perturbed and control (1×4) DCLs after 7 days incubation. **5a** has been amplified, but only **5b** shows significant suppression.

Table 2. Summary of results from libraries containing **5d**

Components	Control %TAPA			Perturbed %TAPA			% Amplification		
5a+**5c**+(**5b** and **5d**)	23	48	29	32	53	15	+39	+10	−48
5a+**5d**	75	25		76		24	+1		−4

N-Acetyl neuraminic acid aldolase (EC 4.1.3.3, cat. 153493, 22.2 U mg^{-1}) and wheat germ agglutinin (cat. 152266, lyophilised powder) were purchased from ICN Biomedicals. Substrate sugars and other chemicals were purchased from Sigma.

3.1. Large-scale preparation of two-component DCL

A stock solution was made up containing 29 mg **6b** (160 μmol), 38 mg **6a** (160 μmol), 35 mg **7** (320 μmol) and 1 mg sodium azide in 50 mM phosphate buffer, pH 7.6. To 50 μL of this stock was added 50 μmol NANA aldolase stock solution (2 U mL^{-1} in 50 mM phosphate buffer, pH 7.6), and the mixture incubated at 37 °C overnight. The solution was heated to 80 °C for 2 min, cooled, and diluted to give 600 μL of a 10% D_2O in water solution for NMR analysis.

δ_H (600 MHz) inter alia 8.02 (1H, br. d J=9.3 Hz, **5a** NH), 7.88 (1H, br d J=10.3 Hz, **6a** NH), 5.16 (1H, s, **6b** 1-H_α), 5.11 (1H, s, **6a** 1-H_α), 5.01 (1H, s, **6a** 1-H_β), 4.88 (1H, s, **6b** 1-H_β), 2.20 (1H, dd J=13.2, 4.9 Hz, **5a** 2-H_{ax}), 2.15 (1H, dd J=12.7, 4.9 Hz, **5b** 2-H_{ax}), 1.83 (1H, t J=12.2 Hz, **5a** 2-H_{eq}), 1.78 (1H, t J=12.2 Hz, **5b** 2-H_{eq}) ppm.

3.2. General procedure for the preparation of small-scale DCLs

Phosphate-buffered saline (PBS) contained 150 mM sodium chloride, 10 mM sodium phosphate, and 0.02% w/v sodium azide, adjusted to pH 7.5. NANA aldolase stock was 20 U mL^{-1} in PBS. WGA stock was 50 mg mL^{-1} in PBS. Substrate stock solutions contained 50 μmol of each substrate sugar (12 mg **6a**, 9 mg **6b**, 7.5 mg **6c**, and/or 9 mg **6d** as required) and 100 μmol **7** in 1 mL PBS. Control incubations contained 10 μL substrate stock solution, 20 μL PBS, and 10 μL aldolase stock. For the generation of perturbed DCLs 20 μL WGA stock was substituted for PBS. These mixtures were centrifuged for 1 min at 5000 rpm, 20 μL paraffin oil was added, the mixtures were centrifuged again, and incubated at 37 °C. At intervals 5 μL aliquots were withdrawn, added to 495 μL water, and the diluted solutions immediately analysed by HPLC.

3.3. Microcalorimetry

Isothermal Titration Calorimetry (ITC) was performed by staff at the BBSRC/EPSRC Biological Microcalorimetry Facility at the University of Glasgow, according to standard procedures. WGA (Sigma) was used without further purification and was dissolved in PBS (0.15 M NaCl, 0.1 M P_i, 0.02% NaN_3, pH 7.5) and degassed before use. Ligands **5a** and **5b** were dissolved in the same buffer. Protein concentrations in the ITC cell were determined from UV absorbance measurements at 280 nm using an absorption coefficient of 1.5 $mg^{-1}cm^2$.[15] Control experiments were performed under identical conditions by injection of ligand into buffer alone (to correct for heats of ligand dilution).

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An alternative bioreactor concept for application of an isolated oxidoreductase for asymmetric ketone reduction

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Abstract—In this paper an isolated NADH dependent ketone reductase has been used to synthesise (*S*)-6-bromo-β-tetralol from 6-bromo-β-tetralone, together with commercially available formate dehydrogenase (FDH) as a recycle enzyme to produce preparative quantities of the product. Furthermore, initial experiments indicate potential for an alternative bioreactor concept via the use of a resin (XAD L-323) to bind the product (and residual substrate) of the conversion rather than the cofactors or enzymes, thus allowing a new method of recycle, potentially overcoming existing problems.

1. Introduction

Regio- and stereoselective oxidation and reduction are of paramount importance in synthetic strategies and a range of biocatalysts are available to assist.[1,2] Oxidoreductases are amongst the most useful of all the classes of enzyme to assist organic chemists.[3,4] However, there are practical problems which still prevent widespread implementation. A good example which illustrates some of these limitations is the application of a ketone reductase recently found and isolated from *Trichosporon capitatum*.[5,6] The enzyme can be used in whole cell format to effect the reduction of 6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol (a potential pharmaceutical precursor[7]) with 98% ee (Fig. 1). While the NADH cofactor, which is required, can be recycled in the cell via judicious choice of cosubstrate, other problems are still evident. The reactant is highly insoluble in aqueous environments (0.5 g/L in water) and the rate of dissolution into the reaction mix is too slow to use it as a solid. A solvent is, therefore, required to solubilise the reactant for the reaction. In addition, although the cells displayed high activity, the reactant and product adhered to the cell. Recovery of the product (6-bromo-β-tetralol) was achieved only by the addition of ethanol, which subsequently caused irreversible cellular damage and prevented the cells from being recycled. The limiting factor in the whole cell system was the reactant and product adherence to the cell. However, the solvent, which was used to dissolve 6-bromo-β-tetralone also reduced cell viability. The cells achieved a maximum productivity of 1.6 g of product per gram of cell mass, with a conversion of 63%.

As a result of these initial findings, we have more recently been investigating the isolation of this enzyme with a view to running an isolated immobilised enzyme process with a second auxiliary enzyme, FDH,[8] for NADH recycle. Formate dehydrogenase can be produced in bulk quantities from *Candida boidinii*,[9] and to date, only the use of FDH from *C. boidinii* for the regeneration of NADH has been demonstrated on a technical scale.[4] The NADH dependent

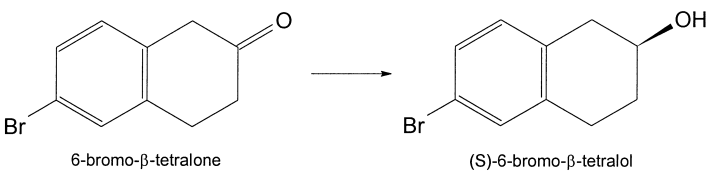

Figure 1. Conversion of 6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol by ketone reduction in *Trichosporon capitatum* (MY 1890).

Keywords: Oxidoreductase; 6-Bromo-β-tetralone; *Trichosporon capitatum*.
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tetralone reductase was successfully isolated from the yeast cells of *T. capitatum* (MY 1890).[6] The enzyme exhibited a half-life of 7 h at 22 °C after the Q-Sepharose chromatography column. The half-life was increased to 90 h at 22 °C by removing the EDTA, which was a component of the protease inhibitor. The enzyme could be further purified via chromatography using hydroxy apatite and toyopearl resins sequentially.[6] However, the isolated ketone reductase did not bind effectively to the immobilisation support, while the substrate did, and this therefore, led to a re-evaluation of the logic for immobilisation.

Figure 2 indicates three schemes which can be used downstream of an isolated enzyme reactor for separation of the components. Figure 2, Scheme I employs immobilised biocatalysts(s) (B) which can be easily separated by filtration followed by a subsequent separation of cofactor (C) and product (P). This second step is not straightforward and the use of NADH attached to PEG for example, has been shown to be effective in aiding filtration.[10] In fact with such a system, both biocatalyst and cofactor can be isolated with filtration and retained for recycle to the reactor (Fig. 2, Scheme II). Clearly attaching PEG to the NADH has consequences, as does immobilisation resulting in reduced activity unless very small beads can be used to overcome diffusional limitations (and these are then difficult to filter). An alternative approach is to retain the product on a support and allow the biocatalyst(s) and cofactor to leave the reactor

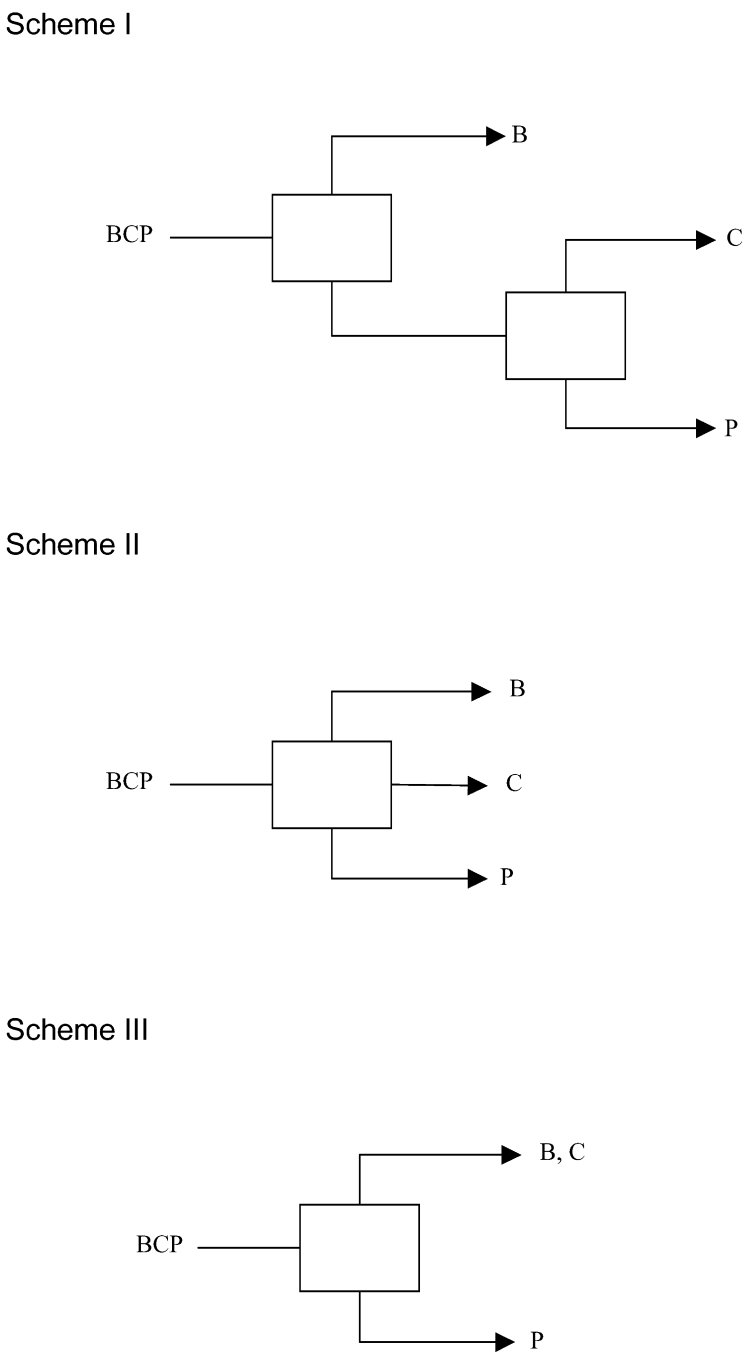

Figure 2. Possible schemes for separation of biocatalyst (B), cofactor (C) and product (P) from an isolated oxidoreductive conversion.

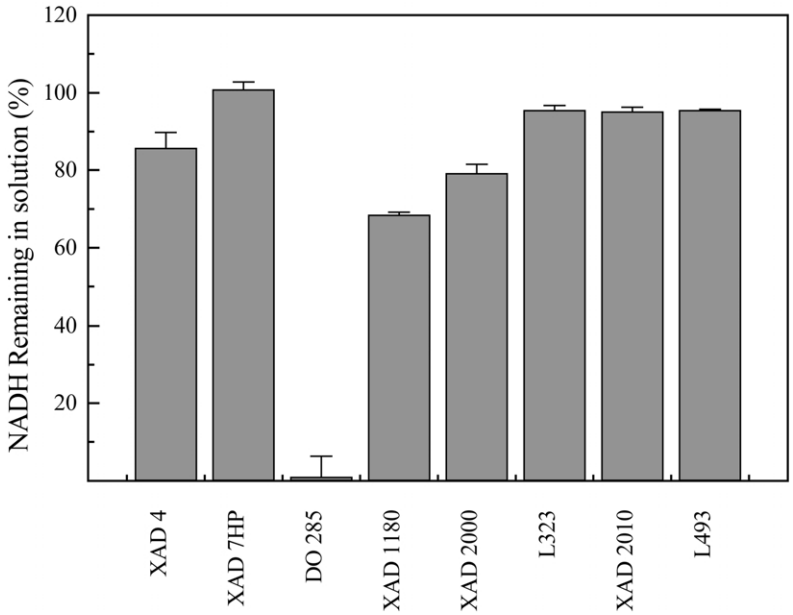

Figure 3. Resin screen for NADH binding. Error bars were calculated as standard deviations of multiple reactions.

ready for recycle. Subsequently product can be eluted. In many cases finding a product-selective carrier is particularly difficult[11,12] but a non-selective carrier will still allow effective separation for downstream processing providing a high conversion is achieved in the reactor (Fig. 2, Scheme III). In this paper, we explore such an approach using the reduction of (*S*)-6-bromo-β-tetralone as an example.

2. Results and discussion

2.1. Immobilisation of tetralone reductase

Immobilisation of tetralone reductase was ineffective as the reactant bound to the Eupergit C® beads and therefore the activity of the immobilised enzyme could not be measured. This binding property observed with Eupergit C was subsequently used as the basis for the alternative reactor concept.

2.2. Resin screen for NAD(H) binding

A range of cheap XAD resins were obtained and screened for their binding of NAD(H). If the cofactor, NADH was to be reused, then it must not adsorb on the resins (Fig. 2). Results for these trials are shown in Figure 3. Dowex optipore (DO 285) showed significant adsorbance of NADH. For effective product recovery, the resins needed to adsorb the reactant and product, not the enzymes or cofactor. The resins, which were found to be the most successful at retaining NADH in solution were XAD 7HP, XAD L-323, XAD 2010 and XAD L-493.

