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Extractive Biocatalysis: a Powerful Tool in Selectivity Control in Yeast Biotransformations

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Abstract: The effect of absorbing resins on the yeast reduction of α,β-unsaturated carbonyl compounds is reported. Enantioselectivity, chemoselectivity and space-time yields of the biotransformation are impressively enhanced. The distribution of substrates and products between the resin and the water phase shows that the improved selectivity has to be attributed to the control of substrate concentration.

Keywords: Baker's yeast; reduction; absorbing resins; in situ product removal.

Introduction

The utility of biocatalysts in organic synthesis is well established. While hydrolytic biocatalysts are usually employed in the form of crude or purified proteins, enzymes with oxidoreductase activity are seldom used in large scale applications due to the cofactor requirement and its recycling problem. It has recently been claimed that cofactor recycling can be brought about economically [1,2]. However this requires a technological approach which needs to be tuned for every different reduction case. In contrast the use of whole cell biocatalysts for this purpose is expanding particularly in the field of enantiomerically pure pharmaceutical intermediates or products [3]. Associated with the use of such biocatalysts are a number of other problems which must be overcome before the process becomes economically convenient and industrially compatible. Besides confining the reaction in the minimum possible volume, selectivity and efficient product recovery is becoming the crucial issue, once a cooperative microorganism has been found. In most synthetic laboratories the choice of microorganisms is often limited to the use of non-pathogenic, easy-to-grow yeasts and fungi which are known to readily develop enzymes for the catabolism of xenobiotics, in this case non-conventional substrates. Baker's yeast is the most popular and synthetically useful of them all [4].

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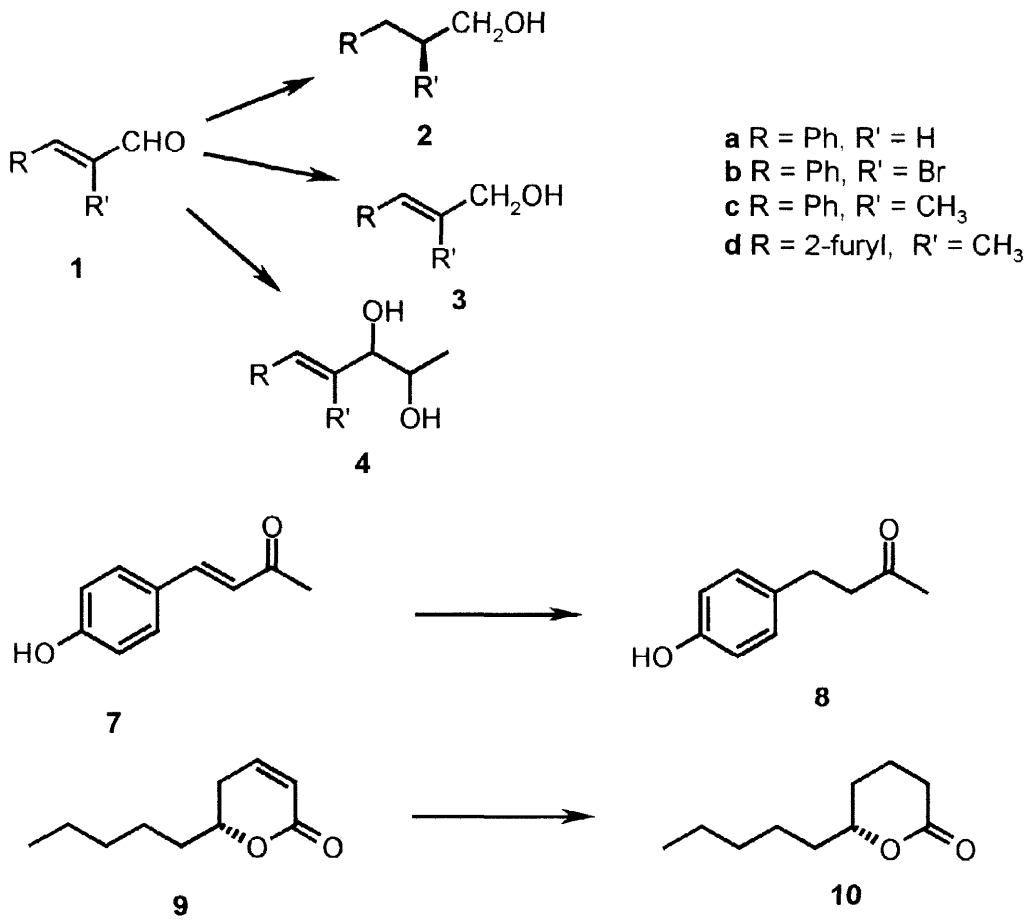
Absorbing resins have recently been used for *in situ* product removal in fermentations. Advantages are improved production due to the (partial) removal of the products from the solution and the cells surfaces, thus avoiding inhibition effects and cell toxicity [5-10]. Together with other techniques this approach constitutes what is defined as *extractive bioconversion* [11]. These concepts have been applied in fermentation processes in which the product is completely or largely water soluble. Recently the application to the biotransformation of a hydrophobic non-natural substrate by the yeast *Zigosaccharomyces rouxii* has been reported [12]. In that instance the yield of the product was dramatically improved by suppression of phenomena of substrate-inhibition in the biotransformation. The process in which an absorbing resin was used to control the substrate concentration has been scaled up to pilot plant scale [13]. This biotransformation could be better defined as a case of *extractive biocatalysis*. Advantages in controlling substrate and product concentration in the medium should be particularly useful in baker's yeast (b.y.) biotransformations where the problem of selectivity and limited yields due to substrate/product inhibition is often crucial. Since the decreased substrate concentration in a multienzymatic system favours the reaction catalysed by the enzyme with the lower K_m for the substrate, the control of substrate concentration will ultimately influence the enantiomeric excess of the product [14, 15]. We have recently shown how the use of absorbing resins can improve the enantiomeric excess in the well known b.y. reduction of 3-oxo-butyrate [16]. In this article we report on the multiple effects exerted by the use of extractive resins on b.y. biotransformations of different substrates. In all the reported cases the outcome of the bioconversion is profoundly influenced and the products are always formed and isolated in higher yields, with improved stereo- and chemoselectivity. With this additional tool for efficiency control, b.y. biotransformations should acquire improved application in organic synthesis.