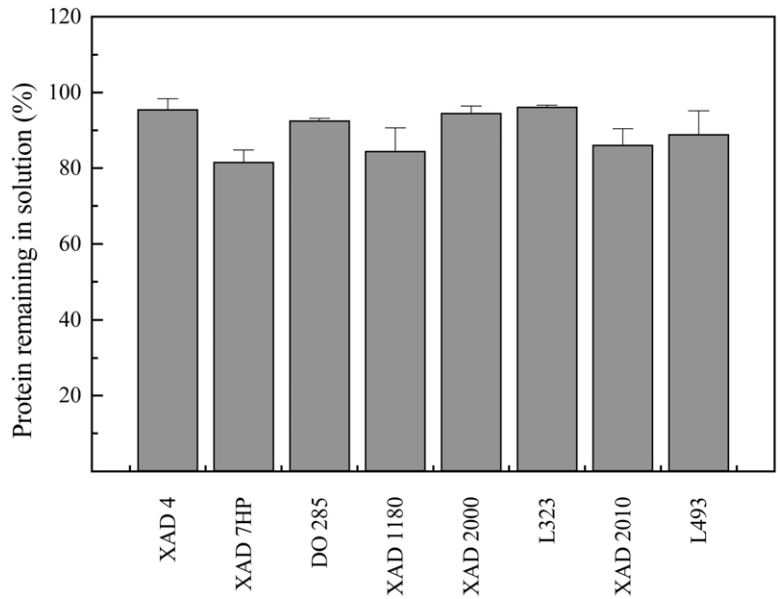

Figure 4. Resin screen for protein binding. Error bars were calculated as standard deviations of the multiple reactions.

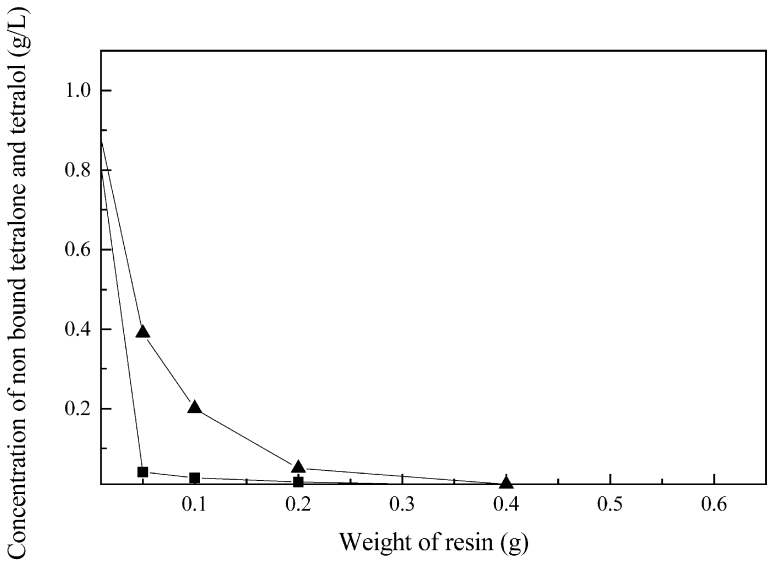

Figure 5. Adsorbance of 6-bromo-β-tetralone (■) and 6-bromo-β-tetralol (▲) on XAD L-323.

2.3. Resin screen for protein binding

The resins were also screened for their ability to adsorb the enzymes (ketone reductase and FDH) in the samples (Fig. 4). If the enzymes were to be reused, then they should not adsorb onto the resin. Little adsorbance of the enzymes was observed on the resins tested.

2.4. Binding of reactant and product on XAD L-323

If product removal was to be employed in a process, the resin used to adsorb the product and unconverted reactant must have a high affinity towards these compounds, but must reject the cofactors and the enzymes. 6-Bromo-β-tetralone was easily adsorbed onto the XAD resin L-323 (Fig. 5). An unoptimised mass of 50 mg of the resin adsorbed the 10 mg of tetralone in the solution. 6-Bromo-β-tetralol was less easily adsorbed onto the XAD resin (Fig. 5). An unoptimised mass of 200 mg of the resin adsorbed the 10 mg of 6-bromo-β-tetralol in the solution.

2.5. Bioconversion

A bioconversion was initiated using highly concentrated 6-bromo-β-tetralone dissolved in methoxyethanol, which had previously been found to be the most effective solvent.[6] This would reveal whether an efficient bioconversion could be achieved using low volumes of solvent, but high reactant concentrations. The regenerating enzyme and cosubstrate were both added to the reaction mixture in excess. Results revealed that the recycling enzyme system worked effectively. Using the initial reactant concentration of 1 g/L gave a high initial activity for the enzyme (Fig. 6) and a high conversion (Fig. 7). This initial reactant concentration of 1 g/L was used in subsequent bioreactor experiments.

2.6. Recycle bioreactor (10 mL)

A 10 mL batch bioreactor was run to prove that the bioconversion could be implemented, with subsequent cofactor and enzyme recycling, and regeneration. The

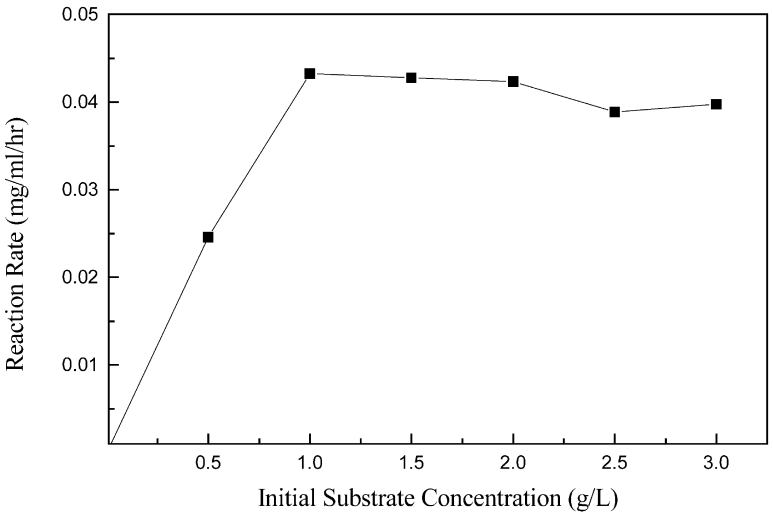

Figure 6. Effect of initial substrate concentration on the rate of reaction. 100 mL of methoxyethanol was used to dissolve 6-bromo-β-tetralone in 10 mL of reaction mixture.

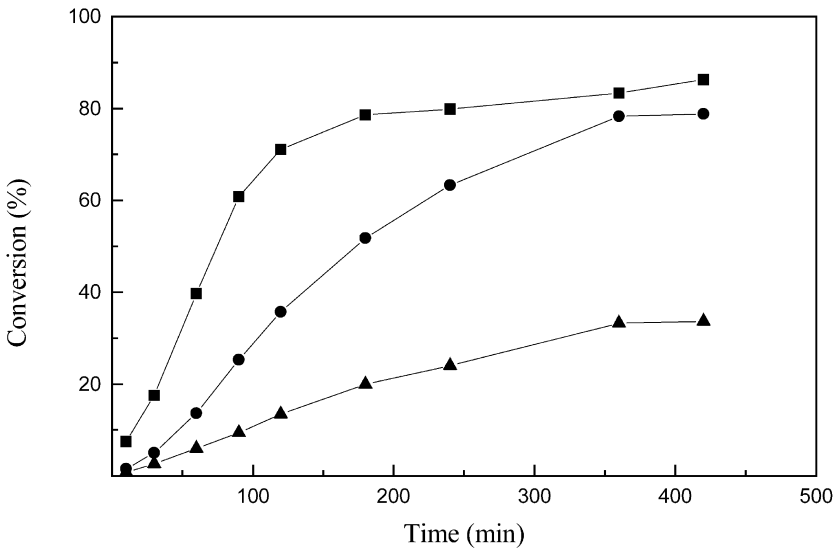

Figure 7. Bioconversion profile of 6-bromo-β-tetralone to 6-bromo-β-tetralol using methoxyethanol as the solvent with an initial reactant concentration of 1 g/L (■), 5 g/L (●), and 10 g/L (▲).

method used a batch reactor, in which all the reaction components were added simultaneously. Prior to filtration, the resin was added to the reaction components and left to adsorb the residual reactant and product, as shown in the method diagrammatically represented in Figure 8. The conversion-time profile is shown in Figure 9. A second pass was run with the enzyme and cofactor from the first batch and the reaction followed a similar reaction profile (Fig. 9),

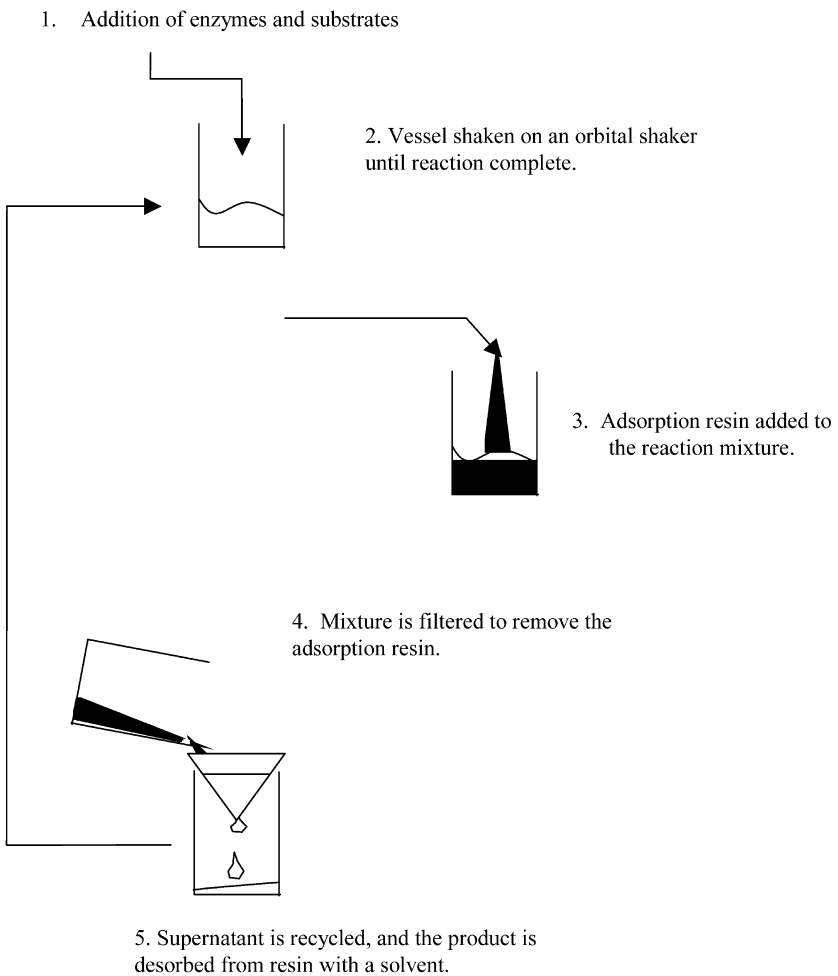

Figure 8. Reaction methodology for the batch production of 6-bromo-β-tetralol.

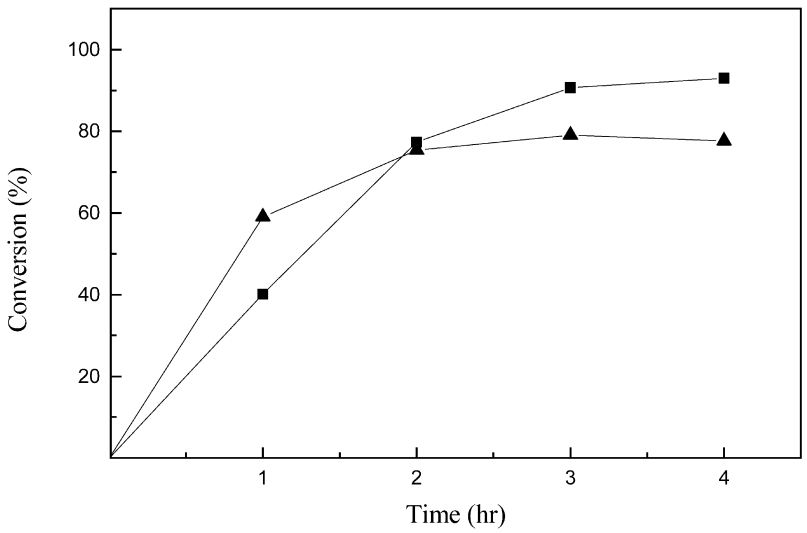

Figure 9. Bioconversion profile for a first (■) and second batch (▲), where the product and remaining reactant have been separated from the enzymes and cofactors, which have already completed a first batch reaction.

although top up enzyme was added. Complete conversion was not met in either case, with the second pass showing lower conversion than the first pass.

3. Conclusions and outlook

In this paper a new concept for operation of an isolated oxidoreductase has been outlined using immobilisation of (reactant and) product rather than enzyme(s) and cofactor. The concept has been briefly illustrated using the reduction of a ketone with an isolated reductase from yeast. The system is unoptimised but has potential for operation providing the isolated enzyme is sufficiently stable. Three options exist for implementation. First, the potential configuration of the bioreactor could be a (continuous) stirred tank reactor (Fig. 10). This would be particularly attractive since the enzyme is not immobilised onto a resin,

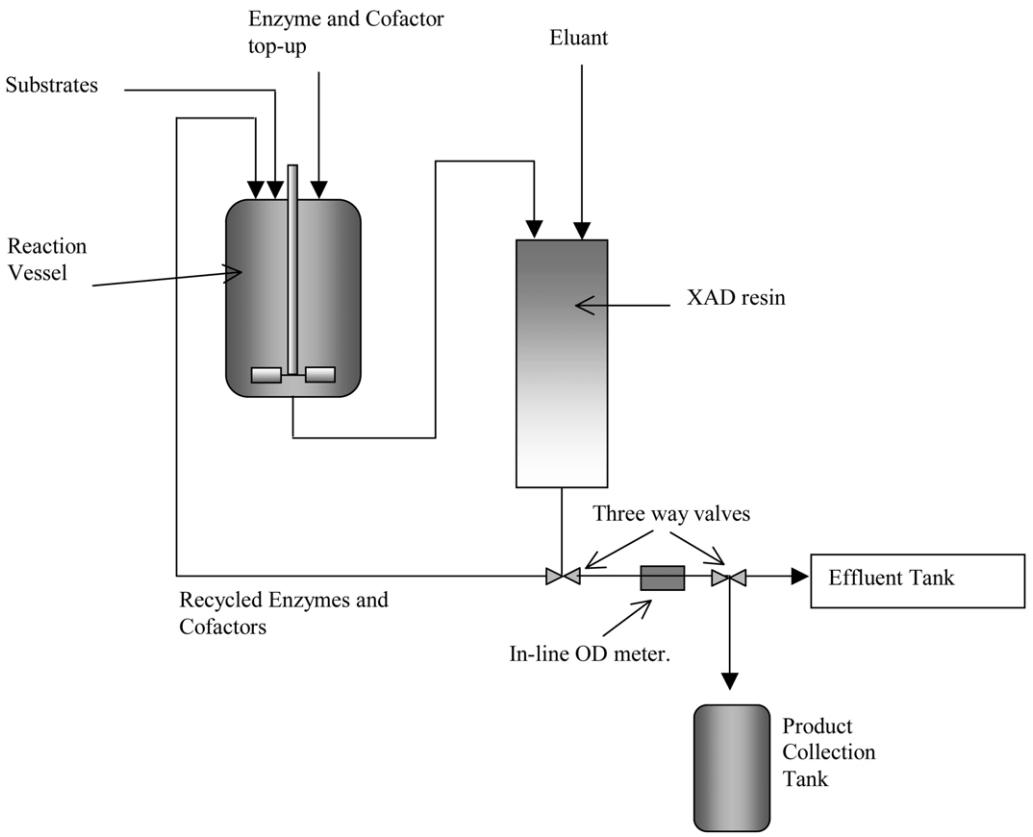

Figure 10. Possible design of the bioreactor with product removal and cofactor recycle.

and need not be retained behind a membrane. The reactants could be added to the bioreactor, whereby the enzymes and cofactors carry out the asymmetric synthesis. Since, the concentration of reactant in the reactor is the same as that in the leaving stream, toxic reactants could be handled effectively.[13] After the reaction is complete, the reaction mixture could then be passed through a column of XAD-323 resin where the product (and residual reactant) would be retained and the enzyme(s) and cofactors recycled back to the reactor where more reactant would be added. More enzyme and cofactor could be added with the reactant to top up the volume, if there are losses in the XAD column. Separately, the product would be eluted from the XAD resin using a solvent, in this case, ethanol. Based on initial experiments, using ethanol as an eluant would give a final product concentration of approximately 10 g/L. A higher product concentration could be achieved by using a solvent with a higher solubility of 6-bromo-β-tetralol, and hence a smaller volume.

Secondly, if the enzyme could be stabilised using a resin which 6-bromo-β-tetralone and 6-bromo-β-tetralol did not adhere to, the same bioreactor concept could still be used. The immobilised enzyme(s) could be retained in a first column (instead of an STR), and the cofactor and other reaction components could leave the reactor to the XAD resin on a second column, as before, and the cofactors recycled back into the bioreactor in continuous operation. This might give added stability to the enzyme(s), where operation with the isolated enzyme was not possible.

Finally, the third option could be to use an adsorbent resin as a method of substrate delivery and removal. This has previously been described for whole cell yeast mediated stereoselective ketone reductions.[14–16] In one example,[15] at scale, 3,4-methylene-dioxyphenyl acetone was reduced to the corresponding (*S*)-3,4-methylene-dioxyphenyl isopropanol in greater than 95% isolated yield and 99.9% enantiomeric excess. The supply of the reactant and the removal of the product, via a polymeric hydrophobic resin allowed the reaction concentration to be increased from 6 to 40 g/L, with an overall productivity of 75 g/L/day. In cases as just described, the properties of the reactant and the product must be such that the reactant can be easily desorbed from the adsorbent resin and the product easily adsorbed back onto the resin. The limitations of this method would depend upon the affinity the reactant and product have for the resin.[12] Further whole cell examples using this approach are now forthcoming,[17,18] but the results presented here may additionally provide options for sufficiently stable isolated enzymes.

This final analysis of the optimal reactor configuration will be dependent upon the properties of the reactants, products and enzymes, but significant productivity enhancements may be possible.

4. Experimental

4.1. Analytical

HPLC (Gilson, Middletown, WI, USA) separation was achieved on a Zorbax RX-C8 column (ManMod Analytical, Chadds Ford, PA, USA) by isocratic elution using a 50% acetonitrile and 50% acidified water (0.1% H3PO4) mobile phase at 1.5 mL/min. Tetralone and tetralol had retention times of 4.70 and 6.20 min, respectively. Detection was by UV at 220 nm.

4.2. Production of tetralone reductase

Tetralone reductase was isolated by centrifugation and homogenisation from cells of *T. capitatum* (MY 1890) (kindly donated by Merck Research Laboratories, Rahway, NJ, USA). Subsequent purification was through Q sepharose, hydroxy apatite and toyopearl chromatography columns.[6]

4.3. Immobilisation of tetralone reductase

Dry Eupergit C (Röhm, Darmstadt, Germany) beads (1 g) were mixed with tetralone reductase solution (5 mL) in 1 M potassium phosphate buffer pH 7.5 and left at room temperature for 72 h.

4.4. Resin screen for NADH binding

0.5 g/L NAD(H) solution dissolved in water (2 mL) was added to resin (0.2 g) (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, XAD L-323, XAD 2010, or XAD L-493) (Supelco, Poole, Dorset, UK). The suspensions were shaken continuously for 30 min and then filtered using Puradisc™ syringe filters to remove the resin (Whatman, Maidstone, Kent, UK). The remaining NADH in solution was assayed by spectrophotometer at 340 nm.

4.5. Resin screen for protein binding

XAD resin (1 g) (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, XAD L-323, XAD 2010, or XAD L-493) (Supelco, Poole, Dorset, UK) was added to 0.2 g/L tetralone reductase solution (2 mL). After 30 min of continuous shaking, the samples were filtered using Puradisc™ syringe filters to remove the resin. The filtered samples were then assayed for total protein using the Bradford spectrophotometric assay.[19]

4.6. Binding of 6-bromo-β-tetralol on XAD L-323

Solutions of water (5×10 mL) containing 100 g/L 6-bromo-β-tetralone solution (100 μL) in methoxyethanol (final concentration 1 g/L), were added to 0.05, 0.1, 0.2, 0.4 or 0.6 g of XAD L-323. The samples were shaken continuously for 30 min. Subsequently, samples were filtered using Puradisc™ syringe filters to remove the resin and the filtrate assayed by HPLC for 6-bromo-β-tetralol.

4.7. Binding of 6-bromo-β-tetralone on XAD L-323

Solutions of water (5×10 mL) containing 100 g/L 6-bromo-β-tetralol in methoxyethanol (100 μL) (final concentration 1 g/L), were added to 0.05, 0.1, 0.2, 0.4 and 0.6 g of XAD L-323. The samples were shaken continuously for 30 min. Subsequently, samples were filtered using Puradisc™ syringe filters to remove the resin and the filtrate assayed by HPLC for 6-bromo-β-tetralone.

4.8. Bioconversion

Formate (8 mg), NAD (0.43 mg) and FDH (8 mg) were dissolved in the tetralone reductase solution (2970, 2850 and 2700 μL). The reactions were initiated through the addition of a 100 g/L tetralone solution in 30, 150 and 300 μL (final concentrations 1, 5 and 10 g/L, respectively). Formate was added in excess. The reactions were monitored over time by HPLC analysis for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.9. Bioconversion with solid phase product removal 1

Tetralone reductase (10 mL) was added to NAD (1.43 mg), FDH (20 mg) and formate (4.5 mg). The reaction was initiated with 100 g/L tetralone in methoxyethanol (100 μL) of a (final reaction concentration 1 g/L) solution. To assay the reaction mix, 200 μL samples were taken (a total of 1 mL of the reaction mixture was removed for assaying). XAD L-323 (0.5 g) was added to the reaction components to facilitate product removal. The reaction components were then filtered using a syringe filter, into a new sterile Sterilin™ reaction vessel (Bibby Sterilin, Stone, Staffs, UK). A sample of the filtered mix was assayed to check that the product and remaining substrates had been removed. The reaction was assayed at defined time points by HPLC for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.10. Bioconversion with solid phase product removal 2

Tetralone reductase (1 mL in total) was removed in the assaying of the first reaction. Extra tetralone reductase (1 mL) was added to the filtered reaction components with formate (4 mg), FDH (2 mg) and NAD (0.143 mg). The reaction was initiated again with 100 g/L 6-bromo-β-tetralone in methoxyethanol (100 μL). The reaction was assayed at defined time points by HPLC for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

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Rapid identification of enantioselective ketone reductions using targeted microbial libraries

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Abstract—A collection of about 300 microbes was surveyed for the ability to generate chiral secondary alcohols by enantioselective reduction of a series of alkyl aryl ketones. Microbial cultures demonstrating utility in reducing model ketones were arrayed in multi-well plates and used to rapidly identify specific organisms capable of producing chiral alcohols used as intermediates in the synthesis of several drug candidates. Approximately 60 cultures were shown to selectively reduce various ketones providing both the *R* and *S* enantiomers of the corresponding alcohols in 92–99% ee with yields up to 95% at 1–4 g/L. An alternative approach to chiral alcohols based on selective microbial oxidation of racemic alcohols is also reported. This study provides a useful reference for generating chiral alcohols by selective microbial bioconversion.

1. Introduction

It is well recognized that the pharmacological activity of a drug often depends on the specific configuration of the substituent around its chiral center(s). During the early stages of drug candidate selection, the effect of chirality on biological activity must be ascertained, prompting the need to generate chiral intermediates such as secondary alcohols. Chiral alcohols are readily obtained by enantioselective reduction of ketones using both chemical and biological means. Although hydrogenation of ketones with Rh- and Ru-diphosphine catalysts has been frequently utilized for the synthesis of chiral alcohols on a large scale,[1] biological reductions catalyzed by isolated dehydrogenases or whole microbial cells continue to provide an attractive means to reduce a broad range of ketones selectively.[2] Indeed, biological reductions of carbonyl groups have found important applications in the synthesis of several drug intermediates including Taxol (anticancer),[3] Montelukast (anti-asthma),[4] Zetia (cholesterol absorption inhibitor),[5,6] Trimegestone (hormone mimetic),[7] and Trusopt (anti-glaucoma).[8] Despite a large number of examples of microbial ketoreductions reported in the literature (for reviews see refs. 2,9–14) the task of choosing a microbe capable of selectively reducing a particular intermediate is still largely based on screening hundreds of microbial cultures in flask fermentations. This time consuming process often prohibits practical integration of the bioreduction with process development. To avoid this drawback, the development of rapid screening and selection methodologies is essential. Identified in this study are approximately 60 cultures, selected from a library of over 300 bacteria, yeast and filamentous fungi, for their ability to reduce numerous model ketones in a highly efficient and enantioselective manner. The design of the culture library was based on a literature survey of microbial reductions of ketones reported in the last 20 years. Initial results of a subset of the collection were reported earlier by Dodds et al.[15] By using libraries of pre-selected organisms arrayed in multi-well plate formats, screens could be shortened significantly and the desired organism identified within days. Rapid identification of enantioselective cultures allowed for the successful utilization of a bioreduction step in the synthesis of several drug intermediates.

2. Results

The bioconversions of alkyl aryl substrates **1a**–**f**, **3a**–**d**, **5a**–**d**, **7**, **9**, **11a**–**b**, and **13** (Fig. 1) were carried out at 2 g/L with cultures grown in complex medium. The initial screen was focused on simply identifying active and enantioselective cultures. No systematic attempts were undertaken to maximize either enantioselectivity or efficiency, although it should be emphasized that in many cases the culture productivity and selectivity was affected by the substrate

Keywords: Enantioselective reductions; Chiral alcohols; Microbial bioconversion; Ketone reduction.
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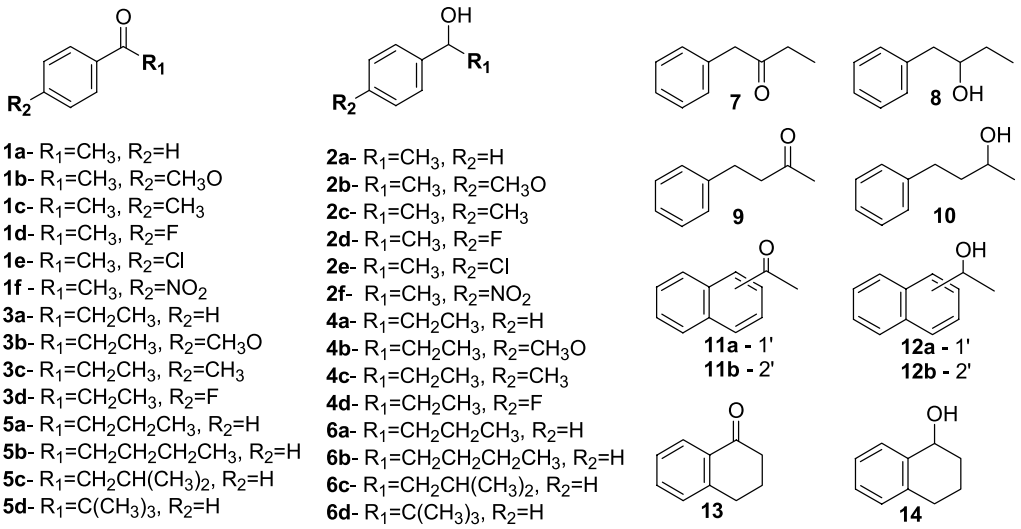


Figure 1. Ketones and alcohols used in microbial panel selection.

concentration, growth medium, and fermentation conditions. Cultures providing high selectivity and yield for each of the ketones surveyed were used to generate material in shake flask bioconversions for structural confirmation following purification (see Section 5).