Results and Discussion

We studied the biotransformation of α,β-unsaturated carbonyl compounds **1a-d** and **7** (scheme 1), and compared the outcome of the bioconversion in fermenting yeast with and without the resins. We chose those substrates because we had previously shown that several reaction events can simultaneously take place with them, namely reduction of the carbonyl group, C-C double bond reduction and, in the case of aldehydes, acyloin condensation [17,18].

Moreover, since there is an industrial interest in the biotransformation leading from extractive **9** (Massoia lactone) to *natural* **10**, the possibility of performing the biotransformation on a large scale with higher space-time yields was explored [19].

Although each of the obtainable products in the above biotransformations is synthetically of interest, the parameters governing the selectivity of the transformation are fundamental since separation of products formed in low yields from complex mixtures is of little practical interest. We compare the selectivity observed with 24 hours biotransformation time.



Scheme 1

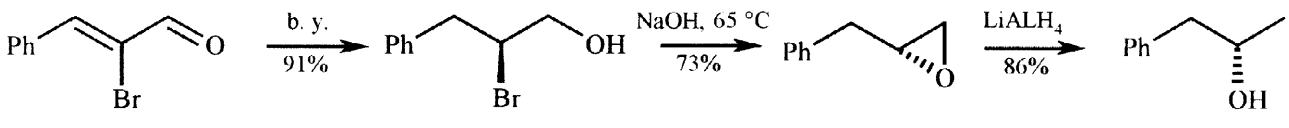
For higher incubation times the completely reduced compound (carbonyl and C-C double bond) is usually obtained both with and without the resin. Since both the substrates and the products are highly lipophilic compounds, the choice of the resin is rather unimportant, the organic compounds being largely on the solid phase. However simple reduction of the C-C double bond and of the carbonyl can change considerably the partition coefficient between the resin and the water phase, as shown in figure 3 for substrate **1b**. We thus describe the experiments obtained with XAD 1180. A number of other hydrophobic resins behaved equally well. The operational conditions are such that the substrate is added to the fermenting yeast absorbed on the resin or directly to the yeast fermentation to which the resin has been previously added. The biotransformation is monitored by conventional methods. At the end of the biotransformation the resin is separated from the biomass by simple filtration through a sintered glass filter of appropriate porosity. Washing the resin with an appropriate solvent allows the recovery of the product and unreacted substrate in yields usually higher than 90%. The resin recovered can be reused for the same purpose without further treatment. Collective data concerning the selectivity are gathered in table 1.