Bioconversion of several 4′-substituted analogues of acetophenone and propiophenone were studied to investigate whether the presence of various substituents predictably alters the efficiency of microbial reduction. Cultures providing the highest enantioselectivity and yield are listed in Tables 1 and 2. Overall, cultures capable of reducing ketones without 4′-substituents were able to reduce the ketones with substituents in most cases. Not surprisingly, reduction of derivatives with electron withdrawing substituents such as nitro-, chloro- and fluoro- was more efficient than that of the derivatives with electron donating groups methyl- or methoxy. The effect of 4′-substitutions on enantioselectivity was culture specific and not predictable. Relatively few microbes were capable of selectively reducing aryl ketones with longer alkyl chain substituents (**5a**,**b**), branched alkyl substituents (**5c**,**d**), or tetralone (**13**) (Table 3). It is worth mentioning, however, that several cultures were identified that did reduce the highly hindered

Table 1. Selective microbial reduction of model 4′-analogues of acetophenone

Substrate	Yield (%)	ee (%)	Culture—ATCC
Acetophenone (**1a**)	59	>99 *S*−	*Rhodotorula glutinis 16740*
	24	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	24	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	39	97 *S*−	*Pichia subpelliculosa 16766*
	43	>99 *R*+	*Geotrichum klebahnii 20001*
	22	>99 *R*+	*Geotrichum candidum 34614*
4′-Methoxy- (**1b**)	61	>99 *R*+	*Yarrowia lipolytica 8661*
	37	>99 *R*+	*Pichia methanolica 58403*
	23	>99 *R*+	*Rhodotorula mucilaginosa 64684*
	20	>99 *R*+	*Mucor racemosus 7924*
	17	>99 *S*−	*Fusarium caucasicum 18791*
	15	>99 *S*−	*Aspergillus niveus 12276*
4′-Methyl- (**1c**)	53	>99 *S*−	*Pichia subpelliculosa 16766*
	77	73 *R*+	*Yarrowia lipolytica 8661*
4′-Fluoro- (**1d**)	75	>99 *S*−	*Pichia subpelliculosa 16766*
	62	>99 *S*−	*Rhodotorula glutinis 16740*
	68	95 *S*−	*Rhodotorula mucilaginosa 64684*
	79	97 *R*+	*Geotrichum candidum 34614*
	87	85 *R*+	*Yarrowia lipolytica 8661*
4′-Chloro- (**1e**)	80	>99 *S*+	*Rhodotorula glutinis 16740*
	75	>99 *S*+	*Rhodotorula mucilaginosa 64684*
	64	>99 *S*+	*Rhodotorula mucilaginosa 4056*
	52	>99 *S*+	*Aspergillus niveus 12276*
	48	>99 *S*+	*Aspergillus carneus 16798*
	39	>99 *S*+	*Pichia methanolica 58403*
	34	>99 *S*+	*Mucor racemosus 7924*
	86	>99 *R*−	*Yarrowia lipolytica 8661*
	34	>99 *R*−	*Fusarium caucasicum 18791*
4′-Nitro- (**1f**)	94	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	91	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	68	>99 *S*−	*Rhodotorula glutinis 16740*
	84	94 *R*+	*Geotrichum candidum 34614*

Table 2. Selective microbial reduction of model 4′-analogues of propiophenone

Substrate	Yield (%)	ee (%)	Culture—ATCC
Propiophenone (**3a**)	59	>99 *S*−	*Rhodotorula glutinis 16740*
	32	>99 *S*−	*Pichia subpelliculosa 16766*
	20	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	20	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	13	>99 *S*−	*Candida albicans 11006*
	22	97 *S*−	*Williopsis saturnus 20128*
	30	98 *R*+	*Debaryomyces hansenii 20220*
	19	87 *R*+	*Yarrowia lipolytica 8661*
4′-Methoxy- (**3b**)	25	>99 *S*−	*Pichia subpelliculosa 16766*
	17	>99 *S*−	*Aspergillus niveus 12276*
	10	>99 *R*+	*Yarrowia lipolytica 8661*
4′-Methyl- (**3c**)	31	>99 *S*−	*Mucor racemosus 7924*
	25	>99 *S*−	*Rhodotorula glutinis 16740*
	21	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	19	>99 *S*−	*Aspergillus carneus 20231*
	12	>99 *S*−	*Aspergillus carneus 16798*
	35	87 *R*+	*Yarrowia lipolytica 8661*
4′-Fluoro- (**3d**)	54	>99 *R*+	*Yarrowia lipolytica 8661*
	50	>99 *R*+	*Aspergillus carneus 20231*
	37	>99 *R*+	*Aspergillus carneus 16798*
	26	>99 *R*+	*Pichia methanolica 58403*
	20	>99 *S*−	*Rhodotorula mucilaginosa 4056*

Table 3. Microbial reduction of model phenone and naphthone substrates

Substrate	Yield (%)	ee (%)	Culture—ATCC
Butyrophenone (**5a**)	29	>99 *S*−	*Aspergillus niveus 12276*
	28	97 *S*−	*Williopsis saturnus 20128*
	19	>99 *S*−	*Aspergillus carneus 16798*
	15	>99 *S*−	*Mucor racemosus 7924*
Valerophenone (**5b**)	35	>99 *S*−	*Aspergillus niveus 12276*
	33	>99 *S*−	*Aspergillus carneus 16798*
	30	>99 *S*−	*Aspergillus carneus 20231*
	29	>99 *S*−	*Mucor circinelloides 7941*
	24	>99 *S*−	*Mucor racemosus 7924*
Isobutyrophenone (**5c**)	46	>99 *S*−	*Geotrichum klebahnii 20001*
	27	>99 *S*−	*Geotrichum candidum 34614*
	28	96 *S*−	*Fusarium caucasicum 18791*
	17	99 *S*−	*Geotrichum marinum 20614*
	12	>99 *S*−	*Pichia subpelliculosa 16766*
	18	>99 *S*−	*Candida albicans 20032*
	22	98 *R*+	*Debaryomyces hansenii 20220*
2,2-Dimethylpropiophenone (**5d**)	28	>99 *S*−	*Pichia subpelliculosa 16766*
	18	>99 *S*−	*Yamadazyma haplophila 20321*
	12	>99 *S*−	*Torulaspora delbrueckii 20100*
	9	>99 *R*+	*Debaryomyces hansenii 20220*
	17	75 *R*+	*Fusarium caucasicum 18791*
1-Phenyl-2-butanone (7)	56	>99 *S*+	*Pichia subpelliculosa 16766*
	14	>99 *S*+	*Candida albicans 11006*
	15	>99 *R*−	*Debaryomyces hansenii 34022*
	34	99 *R*−	*Mucor racemosus 7924*
	30	95 *R*−	*Geotrichum klebahnii 20001*
	33	94 *R*−	*Aspergillus niveus 12276*
Benzylacetone (**9**)	58	96 *S*+	*Zygosaccharomyces bailii 38924*
	20	96 *S*+	*Torulaspora delbrueckii 10662*
	28	>99 *R*−	*Trigonopsis variabilis 58377*
	15	>99 *R*−	*Candida parapsilosis 7330*
1-Acetonaphthone (**11a**)	79	>99 *S*−	*Rhodotorula glutinis 16740*
	66	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	61	>99 *S*−	*Geotrichum marinum 20614*
	61	99 *S*−	*Geotrichum candidum 34614*
	56	>99 *S*−	*Geotrichum klebahnii 20001*
	47	>99 *S*−	*Rhodotorula mucilaginosa 64684*
2-Acetonaphthone (**11b**)	41	>99 *S*−	*Rhodotorula glutinis 16740*
	39	>99 *S*−	*Rhodotorula mucilaginosa 4056*
	36	>99 *S*−	*Rhodotorula mucilaginosa 64684*
	21	>99 *S*−	*Pichia methanolica 58403*
α-Tetralone (**13**)	35	>99 *S*+	*Pichia methanolica 58403*
	14	>99 *S*+	*Rhodotorula glutinis 16740*
	13	>99 *S*+	*Williopsis saturnus 20128*

ketone **5d** with high enantioselectivity. Likewise, increasing the distance of the carbonyl moiety from the phenyl ring resulted in a significant decrease in the frequency and types of cultures identified which could selectively reduce phenones **7** and **9**. Carbonyl position had less influence on selective reduction of naphthones **11a** and **11b**. Cultures providing the highest selectivity and yield for reduction of aryl ketones **5a**–**d**, **7**, **9**, **11a**–**b** and **13** are listed in Table 3.

As presented in Tables 1–3, a higher number of cultures exhibited *pro-S* selectivity over *pro-R*. It was of interest, therefore, to examine whether alcohols with the opposite, (*R*)-stereochemistry, could be obtained by a selective oxidation of racemic alcohols. This approach has been found to be useful for the preparation of a variety of enantioenriched secondary alcohols.[16–18] A panel of microbes was screened for their ability to selectively oxidize racemates of **2a**, **4a**, **6a**–**d**, **8**, **10**, **12a**–**b** and **14** (Fig. 1) at 2 g/L. Bioconversions were conducted with cells suspended in TNC medium, ammonium acetate or sodium citrate buffers (see Section 5). Racemate resolution by oxidation was achieved with high selectivity for **2a**, **4a**, **10** and **14**. As expected, racemate resolution by oxidation resulted in the production of (*R*)-alcohols (with one exception) with good to excellent selectivity. Culture and media specific effects were observed and conditions affording maximum productivity are summarized in Table 4 (Fig. 1).

All cultures demonstrating selective conversion of one or more model compounds were incorporated into 24-well plate panels and used in reducing intermediates of several drug candidates—**15**, **17**, **19**, **21**, and **23** (Fig. 2). Remarkably, 5–25% of the cultures in the assembled panels reduced at least one of the five ketones with high selectivity, yielding the corresponding alcohols with ee>95%. A subset of cultures exhibiting high selectivity in reduction of the above ketones at 1 g/L is listed in Table 5. Ketones **15**, **17**, and **19** were reduced by a number of microbes into either the (*R*)- or (*S*)-enantiomer of the corresponding alcohol with varying yield and excellent enantioselectivity (ee 93–>99%). The yield and volumetric productivity were further improved by conducting the bioconversions with highly concentrated resting cells. For example, an 8 h

Table 4. Resolution of model racemic alkyl aryl alcohols by microbial oxidation

Racemate	Culture—ATCC	Media	Yield (%)	ee (%)
1-Phenyl-1-ethanol (**2a**)	*Yarrowia subpelliculosa* 16766	SCT	45	>99 *S*−
	Geotrichum candidum 34614	AMA	33	>99 *R*+
	Yamadazyma haplophila 20321	TNC	24	>99 *R*+
		SCT	48	95 *R*+
1-Phenyl-1-propanol (**4a**)	*Geotrichum candidum* 34614	TNC	24	96 *R*+
	Candida parapsilosis 16632	SCT	20	88 *R*+
1-Phenyl-3-butanol (**10**)	*Yarrowia lipolytica* 8661	AMA	22	95 *R*−
	Rhodotorula mucilaginosa 4056	TNC	41	89 *R*−
	Candida parapsilosis 16632	TNC	32	85 *R*−
α-Tetralol (**14**)	*Rhodotorula glutinis* 16740	SCT	34	98 *R*−
		TNC	37	95 *R*−
	Geotrichum candidum 34614	SCT	24	95 *R*−
		TNC	31	92 *R*−
	Rhodotorula mucilaginosa 4056	SCT	41	93 *R*−
		TNC	42	91 *R*−
	Pichia methanolitica 58403	SCT	33	91 *R*−

incubation of **15** (4 g/L) with *Candida apicola* 24616 cells (1 kg/L) in TNC medium at 30 °C produced (*R*)-**16** in 95% yield (ee>98%). Resting cells of *Debaryomyces hansenii* 10619 and *Candida bombicola* 22214 were also capable of reducing 4 g/L of **15** into (*R*)-**16** in 50–67% yield with high selectivity (ee 96–98%).

To prevent evaporation of the highly volatile ketone, **21**, a panel of 45 microbes selected from the initial plate screen was propagated in flasks and then screened in a resting state format employing screw cap tubes (Table 5). While a number of cultures produced **22** in the (*S*)-configuration in excellent yield and enantiomeric purity, the cultures with the *pro-R* preference were only moderately selective (ee of *R*-**22** was only in 84–92% range). In a similar trend, a number of cultures exhibited *pro-S* selectivity in the reduction of ketone **23**, while none was pro-*R* selective. Additionally, an increase in the substrate concentration up to 10 g/L did not affect enantioselectivity of several *pro*-(*S*) selective cultures. As a result, (*S*)-**24** was obtained in 66% yield (ee >99%) following a 48 h incubation of **23** at 10 g/L with resting cells of *M. plumbeus* 4740.

3. Discussion

Microbial fermentations provide a diverse renewable supply of dehydrogenase activities with inherent capabilities for cofactor regeneration. Numerous examples of selective microbial reductions have been reported for a wide variety of ketones (for recent examples see Refs. 11,19–28). Nevertheless, no algorithms have been developed yet which

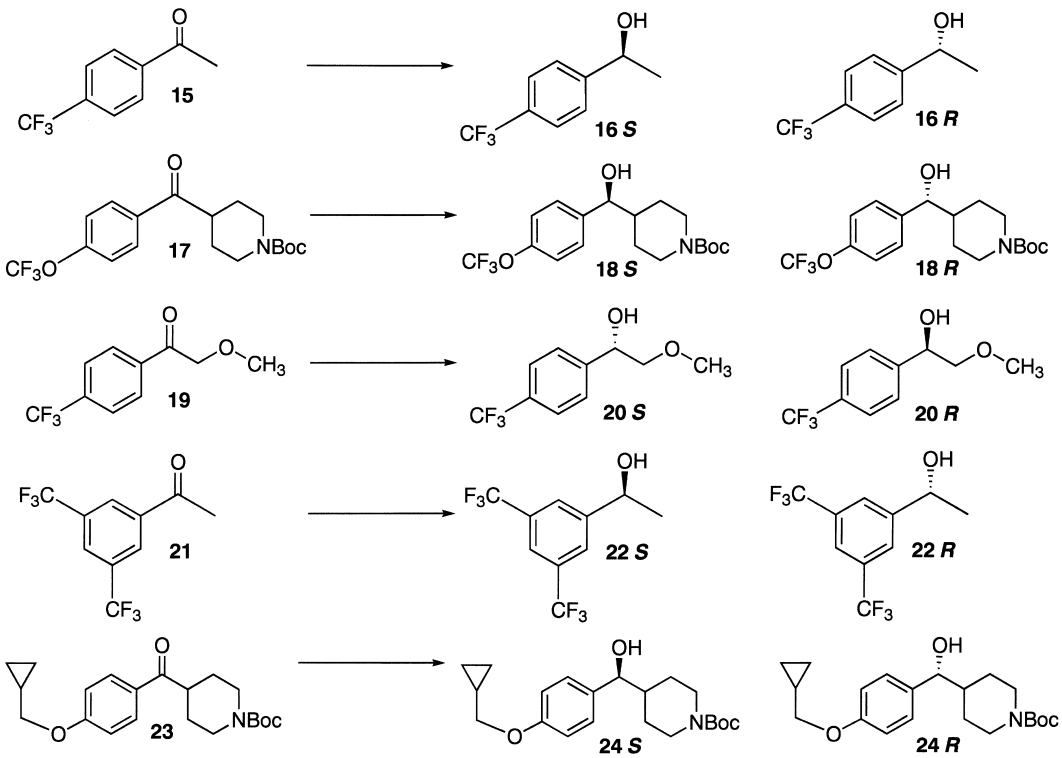


Figure 2. Selective reduction of ketone intermediates for synthesis of drug candidates.

Table 5. Generation of chiral alcohol intermediates by selective microbial reduction

Substrate	Yield (%)	ee (%)	Culture—ATCC
4′-Trifluoromethylacetophenone (**15**)	72	>99 *S*	*Rhodotorula glutinis*16740
	77	>99 *S*	*Rhodotorula mucilaginosa 4056*
	67	>99 *S*	*Rhodotorula pilimanae 32762*
	43	>99 *S*	*Torulaspora fermentati 46935*
	33	>99 *S*	*Torulaspora delbrueckii 20100*
	45	>99 *S*	*Saccharomyces cerevisiae 58520*
	50	96 *S*	*Aspergillus carneus 20231*
	40	95 *S*	*Mucor circinelloides 7941*
	22	98 *S*	*Mucor mucedo 20094*
	48	96 *S*	*Mucor hiemalis 16636*
	37	96 *S*	*Paecilomyces variotii 14572*
	56	95 *R*	*Yarrowia lipolytica 46482*
	72	>99 *R*	*Candida apicola 24616*
	38	99 *R*	*Candida bombicola 22214*
	66	96 *R*	*Candida parapsilosis 20224*
	81	98 *R*	*Debaryomyces hansenii 10619*
	88	95 *R*	*Trigonopsis variabilis 58377*
	88	96 *R*	*Torulaspora vanriji 56221*
4-(4-Trifluoromethoxy-benzoyl)-piperidine-1-carboxylic acid *tert*-butyl ester (**17**)	66	>99 *S*	*Candida guilliermondii 9058*
	46	>99 *S*	*Pichia anomala 66346*
	34	>99 *S*	*Torulopsis species 66815*
	33	>98 *S*	*Hansenula anomala 20211*
	87	97 *S*	*Candida fumata 26418*
	21	93 *R*	*Rhodotorula glutinis 26085*
	15	90 *R*	*Mortierella ramanniana 38191*
2-Methoxy-4′-trifluoromethylacetophenone (**19**)	85	87 *S*	*Debaryomyces hansenii 20220*
	60	88 *S*	*Candida polymorpha 20213*
	77	95 *S*	*Hansenula polymorpha 26012*
	71	99 *R*	*Rhodotorula mucilaginosa 64684*
	66	96 *R*	*Rhodotorula pilimanae 32762*
	66	95 *R*	*Schizosaccharomyces pombe 20130*
	79	97 *R*	*Williopsis saturnus 42306*
	73	99 *R*	*Trigonopsis variabilis 58536*
3′,5′-Bis-trifluoromethylacetophenone (**21**)	66	85 *R*	*Candida kefyr 14244*
	93	92 *R*	*Candida tropicalis 46491*
	>99	84 *R*	*Torulaspora etchellsii 20126*
	61	87 *R*	*Fusarium caucasicum 18791*
	64	86 *R*	*Trichosporon cutaneum 46446*
	66	>99 *S*	*Rhodotorula glutinis 26085*
	50	>99 *S*	*Rhodotorula mucilaginosa 64684*
	41	>99 *S*	*Rhodotorula pilimanae 32762*
	88	>99 *S*	*Williopsis saturnus 18119*
	97	95 *S*	*Aspergillus carneus 16798*
	70	>99 *S*	*Aspergillus terreus 24839*
	92	>99 *S*	*Geotrichum candidum 90685*
4-(4-Cyclopropylmethoxy-benzoyl)-piperidine-1-carboxylic acid *tert*-butyl ester (23)	37	>99 *S*	*Debaryomyces hansenii 20220*
	38	>99 *S*	*Rhodococcus species 21146*
	46	>99 *S*	*Fusarium caucasicum 18791*
	52	>99 *S*	*Geotrichum candidum 74487*
	47	>99 *S*	*Mortierella isabellina 44853*
	59	>99 *S*	*Mucor plumbeus 4740*

are capable of predicting the activity and selectivity of a culture on a ketone of interest with even moderate accuracy. The process of selecting a microbe for a particular biotransformation is still highly empirical and time consuming. A typical 1000-culture screen conducted in a classical flask fermentation format is estimated to take 4 weeks on average. The excessive amount of resources and the length of time required to complete such a screen often creates a barrier on the way to incorporating a biological step into a multi-step chemical synthesis. To overcome this limitation, an efficient approach allowing for the rapid identification of cultures with the desired activity was needed. In order to accelerate the microbial screening program we created a 'targeted' library of microbes enriched for the desired activity that was based on an extensive and critical evaluation of prior work on bioreductions. As a result, a collection of about 300 cultures representing 55 genera of bacteria, yeast and filamentous fungi, were assembled and tested against 19 commercially available UV active ketones.

Cultures demonstrating the highest selectivity and yield for each of the nineteen alkyl aryl ketones surveyed are listed in Tables 1–3. Notably absent from the compilation of the primary screen results were representative bacteria. Although various strains of *Lactobacilli*,[29–32] *Gluconobacter*,[33–35] and *Rhodococci*[19,36–39] have been shown to catalyze selective reductions of a variety of ketones, these cultures were not among the top performers in our library survey. In fact, the most efficient and selective cultures were

those of fungal origin (both yeast and filamentous fungi). Most cultures capable of reducing the alkyl aryl ketone series generated alcohols in the (*S*)-configuration, the pattern consistent with Prelog's Rule.[40] Nevertheless, the Prelog model was not a useful predictor for the outcome of whole cell bioconversions as was evident from the results presented in Tables 1–5. For example, *Yarrowia lipolytica*[16,17,41] and *Debaryomyces hansenii* were found to primarily generate (*R*)-alcohols. In the case where the ketoreduction failed to produce an alcohol in the (*R*)-configuration an alternative approach, based on resolution of the racemic alcohol by selective oxidation of one enantiomer proved to be quite useful (Table 4).

To demonstrate the utility of the targeted library approach, about 300 microorganisms were incorporated into a 24-well plate format and used to identify cultures capable of selective reduction of several target ketones (Table 5). The chiral alcohol products formed as a result of bioreductions of ketones **15**, **17** and **19** were used as chiral intermediates in the preparation of three antiviral CCR5 antagonists.[42–44] Selective reduction of **21** and **23** were investigated in conjunction with the synthesis of an antidepressant NK1 receptor antagonist[45–47] and an anti-muscarinic M2 receptor antagonist[48,49] respectively. The primary objective of these screens was to rapidly identify highly selective bioconversions to produce chiral alcohol intermediates for the synthesis of putative drug candidates. Since only gram quantities of the chiral alcohols were needed to support the synthesis, little optimization was conducted. Using multi-well plates prepared in advance containing grown cultures, the bioconversions of all five ketone intermediates were identified within just a few days, representing a 5–10-fold improvement in the average screen time (Table 5). Furthermore, by altering the time of substrate addition or by using high concentration of resting cells, further improvements in reaction productivity were achieved. For example, (*R*)-**16** was produced in 95% yield (ee >98%) in the presence of *Candida apicola* 24616 with 4 g/L of **15**; the yield of (*S*)-**18** (ee>99% ee) obtained with yeast *Pichia anomala* 66346 was improved from 46% to 71% and finally (*S*)-**24** (ee>99%) was obtained in 66% yield following the incubation of **23** at 10 g/L with *M. plumbeus* 4740.

4. Conclusion

Quick access to microbial bioreductions can prove to be useful at various stages of development, from supporting the synthesis of putative drug candidates at the laboratory level, to manufacturing intermediates and active pharmaceutical ingredients at commercial scale. This study has demonstrated the utility of using targeted libraries for rapid identification of microbial cultures capable of reducing a variety of ketones in a highly enantioselective manner. A library composed of about 300 commercially available cultures, arrayed in multi-well plates yielded a set of 60 microbes that reduced a series of 24 ketones with excellent selectivity. It is estimated that the described plate format afforded a 5–10-fold reduction in the average screen time compared to a conventional flask-based screening protocol. The abundance of microbial cultures capable of enantioselective reductions and their broad substrate tolerance provides a general and reliable approach to the synthesis of chiral alcohol enantiomers.

5. Experimental

5.1. Cultures, chemicals, media and laboratory ware

All microbial cultures used in this study were obtained from the American Type Culture Collection (Manassas, VA). Alkylaryl ketones (**1a–f**, **3a–d**, **5a–d**, **7**, **9**, **11a–b**, **13**, **15**, **21**) were purchased from Aldrich Chemicals (Milwaukee, WI). Most racemic alcohol-standards were either purchased from Aldrich or obtained by reducing the corresponding ketones with sodium borohydride or lithium aluminum hydride following standard procedures. Ketones **17**, **19**, and **23** and the corresponding racemic and chiral alcohols **18**, **20**, and **24** used as analytical standards were supplied by the Chemical Research Department, SPRI. Analytical grade solvents for extraction and HPLC were purchased from Fisher Scientific (Fairlawn, NJ). Medium components were purchased from Difco/Becton Dickson (Sparks, MD), Sensient (Juneau, WI), ICN Biomedical (Aurora, OH), Avebe (Veendam, Netherlands) and Fisher Scientific (Fairlawn, NJ). Silica Gel 60 was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) and TLC plates (Silica Gel GF plates 20×20 cm 1000u) from Analtech Inc. (Newark, DE). Polypropylene 24 and 384 deep well plates, 24-well cap mat and plastic lids were purchased from Whatman Inc. (Clifton, NJ). Slotted 384 well cap mats were purchased from Greiner Bio-One, Inc. USA (Longwood, FL).

5.2. Culture propagation, bioconversion and sample preparation

Filamentous fungi were grown in SIM-6 medium (35 g/L soy flour, 50 g/L white potato dextrin, 5 g/L dextrose, 2 mg/L cobalt chloride, 5 g/L calcium carbonate, pH 5.0). Yeast were grown in YPD (10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L dextrose, pH 5.5–6.0) or TNC (10 g/L Tastone 154, 20 g/L NZ-amine, 30 g/L cerelose at pH 5.0–5.5) media. Bacteria were grown in NYC medium (8 g/L nutrient broth, 11 g/L cerelose, 20 g/L Tastone 154, pH 7.0). Seed cultures were grown at 24–30 °C in 25 mL of medium in 125 mL flasks for up to 72 h with agitation (175–250 rpm). Seed cultures (1 mL) were transferred into a second stage flask fermentation for bioconversion. Cultures selected for panel screening were also transferred from the seed cultures into a series of 24-deep well plates along with glycerol (20% final conc.) and sealed with 24 well cap mats. The plates were stored at −80 °C. Frozen culture plates were thawed at room temperature followed by addition of 1.0 mL of pre-sterilized TNC or NYC media per well. The plate was covered with a loose fitting pre-sterilized plastic lid, followed by incubation at 24–30 °C for up to 72 h with agitation (180 rpm). Ketone or racemic alcohol substrates dissolved in methanol, ethanol or dimethyl sulfoxide were added to cultures in flasks or 24-deep well plates to a final concentration of 0.5–2 g/L following 24 h of culture growth. Flask fermentation

samples (2 mL) taken following 24–72 h of incubation, were extracted with 6 mL of MTBE. A 0.5 mL aliquot of extract was transferred into a 1 mL vial and analyzed by HPLC. For culture panels in screens, 2 mL bioconversions in 24 well plates were extracted by addition of 6 mL of MTBE per well. Sample plates were sealed with cap mats and a foil overlay and stored at −20 °C until sampled for HPLC analysis. Samples (0.3 mL) from each reaction extract were transferred and consolidated for analysis into 384 well plates using a MultiProbe EX II robotic liquid handling station (PerkinElmer Life Sciences, Boston, MA). Assay plates were sealed with 384-well cap mats prior to automated HPLC analysis using an Alliance 2795 fluidics module (Waters Corporation, Milford, MA) as described below. Evaluation of high cell density bioconversions was conducted by harvesting cells from standard flask fermentations by centrifugation and suspending the cell pellet in ammonium acetate buffer pH 7.0 (AMA; 30 g/L), sodium citrate buffer pH 7.0 (SCT; 30 g/L), TNC medium or 50 mM potassium phosphate buffer pH 7.0 containing glucose (100 g/L) at equal or reduced volumes to achieve the desired concentration of cells. Ketone or racemic alcohol substrates dispersed in ethanol were added to cells in flasks at a final concentration of 0.5–10 g/L. Reactions were conducted in flasks and sampled as described above.

5.3. Reverse phase and chiral HPLC analysis

All HPLC analyses were conducted with equipment from Waters Corporation (Milford, MA). Ketones and alcohols were analyzed using 10 μL injections, and monitoring UV at 200 −220 nm. Reverse phase assays were conducted using Symmetry™ C-18 (Waters Corporation, Milford, MA) or Luna™ C-18 (Phenomenex, Torrance, CA) 4.6×150 mm columns at 25–30 °C with isocratic elution using acetonitrile/water (60:40 or 70:30; 1–1.5 mL/min). Chiral assays separating alcohol enantiomers and the corresponding ketone substrates were conducted using Chiracel™ 4.6×250 mm columns (Chiral Technologies Inc., Exton, PA) at 25–30 °C with isocratic elution, employing mixed solvents as follows; OB-H column with hexanes/isopropyl alcohol (90/10), at 1 mL/min for bioconversions **1c** and **5a**; hexanes/isopropyl alcohol 96.5/3.5 (1–1.25 mL/min) for bioconversions **1d**, **3a**,**c**,**d**, **5c**, **11b**; hexanes/isopropyl alcohol 98/2, (1 mL/min) for bioconversions **5b**, **19**; *n*-heptane/ethanol 96:4, (1 mL/min) for bioconversions **1f**; *n*-heptane /ethanol 99/1, (1 mL/min) for bioconversion **15**; OD column with hexanes/isopropyl alcohol at 90/10, (1.5 mL/min) for bioconversion **11a**; hexanes/isopropyl alcohol 95/5 (1–1.25 mL/min) for **3b**, **5d**, and **9**; hexanes/isopropyl alcohol 98/2, (1–1.25 mL/min) for **1b**,**e**, **7** and **21**; OJ column with hexanes/isopropyl alcohol 98/2, (1.25 mL/min) for bioconversions **1a**; hexanes/isopropyl alcohol 95/5, (1.25 mL/min) for **13**; AD column with hexanes/ethanol 95/5, (1 mL/min) for bioconversion **17** and with *n*-heptane/ethanol 93:7 (1 mL/min) for **23**.