Substrate	Sat. Alcohol	ee (%)	Unsat. alcohol	Diols	Sat. carbonyl compound	Conversion
1a	100 (34)	-	(56)	0 (10)	0	100 (100)
1b	86 (48)	98.6 (68.2)	13 (24)	0 (25)	0	99 (97)
1c	94 (2)	98 (98)	3 (18)	0 (10)	0	97 (30)
7	0 (traces)	-	-	-	50 (11)	50 (11)
1d	39 (20)	96 (98)	59 (69)	0 (10)	-	98 (99)

Table 1: % yields concerning yeast extractive biocatalysis (in brackets normal conditions) of various substrates. Typical run: 50 mL H_2O, 2.5 g glucose, 12.5 g pressed yeast, 100 mg substrate, 100 mg XAD 1180, 30 min prefermentation, 24 h.

Bioreduction of α,β-unsaturated carbonyl compounds **1a-d**

Cinnamaldehyde **1a** is completely reduced to the saturated alcohol in quantitative yield with a ratio of resin/substrate = 1. The striking difference with respect with the conventional biotransformation is that no diol from the acyloin condensation is detected in the mixture. In the absence of the extractive phase, a mixture of saturated and unsaturated alcohols is obtained at the same reaction time. The improved efficiency in the present case is proved not only by the better selectivity but also from the fast conversion into the final saturated alcohol **2a** (100% formation of the saturated alcohol vs. 34%). The acyloin condensation is also completely suppressed when the other substrates **1b**, **1c** and **1d** are fermented. The yeast reduction of (Z)-α-bromo cinnamaldehyde **1b** gives the corresponding bromo alcohol **2b** [17] of (*S*) absolute configuration in quantitative yield and very high enantiomeric excess (98.6%). The ee was evaluated from GC on a chiral stationary phase and comparison with a racemic sample. The absolute configuration was established by converting **2b** into 1-phenyl-2-propanol **6** (epoxide formation and $LiAlH_4$ reduction) of (*S*) absolute configuration and comparison of the $[\alpha]_D$ values found in the literature [20] (scheme 2). The absolute configuration is in agreement with a working model for the reduction of triply substituted double bond [4]. Compound **2b** has not been prepared before in enantiomerically pure form. It is an intermediate in the synthesis of the active enantiomer of the anorressant 3-benzyl-morpholine [21].



1b **2b** **5** **6**

Scheme 2

The dramatic improvement of the enantiomeric excess with respect to normal fermenting conditions suggests that this is due to the beneficial effect exerted by the controlled substrate concentration on two competing enzymes. The presence of multiple enzymes with enoate reductase activity with different enantiopreferences has been proposed before from the study of yeast reduction of 7 in the presence of deuterated cofactors [22]. A direct correlation between initial substrate concentration and product enantiomeric excess was obtained by comparing different runs at different substrate concentration in normal fermenting conditions (figure 1).

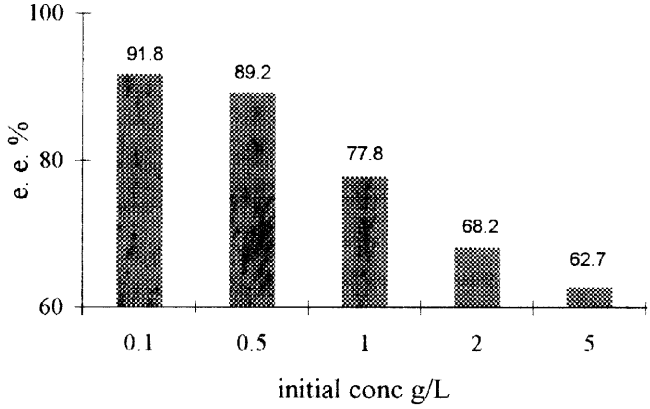


Figure 1. Variation of the enantiomeric excess of **2b** depending from the initial concentration of **1b** during the 24 h b. y. fermentation without the use of absorbing resins.

In the absence of absorbing resins, significant enantioselection is only obtained at concentrations of no preparative value (0.1 g/L). As little as 10% w/w of resin is sufficient to bring the ee of **2b** to 95.6%. With higher resin-substrate ratios the ee increases and then levels off (figure 2).