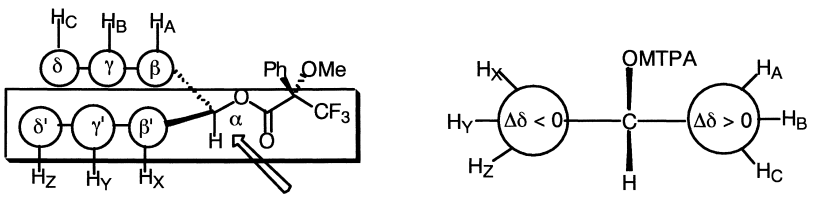
5.3.1. Synthesis of phenylalkanol by microbial bioconversion. The procedures used to generate and purify alcohol products were essentially identical except for the type of microorganism and propagation conditions (as specified above). A representative example based on the synthesis of (*S*)-4′-nitro-1-phenyl-1-ethanol (**2f**) is described. A seed culture of *R. muciaginosa* 4056 (formerly *Rhodotorula rubra*) was grown in a 300 mL flask containing 50 mL of YPD medium at 30 °C for 24 h with agitation (250 rpm). The entire amount of culture was then transferred into a 2 L flask containing 1 L of YPD medium and grown for 24 h at 30 °C with agitation (250 rpm). The substrate, 4′-nitroacetophenone **1f** (2 g) dissolved in methanol (4 mL) was then added and the mixture was incubated under the above conditions for another 48 h resulting in ~94% conversion of the ketone to alcohol product, **2f**. Fermentation broth was then centrifuged at 12,000*g* for 15 min to remove cells and the supernatant was extracted with MTBE (2×500 mL). Cell pellets were suspended in saturated NaCl solution (50 mL) and extracted with MTBE (2×50 mL). The MTBE layers were combined, dried with anhydrous $MgSO_4$, filtered and the solvent evaporated in vacuo. The crude product was purified by preparative TLC (Silica Gel GF plates; 30% EtOAc/hexanes) yielding **2f** as a yellow oil; [Found: C, 57.51; H, 5.43; N, 8.46. $C_8H_9NO_3$ calculated C, 57.48; H, 5.43; N, 8.38]; R_f (30% EtOAc/hexanes) 0.14; $[\alpha]_D^{24.5}=-29.7$ (*c* 2.59, EtOH); ν_{max} (liquid film) 3500–3200 (br), 2970, 1600, 1521, 1347, 1090 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.5 (3H, d, *J*=6.49 Hz, CH*Me*), 1.97–2.18 (1H, br, MeCHO*H*), 5.05 (1H, q, *J*=5.16 Hz, MeC*H*OH), 7.55 (2H, d, *J*=8.57 Hz, Ph-*H*), 8.20 (2H, d, *J*=8.72 Hz, Ph-*H*); δ_C (400 MHz, $CDCl_3$) 25.52, 69.51, 123.77, 126.13, 147.16, 153.10; *m*/*z* (CI, CH_4) 168 (M+1,100%), 150 (55%), 107 (6%).

5.3.2. Optical rotation determination. The specific rotation of each purified alcohol product was measured in EtOH (1.0–2.6 mg/mL) at the temperature indicated (°C). Alcohols generated using *P. subpelliculosa* 16766 (formerly *Hansenula subpelliculosa*) include; **2a** (*S*) $[\alpha]_D^{23.3}=-30.7$, **2c** (*S*) $[\alpha]_D^{23.5}=-40.0$, **2d** (*S*) $[\alpha]_D^{23.6}=-34.6$, **4a** (*S*) $[\alpha]_D^{24.7}=-34.3$, **4b** (*S*) $[\alpha]_D^{24.2}=-24.8$, **8** (*S*) $[\alpha]_D^{24.6}=+18.1$. Alcohols generated using *Y. lipolytica* 8661 include; **2b** (*R*) $[\alpha]_D^{23.7}=+27.7$, **2e** (*R*) $[\alpha]_D^{24.8}=+44.9$, **4d** (*R*) $[\alpha]_D^{24.4}=+33.9$. Alcohols generated using *R. glutinis* 16740 include; **4c** (*S*) $[\alpha]_D^{24.4}=-34.8$, **12a** (*S*) $[\alpha]_D^{25.0}=-79.4$ and **12b** (*S*) $[\alpha]_D^{23.6}=-43.1$. **2f** (*S*) $[\alpha]_D^{24.5}=-29.7$ was obtained using *R. mucilaginosa* 4056 as described above; **6a** (*S*) $[\alpha]_D^{24.6}=-42.5$ was obtained using *A. niveus* 12276; **6b** (*S*) $[\alpha]_D^{24.9}=-26.1$ was obtained using *A. carneus* 16798; **6c** (*R*) $[\alpha]_D^{23.4}=+25.0$ was obtained using *D. hansenii* 20220 (formerly *Torulaspora hansenii*) and **6c** (*S*) $[\alpha]_D^{24.2}=-26.4$ from *C. albicans* 20032; **6d** (*S*) $[\alpha]_D^{23.9}=-29.1$ was obtained using *Y. haplophila* 20321; **10** (*R*) $[\alpha]_D^{23.4}=-18.3$ was obtained with *T. variabilis* 58377 and **14** (*S*) $[\alpha]_D^{24.7}=+18.8$ was obtained using *P. methanolica* 58403.

5.4. Structure configuration of phenylalkanols by paired MTPA esters

The absolute configuration of **2b**–**f**, **4b**–**d**, **6b**,**d**, **8**, **10** was determined by the modified Mosher NMR method.[50] A single enantiomerically enriched alcohol was treated separately with the two enantiomers of the Mosher's acid chloride. The resulting pair of diastereomeric esters was analyzed by high field ^1H NMR in $CDCl_3$. The absolute configuration of the alcohol moiety was assigned on the

Mosher's Model



Difference in chemical shifts of corresponding resonances of the paired Mosher ester diastereomers.

Signal	$\Delta\delta = \delta_S-\delta_R$	Signal	$\Delta\delta = \delta_S-\delta_R$	Signal	$\Delta\delta = \delta_S-\delta_R$
(**2b**)		(**2c**)		(**2d**)	
1-CH, q	-0.039	1-CH, q	+0.028	1-CH, q	+0.039
2-CH_3, d	+0.042	2-CH_3, d	-0.075	2-CH_3, d	-0.058
4'-OCH_3,s	-0.028	4'-CH_3, s	+0.002	Phenyl, m	+0.053
Phenyl, m	-0.015	Phenyl, m	+0.012		+0.053
	-0.014		+0.023		+0.053
			+0.072		
(**2e**)		(**2f**)		(**4b**)	
1-CH, q	-0.054	1-CH, q	+0.043	1-CH, t	+0.067
2-CH_3, d	+0.049	2-CH_3, d	-0.041	2-CH, m	-0.046
Phenyl, m	-0.116	Phenyl, m	+0.069	2-CH, m	-0.044
	-0.106		+0.070	3-CH_3, t	-0.103
				4'-OCH_3, s	+0.003
				Phenyl, m	+0.050
					+0.057
(**4c**)		(**4d**)		(**6b**)	
1-CH, t	+0.061	1-CH, t	-0.074	1-CH, q	+0.068
2-CH, m	-0.050	2-CH, m	+0.034	2-CH_2, m	-0.046
2-CH, m	-0.032	2-CH, m	+0.269	3-CH_2, m	-0.031
3-CH_3, t	-0.111	3-CH_3, t	+0.093	4-CH, m	-0.110
4'-CH_3, s	+0.005	Phenyl, m	-0.064	4-CH, m	-0.065
Phenyl, m	+0.032		-0.064	5-CH_3, t	-0.054
	+0.030		-0.064	Phenyl, m	+0.050
			-0.064		+0.085
			-0.064		+0.084
					+0.083
					+0.082
					+0.077
					+0.064
					+0.057
(**6d**)		(**8**)		(**10**)	
1-CH, s	+0.134	1-CH_2, m	+0.079	1-CH, m	-0.062
2-CH_3, s	-0.052	2-CH, m	+0.040	2-CH, m	-0.036
Phenyl, m	+0.105	3-CH_2, m	-0.061	2-CH, m	-0.000
	+0.099	4-CH_3, t	-0.082	3-CH, m	+0.040
	+0.100	Phenyl, m	+0.115	4-CH_3, d	+0.108
	0.104		+0.128	Phenyl, m	-0.040
	+0.033		+0.117		-0.035

Figure 3. Structure configuration of phenylalkanols by paired MTPA esters.

basis of the difference in the chemical shifts of corresponding resonances of the paired diastereomers (see Fig. 3).

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Effect of the reaction temperature on the transglycosylation reactions catalyzed by the cyclodextrin glucanotransferase from *Bacillus macerans* for the synthesis of large-ring cyclodextrins

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Abstract—The synthesis of cyclodextrins with from 6 to more than 50 glucose units by cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from *Bacillus macerans* was investigated. Analysis of the synthesized cyclic α-1,4-glucan products showed that a higher yield of large-ring cyclodextrins were obtained with a reaction temperature of 60 °C compared to 40 °C. The yield of large-ring cyclodextrins obtained at 60 °C represented about 50% of the total glucans employed in the reaction. Analysis of the cyclodextrin-forming cyclization reaction and of the coupling reaction of the CGTase resulting in the degradation of mainly the larger cyclic α-1,4-glucans indicated higher rates of the cyclization reaction at 60 °C compared to 40 °C while the opposite was found for the coupling reaction.

1. Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme involved in the conversion of starch. The enzyme is a member of the α-amylase family of glycosyl hydrolases (family 13).[1] CGTase can also hydrolyze glucan chains to some extent similar to α-amylases, but differs in its ability to form cyclodextrins (CD), cyclic α-1,4-glucans, as reaction products. While CGTase has been previously employed for the industrial production of CD_6, CD_7, and CD_8 which are composed of six, seven and eight glucose units (α-, β-, and γ-CD), the enzyme is also capable of synthesizing much larger rings. The CGTases from various *Bacillus* species including *Bacillus macerans* convert starch into a mixture of CD with a degree of polymerization from 6 to more than 60.[2,3]

The synthesis and properties of industrially produced CD have been studied intensively in the last years (for a review, see Szejtli 1998).[4] The molecular structures of CD resemble a hollow, truncated cone with a hydrophobic cavity.[5,6] Due to this structure, CD can form inclusion complexes with suitable guest molecules. Various properties of the guest molecules, such as their water solubility, stability, and bioavailability can thereby be manipulated. The commercially available CD are therefore widely applied in the food and pharmaceutical industries. The structure and properties of large-ring CD composed of nine to 21 glucose units have only been characterized recently (for a review, see Endo et al. 2002).[7] The molecular structure of CD_9 resembles a distorted boat shape because CD_9 is more flexible than CD_6, CD_7 or CD_8.[8,9] CD_{10} and CD_{14} have a butterfly-like structure to reduce steric strain with twisting of some glucose units to form flips and kinks.[10–14] The structure of CD_{26} contains two single helices with 13 glucose units each in antiparallel direction.[15–17] Due to their structural features which are distinct from the small CD, large-ring CD could find applications as novel host compounds in molecular recognition processes.[7]

The formation of CD by CGTase proceeds by an α-retaining double displacement mechanism.[18,19] Two catalytic amino acid residues, the catalytic acid/base residue Glu257 and the nucleophile Asp229 are involved in the reaction (Fig. 1). The glycosidic scissile bond oxygen of the bound α-1,4-glucan donor is protonated by Glu 257 resulting in the formation of an oxo-carbonium ion transition state. The transition state collapses into a stable covalent glycosyl-enzyme intermediate linked to Asp 229. The acceptor activated by Glu257 acting as a base then attacks the intermediate and a new α-glycosidic bond is formed via another oxo-carbonium ion transition state (Fig. 1).

CGTase performs three transglycosylation reactions and a hydrolysis reaction. Cyclic α-1,4-glucans are formed by an intramolecular transglycosylation reaction where the terminal 4-OH group of the intermediate acts as an acceptor (cyclization reaction, Fig. 2A). The reverse reaction also occurs where the CD ring is opened and the linear oligosaccharide is transferred to a linear glucan acceptor (coupling reaction, Fig. 2B). Linear α-1,4-glucan products

Keywords: Cyclodextrin glucanotransferase; Large-ring cyclodextrins; Transglycosylation reactions.
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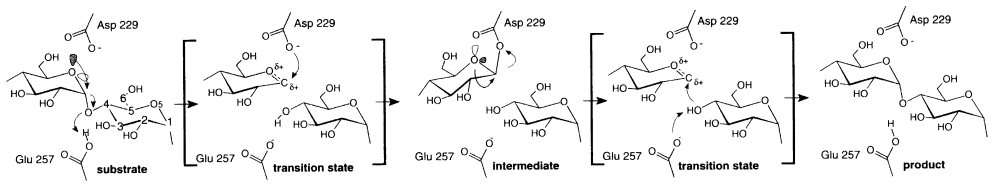

Figure 1. Scheme of the CGTase reaction mechanism. Reprinted from Uitdehaag, J.; van der Veen, B. A.; Dijkhuizen, L.; Dijkstra, B. W. *Enzyme Microb. Technol.*. Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the alpha-amylase family, *30* pp 295–304, Reprinted with permission. Copyright (2002) Elsevier.

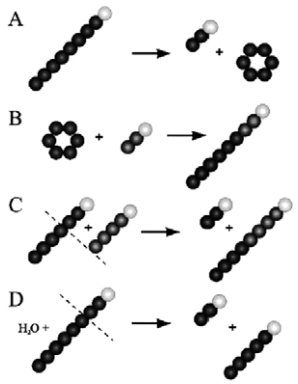

Figure 2. Scheme of the transglycosylation reactions catalyzed by CGTase*. Cyclization (A); coupling (B); disproportionation (C); and hydrolysis (D). The dark circles represent glucose residues, the white circles denote glucose residues with free reducing ends. *Reprinted from van der Veen, B. A.; van Alebeek, G. J.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.*. The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms, *267* pp 658–665, Reprinted with permission. Copyright (2000) Blackwell Publishing Ltd.

are also formed in the third reaction, an inter-molecular transglycosylation where a linear oligosaccharide is transferred to another linear glucan acceptor (disproportionation reaction, Fig. 2C). In a hydrolysis reaction, a glucan is cleaved and the reducing end is transferred to water (Fig. 2D).

The amounts and size distribution of CD formed by CGTase will be strongly influenced by the combined effects of the three transglycosylation reactions, as well as by the hydrolytic activity of the enzyme. CGTases isolated from wild type bacterial strains have been shown to exhibit different product profiles with respect to the size and amount of the CD synthesized.[20–22] A comparison of the large-ring CD synthesis activity of CGTases from *Bacillus* spp. also indicated that the enzymes exhibited different product specificity. The CGTases differed in their hydrolytic activities affecting the ratio of small CD to large-ring CD products synthesized.[3]

The three transglycosylation reactions catalyzed by CGTases show distinct differences in their kinetic mechanisms.[3,23] The differences observed have been explained by the way the substrates bind to the enzyme. Site-directed mutagenesis and kinetic studies have suggested the possibility to independently modify the cyclization and coupling activities of CGTase.[24] Thereby, the product range of CGTase could be manipulated for the synthesis of CD of specific sizes.

In this study, we compare the effect of different reaction temperatures on the cyclization and coupling reactions of the CGTase from *B. macerans* influencing the yield of large-ring CD synthesis products.