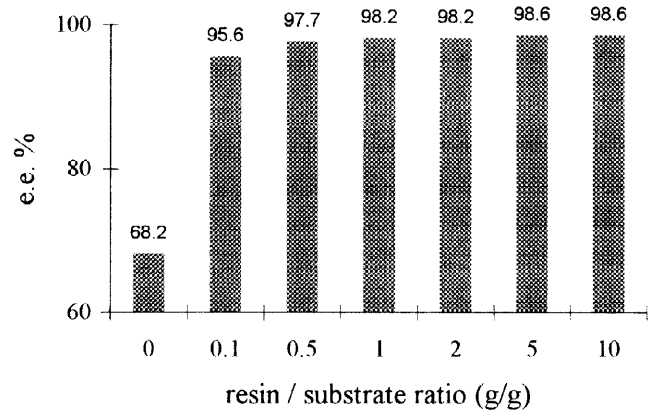


Figure 2. Variation of the enantiomeric excess of **2b**, produced in the 24 h fermentation of **1b** (2 g/L initial concentration) with b. y., with the amount of absorbing resin used. Identical results were obtained at 5 g/L initial concentration (e.e. with no added resin 62.7, see figure 1).

That the improved enantioselectivity of the reaction is due to substrate concentration control is evident from the data in figure 3. At low resin/substrate ratios the resin significantly extracts the substrate, while the product has a much lower affinity for the solid phase and is progressively extracted with growing amounts of resin. Thus the resin ultimately controls the substrate specificity and the stereoselectivity of the biotransformation transforming a multi-product low yielding non-stereospecific reaction into a high yielding process of an enantiomerically pure compound.

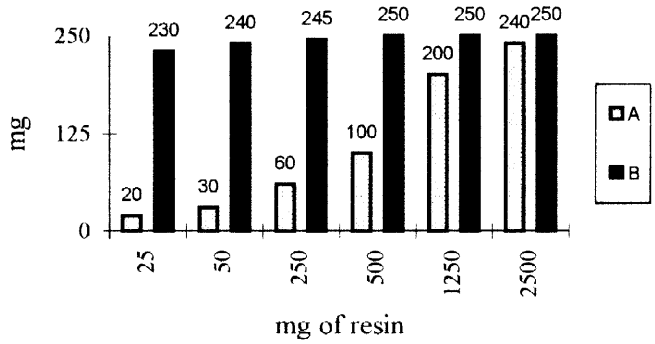


Figure 3. Substrate **B (1b)** and product **A (2b)** recovery from 100 mL of a 2 g/L solution as a function of the amount of resin.

The other chiral saturated alcohols **2c** and **2d** which can already be obtained in high enantiomeric excess under normal fermenting conditions were formed with the same e.e. with or without resin. The ee was within the detection limit of the other enantiomer. Also compound **1d** was transformed with no formation of diols and this is a common feature with all these similar substrates. The yeast reduction of 7 has been reported to give the saturated ketone **8**, the impact flavour of raspberry which is prepared by this method in a *natural way* [23].

Extractive bioconversion of massoia lactone **9**

Massoia lactone is a natural product used as a fragrance in the food industry and employed as the substrate for the bioreduction to *natural* δ-decanolide **10**, an important aroma present in minute amounts in butter fat and in a variety of fruits. Figure 4 shows the conversion obtainable at 24 hours and 48 hours for the reduced compound using a resin/substrate ratio of 5 : 1. In this case the concentration limit in the normal fermenting condition is 2 g/L. At 3 g/L of initial substrate concentration, only 17.5% conversion is observed. If higher substrate concentrations are employed, the conversion progressively lowers, indicating a clear substrate/product inhibition effect. The use of resins can increase the space-time yield up to the values reported in the figures. At

48 hours, conversions higher than 80% can be achieved with an initial substrate concentration of up to 8 g/L. Biotransformation of 25 g can easily be performed in high yield in 144 hours providing that additions of b. y. and glucose were performed every 24 hours (see figure 6). However the conversion is strongly dependent on the stirring and aeration conditions. Best results are usually obtained in horizontal shakers. Moreover figure 5 shows that by increasing the resin/substrate ratio to above 5, the conversion decreases, probably due to the reduced viability of the biomass due to the impact with the resin spheres.