2. Results and discussion

Using synthetic amylose, a linear α-1,4-glucan with an average molecular weight of 280.9 kDa as substrate, large-ring CD are formed at an early stage of the reaction with CGTases from *Bacillus* species.[2,3,22] It has been observed that the amount of large-ring CD decreased during a prolonged incubation with the enzyme. The yield and size distribution of large-ring CD in the course of a synthesis reaction will be strongly influenced by the extent of the coupling and hydrolysis reactions by which the large-ring CD are converted to smaller CD. Since CD_7 and CD_8 were found to be poor substrates for the coupling and hydrolytic reactions, they will accumulate during longer reaction times.[3]

To further investigate the factors influencing the synthesis of large-ring CD by CGTase, we compared the yield and size distribution of CD obtained at different reaction temperatures and incubation times. An analysis of the cyclic α-1,4-glucan products obtained from synthetic amylose as substrate catalyzed by the CGTase from *B. macerans* is shown in Figure 3. Prior to analysis by high performance anion exchange chromatography with pulsed amperometric detection, the reaction mixtures were treated with glucoamylase to convert non-cyclic glucans to glucose. At a reaction temperature of 40 °C, large-ring CD were detected after a reaction time of 1 h (Fig. 3A). After a reaction time of 20 h, almost all of them were converted to CD_6, CD_7 and CD_8 as reported previously.[2] In contrast, at a reaction temperature of 60 °C, large-ring CD were formed after an incubation time of 30 min and could still be detected after a reaction time of 20 h (Fig. 3B). A time course over 480 min of the total production of CD, of CD_6–CD_8, large-ring CD, and of the reducing power at different reaction temperatures is shown in Figure 4. The yield of large-ring CD reached a maximum of 35% of the total glucan after 2 h of incubation at 40 °C and decreased during longer incubation times. Due to the conversion of the large-ring CD to smaller CD by coupling or hydrolysis reactions, their amount increased during longer incubation times keeping the amount of total CD produced almost constant (Fig. 4A). The maximum yield of large-ring CD after 2 h of incubation at 60 °C reached about 50% of the total glucan and remained almost constant during longer incubation times (Fig. 4B).

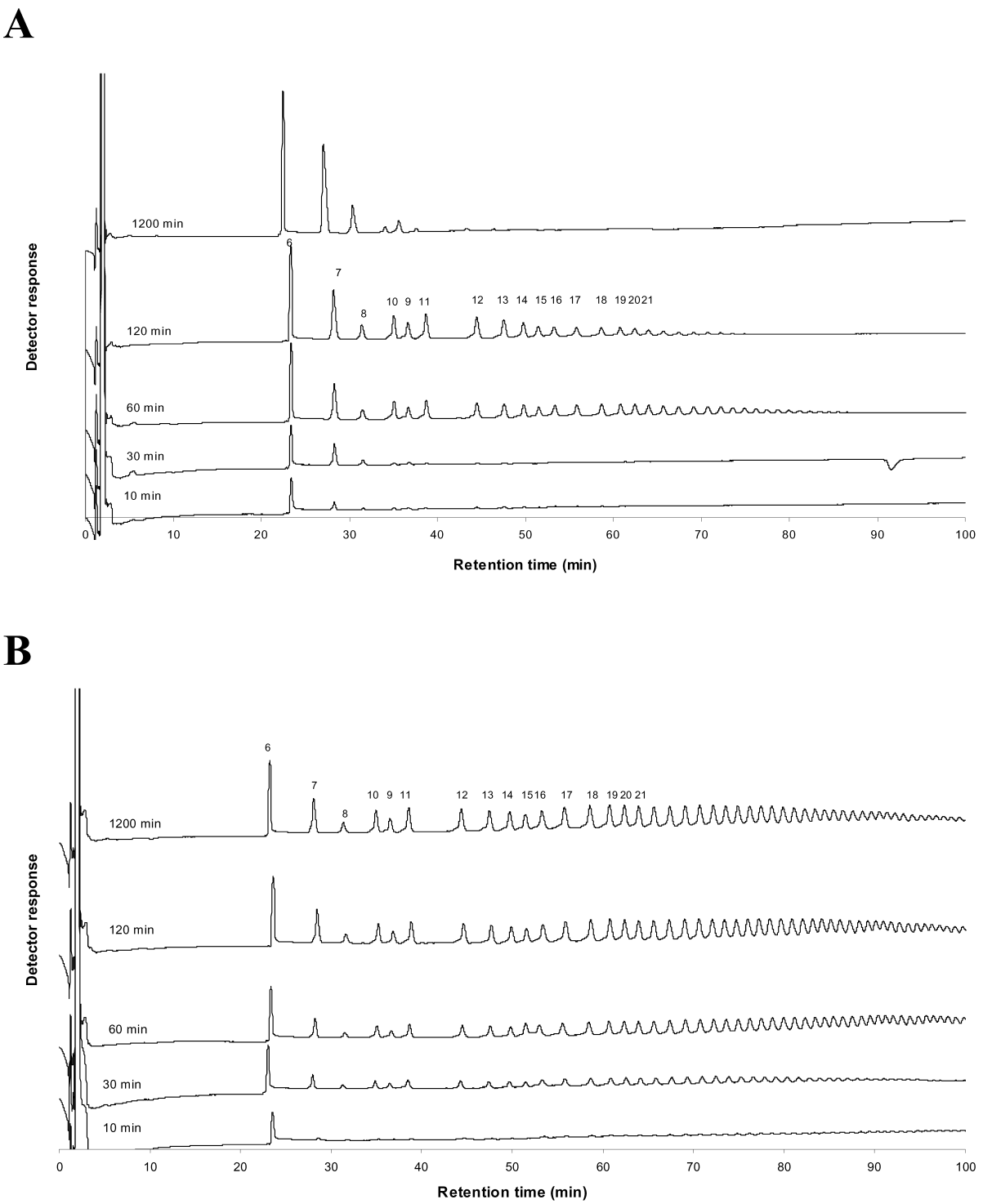

Figure 3. HPAEC analysis of CD synthesized after 10, 30, 60, 120 and 1200 min by the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase. Peak numbers indicate the degree of polymerization of identified CD.

Beside the coupling reaction, the hydrolytic activity of CGTase has also been considered to influence the conversion of large-ring CD to smaller CD.[3] However, it is difficult to distinguish between the hydrolysis and the coupling activity of the enzyme since it catalyzes both reactions at the same catalytic site. After opening of the CD ring, the resulting linear oligosaccharide will be transferred to water in the case of hydrolysis. In the presence of other oligosaccharides or glucose in the enzyme reaction mixture these will also serve as acceptors resulting in a coupling reaction. The amount of reducing power detected in the reaction mixtures representing the amounts of reducing sugars formed also depended on the reaction temperature. In reaction mixtures incubated at 40 °C, it steadily increased and reached higher levels compared to reactions performed at 60 °C. The observed higher amounts of reducing sugars formed at 40 °C may reflect the increased formation of linear oligosaccharides due to hydrolysis reactions.

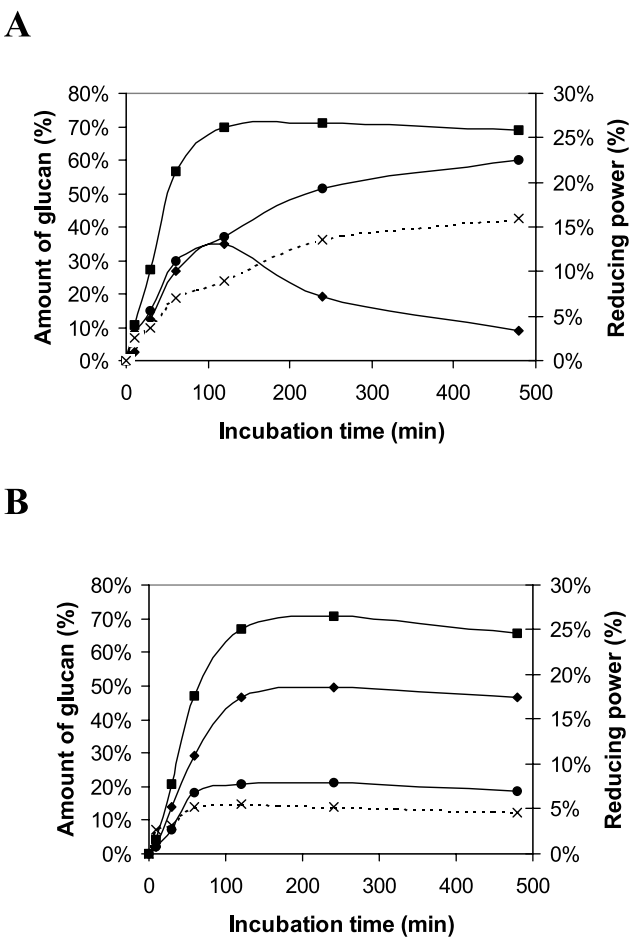

Figure 4. Time course of the amounts of the total CD, CD_6 to CD_8, large-ring CD and the reducing power produced by the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). Total CD, ■; CD_6–CD_8, ●; large-ring CD, ◆; reducing power, ×. Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase.

The amounts of large-ring CD with a degree of polymerisation from 9 to 21 synthesized during 4 h of incubation with the CGTase at the two reaction temperatures were quantified (Table 1). The amounts of each single large-ring CD formed at 60 °C reached about 20–40 nmol ml^{-1}, corresponding to conversion yields of 3–5% of the total glucan employed in the reaction. Reactions performed at 40 °C yielded much lower amounts of large-ring CD, and CD with a degree of polymerisation above 13 could not be detected. A comparison of the size distribution of all CD formed at different temperatures also shows that the ratio of large-ring CD to small CD was higher at 60 °C than at 40 °C (Fig. 5).

Table 1. Amounts of large-ring CD obtained by incubating 0.5% synthetic amylose with CGTase from *B. macerans* at 40 and 60 °C

Cyclodextrin	40 °C (nmol ml^{-1})	60 °C (nmol ml^{-1})
CD_9	34.66	45.71
CD_{10}	11.43	49.03
CD_{11}	4.63	44.33
CD_{12}	4.70	45.35
CD_{13}	2.11	41.59
CD_{14}	nd[a]	29.74
CD_{15}	nd	18.88
CD_{16}	nd	33.58
CD_{17}	nd	45.33
CD_{18}	nd	33.48
CD_{19}	nd	39.68
CD_{20}	nd	24.26
CD_{21}	nd	30.25

[a] nd, not detectable.

To further investigate the influence of the reaction temperature on the amount and size of CD synthesized, the optimum temperature for the cyclization and coupling activities of the CGTase was compared. The results show an optimum of the cyclization reaction at 60 °C, while the optimum for the coupling activity with CD_8 as substrate was found around 45 °C (Fig. 6). A comparison of the kinetic parameters of the cyclization reaction of the CGTase showed a higher rate of CD_7 formation at 60 °C compared to 40 °C (Table 2). The K_m value for synthetic amylose could not be determined since the CD detection assay was not sensitive enough to reliably measure the low amounts of CD_7 obtained at the low amylose concentrations required.

Analysis of the kinetic properties of the coupling activity of the CGTases from *B. circulans* and *Thermoanaerobacterium thermosufurigenes* has revealed a random-order reaction mechanism involving a ternary complex, while the CGTase from *Bacillus sp.* 1101 showed a non-sequential ping-pong reaction mechanism.[23,25–26] Our experimental data for the CGTase from *B. macerans* also indicated a random-order reaction mechanism. The Lineweaver–Burk plots of the kinetic data obtained where the reciprocal of the velocities was plotted against the reciprocal of the CD_8 concentration at different acceptor concentrations did not result in parallel lines (Fig. 7). In a random-order reaction, any of the two substrates, CD_8 and methyl-α-D-glucopyranoside (MαDG), can bind first to the enzyme in a sequential mechanism resulting in two apparent affinity constants: K_m in the absence and K'_m in the presence of the second substrate. The kinetic parameters determined at different temperatures indicated higher rates of the coupling reaction at 40 °C compared to 60 °C (Table 3). The affinity constants K'_m for both substrates in the presence of the other substrate were lower at 40 °C than at 60 °C, while in the absence of the second substrate the reverse effect was found. The affinity for CD_8 was decreased in the presence of the acceptor at both temperatures. The decrease of affinity was more pronounced at 60 °C compared to 40 °C. In contrast, the affinity of the CGTase from *B. circulans* (strain 251) for CD_8 was not affected by the presence of the acceptor.[23]

Structural studies of the CGTases from *B. circulans* strain 251 complexed with CD_8 and of *B. circulans* strain 8 complexed with a CD_7 derivative have indicated different binding modes at the acceptor site for the CD and the linear substrate.[27] These differences may affect the binding of linear oligosaccharides and CD at the active site of the CGTase at different reaction temperatures favouring the coupling reaction at the lower reaction temperature.

The cyclization reaction of CGTase with amylose as substrate has been previously described as an exo-type of attack occurring at the sixth to the eighth glycosidic linkage from the non-reducing end of a α-1,4-glucan chain. However, recent results have shown that CGTases can attack any α-1,4-linkage within the amylose molecule

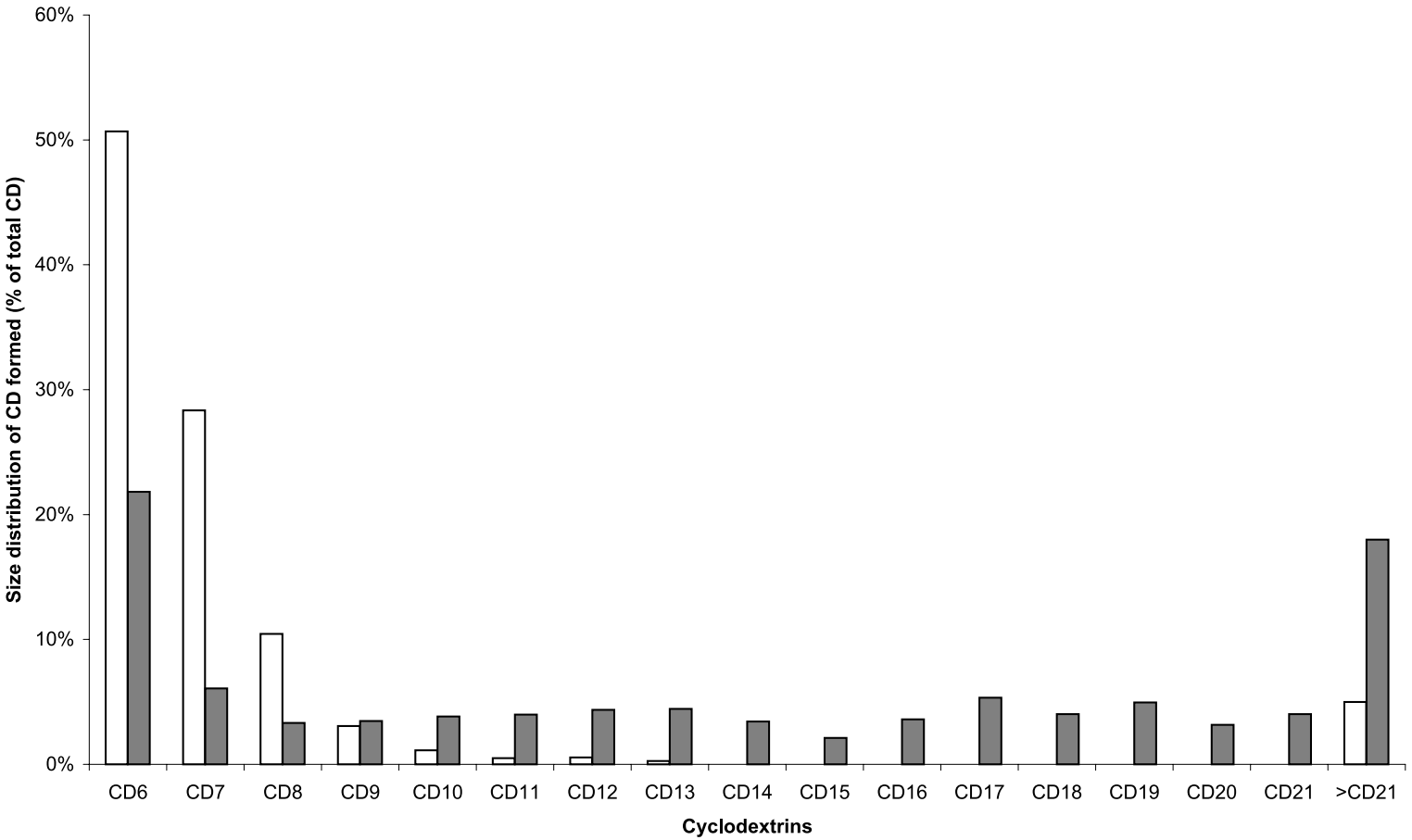

Figure 5. Size distribution of CD synthesized by the CGTase from *B. macerans* at 40 °C (open bars) and 60 °C (solid bars). Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase for 480 min.

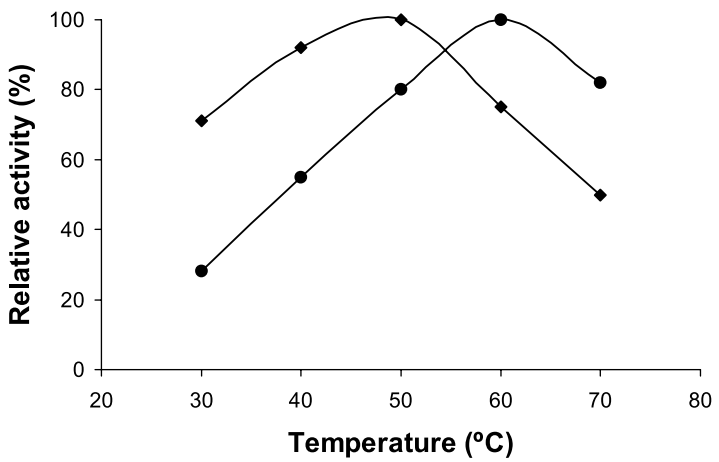

Figure 6. Comparison of the coupling activity (◆) and the cyclization activity (●) of the CGTase from *B. macerans* at different temperatures.

Table 2. Kinetic parameters of the CD_8 cyclization reaction catalyzed by the CGTase from *B. macerans* at 40 and 60 °C

Parameter	40 °C	60 °C
V_{max} (U mg^{-1})	44.6±4.1	56.3±5.2
k_{cat} (s^{-1})	51.2±4.9	64.7±6.0

resulting in the synthesis of a wide range of cyclic α-1,4-glucans by a random cyclization reaction. By adjusting the reaction conditions such as temperature and incubation time, the yield of large-ring CD in the synthesis reaction can be effectively increased.

3. Experimental

3.1. Chemicals and enzymes

Synthetic amylose with an average molecular weight of 280.9 kDa was prepared by the method of Kitamura et al.[28] Soluble starch and α-amylase was from E. Merck AG (Darmstadt, Germany) and methyl-α-D-glucose (MαDG) was from Sigma-Aldrich Chemie GmbH (Munich, Germany). Glucoamylase was from Toyobo Co., Ltd. (Osaka, Japan) and *B. macerans* CGTase was from Amano Enzyme Inc. (Aichi, Japan). The enzyme was further purified using DEAE Sepharose Fast Flow media (Amersham Biosciences Europe GmbH). The enzyme was eluted with a linear gradient between 0 and 0.5 M NaCl in Tris–HCl buffer, pH 7.8. Standards of large-ring CD (CD_9 to CD_{21}) were kindly provided by H. Ueda, Hoshi University, Tokyo, Japan.

3.2. Synthesis of large-ring CD

Synthetic amylose was dissolved in 10 ml of 90% DMSO (v/v). To remove the DMSO, 0.5 ml of amylose solution was mixed with 0.5 ml of distilled water and loaded on a PD10 column (Amersham Biosciences Europe GmbH). After eluting the column with 2 ml of distilled water, the synthetic amylose was eluted with a further 1.5 ml of distilled water and used immediately.[3] The CD synthesis reaction was performed using 0.5% synthetic amylose and 2 U ml^{-1} CGTase in acetate buffer (50 mM, pH 5.5). The reaction was terminated by boiling the mixture for 10 min. Glucoamylase (3.85 U ml^{-1}) was added and the mixture was incubated for 24 h to convert the linear oligosaccharides to glucose. The amount of total CD in the sample was calculated by subtracting the amount of glucose released by glucoamylase from the total amount of glucose released by a combined treatment with glucoamylase and α-amylase.[3]

3.3. Analysis of large-ring CD

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out using a DX-600 system (Dionex Corp., Sunnydale, USA) to analyze and quantify the CGTase synthesis products and to monitor the production process of large-ring CD. A Carbopac PA-100 analytic column (4×250 mm, Dionex Corp., Sunnydale, USA) was used. Samples (25 μl) were centrifuged before analysis. Products were eluted with a linear gradient of sodium nitrate (0–5 min, 1%; 5–49 min, increasing from 1% to 18%; 49–89 min, increasing from 18% to 35%; 89–100 min, increasing from 35% to 39%) in 200 mM NaOH containing 8% MeCN with a flow rate of 1 ml min^{-1}. Retention times (min) of each CD obtained were: CD_6, 21.1; CD_7, 27.3; CD_8, 30.8; CD_9, 36.3; CD_{10}, 34.7; CD_{11}, 38,4; CD_{12}, 44.3; CD_{13}, 47.5; CD_{14}, 49.8; CD_{15}, 51.6; CD_{16}, 53.5; CD_{17}, 53.0; CD_{18}, 59.0; CD_{19}, 61.2; CD_{20}, 62.9; CD_{21}, 64.5). The amounts of CD_6 to CD_{21} obtained were quantified by preparing standard curves with concentrations of 0.1, 0.25, 0.5, 0.75 and 1 mM of each CD.

3.4. Assay for starch-hydrolyzing activity

The starch-hydrolyzing activity of the CGTase was determined by the iodine method with some modifications.[29] The reaction mixture (250 μl) contained 1.2% soluble starch and 50 μl of the enzyme solution. After incubation at 40 °C for 10 min, the reaction was stopped by the addition of 500 μl of 0.5 M acetic acid–0.5 M HCl (5:1, v/v). Then 5 ml of 0.005% I_2 in 0.05% KI solution was added to 100 μl of the mixture and the absorbance was measured at 660 nm. One unit of the enzyme was defined as the amount of enzyme producing a 10% reduction of the intensity of the colour of the iodine complex per minute under the conditions described.

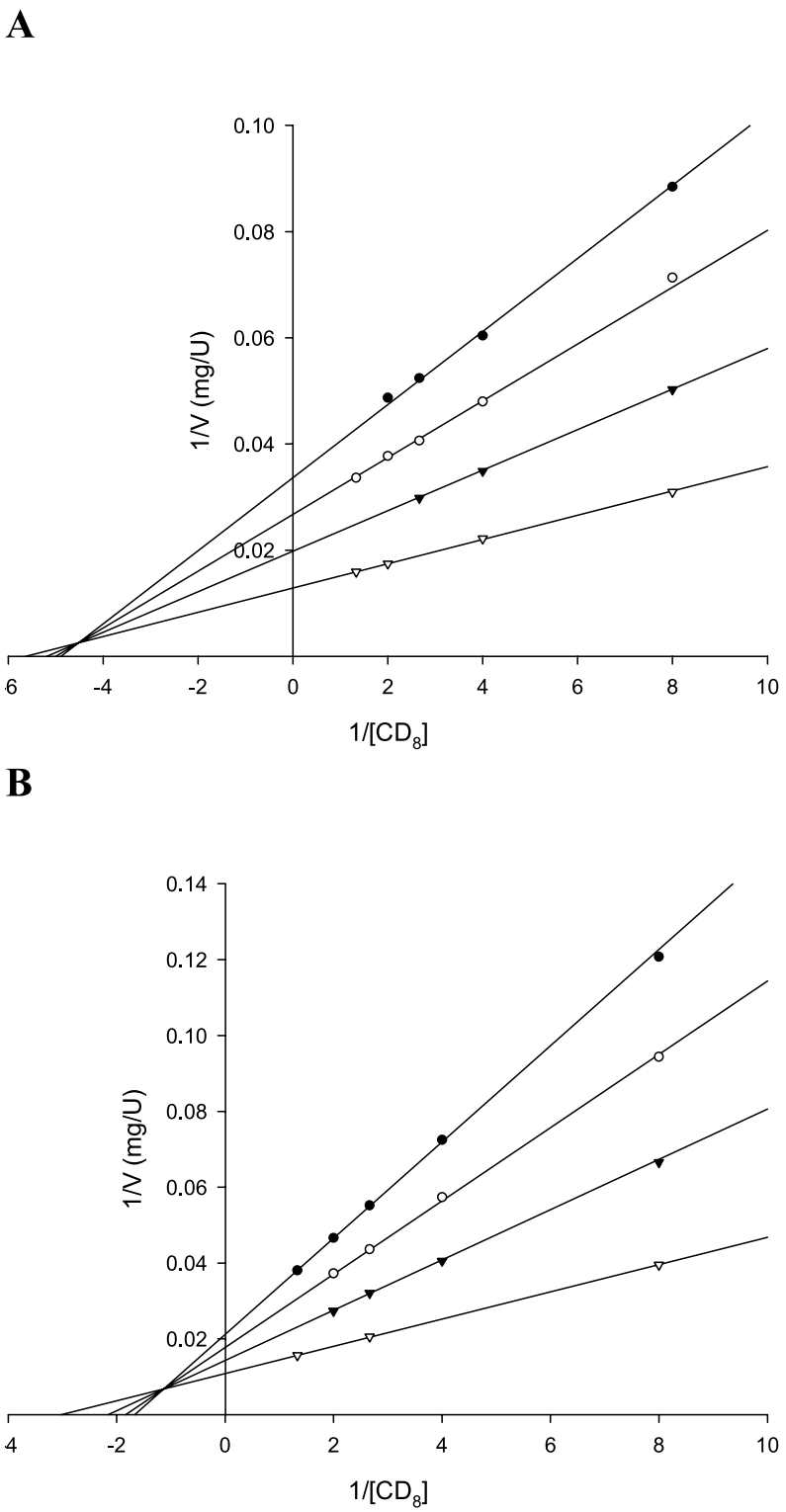

Figure 7. Lineweaver–Burk plots of the coupling reaction of the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). The reciprocal of the velocities is plotted against $1/[CD_8]$ at MαDG concentrations of 15 mM (●), 20 mM (○), 30 mM (▼), and 60 mM (▽).

Protein concentrations were determined using the Bradford method[30] and the reducing power by a modified Park–Johnson method.[31]

3.5. Cyclization activity

Cyclization activity was determined using synthetic amylose (MW 280.9 kDa, 0.5%) as the substrate. The synthetic amylose was incubated in 50 mM acetate buffer (pH 5.5) with appropriately diluted CGTase. At time intervals (0.5–1 min), 100 μl samples were taken and added to a mixture containing 100 μl phenolphthalein in 800 μl sodium hydroxide (0.03 M). One unit of activity was defined as the amount of enzyme able to form 1 μmol of CD_7 per min.

Table 3. Kinetics parameters of the coupling reaction of CD_8 with MαDG catalyzed by the CGTase from *B. macerans* at 40 and 60 °C

Parameter	40 °C	60 °C
$K_{m\ CD8}$ (mM)	0.125±0.01	0.074±0.02
$K'_{m\ CD8}$ (mM)	0.22±0.01	0.88±0.04
$K_{m\ M\alpha DG}$ (mM)	69.87±1.67	27.6±0.7
$K'_{m\ M\alpha DG}$ (mM)	122.8±16.7	326.6±46.0
V_{max} (U mg^{-1})	168.1±5.0	134.3±2.7
k_{cat} (s^{-1})	196.1±5.8	156.6±3.1

Kinetic analysis was performed with Sigmaplot software (SPSS Inc., Chicago, USA) using the Michaelis–Menten equation.

3.6. Coupling activity

The coupling activity between CD_8 and methyl-α-D-glucopyranoside (MαDG) was measured as described previously.[23] CD_8 at concentrations of 1.5, 2, 2.5, 4 and 8 mM was used as donor substrate and up to 60 mM methyl-α-D-glucopyranoside (MαDG) as acceptor substrate. The substrates were incubated in 10 mM phosphate buffer (pH 5.5) for 5 min with appropriately diluted CGTase. The reaction products were incubated with 3.85 U ml^{-1} glucoamylase to convert the linear oligosaccharides formed to glucose. The amount of glucose was determined with the glucose oxidase method.[32] One unit of enzyme activity was defined as the amount of enzyme coupling 1 μmol of CD_8 to MαDG per min.

Kinetic analysis was performed with Sigmaplot software (SPSS Inc., Chicago, USA). The following equation for a random-order reaction mechanism was used to fit the experimental data.[33]

$$v = V_{max}\cdot a\cdot b/(K'_{mA}\cdot K_{mB} + K_{mB}\cdot a + K_{mA}\cdot b + a\cdot b) \quad (1)$$

v=reaction rate, V_{max}=maximum rate; A and B=donor and acceptor substrate; a and b=substrate concentrations; K_m and K'_m=affinity constants for the substrates in the absence and in the presence and of the second substrate.

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