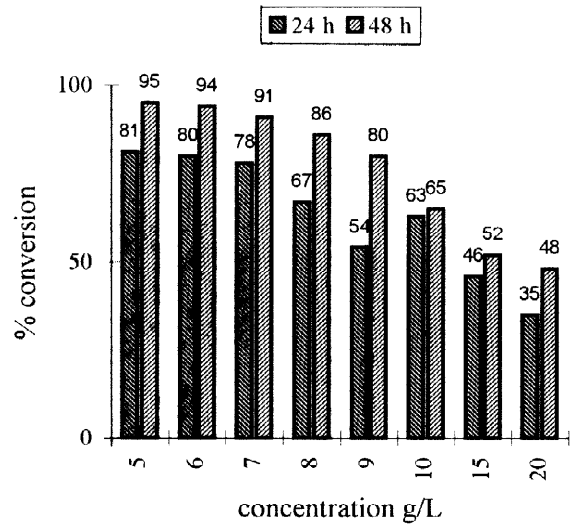


Figure 4. Conversion of Massoia lactone **9** (absorbed on XAD 1180, resin/substrate ratio = 5:1) into **10** by the action of b.y. at 24 and 48 h at different initial concentrations.

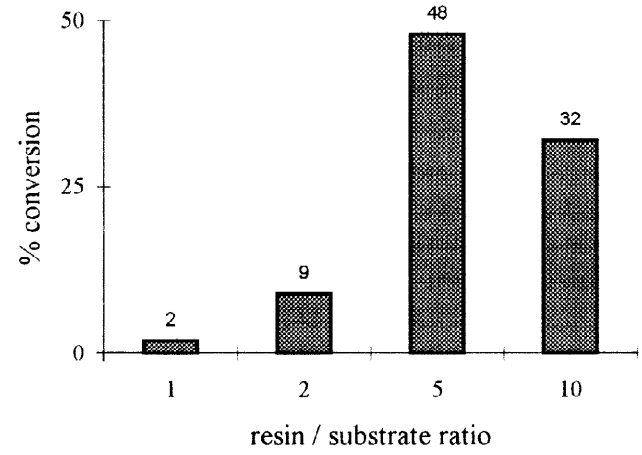


Figure 5. Conversion of Massoia lactone **9** into **10** by b.y. at 20 g/L initial concentration at 48 h as a function of the amount of resin used.

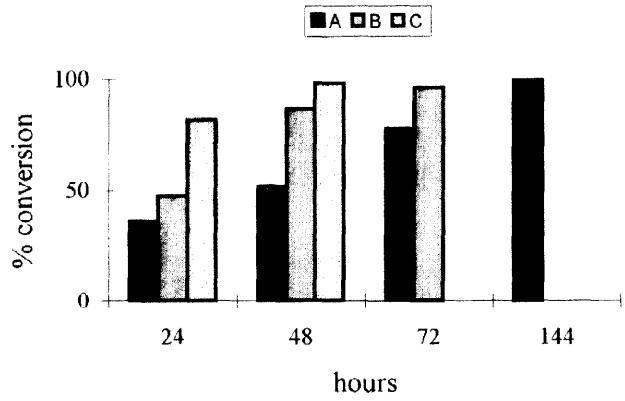


Figure 6. Conversion of Massoia lactone **9** into **10** by the action of b. y. in the presence of absorbing resins (resin/substrate ratio = 5:1) The recovery of **10** is higher than 90%.
A: Beaker 25 L, 50 g of **9**, 10 L water, 500 g glucose, 2.5 kg b.y., mechanical stirrer. Addition of portion of b. y. (500 g, total 2 kg) and glucose (100 g, total 400 g) each 24 h.
B: Erlenmeier conical flask 6 L, 5 g of **9**, 1000 mL water, 50 g glucose, 250 g b. y. horizontal shaker
C: Erlenmeier conical flask 300 mL, 250 mg of **9**, 50 mL water, 2.5 g glucose, 12.5 g b.y. horizontal shaker.

Conclusion

Multiphase techniques have been used in extractive bioconversion in order to increase the yield of products, minimise product inhibition effects or shift the equilibrium to the right. This includes the use of membrane bioreactors and the use of absorbing resins [5-10]. The application has been mainly confined to the extraction of fermentation products. Extractive whole cells biocatalysis is a related application in which the concentration of non-water soluble substrates and products in the water phase is controlled by the addition of a third phase in the form of a hydrophobic low cost extractive resin. This concept applied to the well known baker's yeast bioconversion of α,β-unsaturated carbonyl compounds allows improvements in the stereo- and chemoselectivity and product recovery. The use of low molecular weight enzyme inhibitors for stereochemical control has been reported extensively in yeast reduction [4]. In contrast with that methodology resulting invariably in lowered yields, the use of resins has a beneficial effect both on selectivity and on reaction rates and product recovery resulting in a much improved overall process. This simple technique promises to be of wide applicability in biotransformations with whole cell biocatalysts.

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Experimental

General procedure. Substrates **1a** and **1c** were of commercial origin (Aldrich). Substrates **1b**, **1d** and **7** were prepared following literature procedures [24-26].

Analytical. Chiral analysis was performed on a DANI 8610 with a FID detector, fitted with a glass capillary column, Megadex DACTBSβ-cdx (Mega, Legnano Italy), 25 mt x 0.25 mm i.d., film thickness 0.25 μm. $[\alpha]_D$ were recorded with a Propol automatic digital polarimeter. ^{1}H NMR spectra were recorded on a Varian EMX 250 MHz with TMS as internal standard, coupling constants are measured in Hz.

Absorption of the substrates onto the resin. The crude commercial resin XAD 1180 was previously washed with deionized water (3 mL for 1 mL of resin) and acetone (3 mL for 1 mL of resin). The substrate was dissolved in acetone and the resin, once dried, added to the solution (ex. 1 g of substrate, 10 mL of acetone and 1 g of dry resin). The mixture was shaked for 10 min, avoiding the use of magnetic bar, and then the acetone was evaporated at reduced pressure. The solid so obtained was poured directly into the fermentation vessel.

General fermentation conditions. To a solution of 50 mL of tap water and 2.5 g of D-glucose, 12.5 g of fresh baker's yeast (Distillerie Italiane, Eridania) were added . The mixture was fermented for 30 min at 28 °C and the substrate (100 mg), absorbed onto the resin, was added in one portion. After 24 or 48 h stirring in a horizontal shaker at 120 rev/min at 28 °C, the reaction mixture was filtered through a sintered glass funnel (porosity 0, > 160 μm) and the resin was washed with tap water (3 x 10 mL). The resin was collected and extracted with ethyl acetate (3 x 15 mL). The ethyl acetate was dried with anhydrous Na_2SO_4 and evaporated at reduced pressure so as to obtain crude material (110 mg) which was used directly for analysis.

(*S*)-2-Bromo-3-phenyl-propan-1-ol 2b. Using the conditions reported in the general fermentation conditions **2b** was produced by baker's yeast fermentation. Pure sample was obtained by bulb to bulb distillation, bp 110 °C at 0.1 mmHg, colourless oil. ^{1}H NMR δ ($CHCl_3$) 2.41 (1H, OH, s), 3.20 (2H, CH_2, m), 3.77 (2H, CH_2, m), 4.31 (1H, CH, m) and 7.27 (5H, Ph, m). $[\alpha]_D$ = -22.6 (c 5, $CHCl_3$). Program temperature for the GLC chiral analysis on the MEGA column: 40 °C 1 min, 20 °C / min, 125 °C 2 min, 1 °C / min, 210 °C; injector 250 °C, detector 250 °C. Retention times: **(*R*)-2b** 31.50, **(*S*)-2b** 32.32 (racemic **2b** was synthesised as reported in the literature [20]). Anal calcd for $C_9H_{11}BrO$: C, 50.26; H, 5.15; Br, 37.15. Found: C, 50.25; H, 5.12; Br, 37.19.

(R)-2-Benzyl-oxirane 5. Literature procedure [21] was followed for the preparation of **5**, the reaction was performed on 8 g of **2b**. **5**, a colourless oil, has the following optical rotation value: $[\alpha]_D$ = +25.95 (c 5, benzene), $[\alpha]_D$ = +19.14 (neat), lit [27] +19.19 (neat). ^{1}H NMR δ ($CHCl_3$) 2.55 (1H, CH_2, dd, J=2.48 and 4.95), 2.79 (1H, CH_2, m), 2.87 (2H, CH_2, m), 3.15 (1H, CH, m) and 7.28 (5H, Ph, m).

(*S*)-1-Phenyl-propan-2-ol 6. 3 g (22 mmol) of **5**, dissolved in 15 mL of dry diethyl ether, were added dropwise to a suspension of $LiAlH_4$ 0.23g (6 mmol) in 50 mL of anhydrous diethyl ether. The mixture was stirred at reflux for 2 h, after that time the reaction was quenched with ice and the organic phase separated; the aqueous layer

was extracted twice with diethyl ether and the combined organic phases were dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave 3 g of crude oily material which was purified by bulb to bulb distillation, bp 100 °C at 10 mm Hg, so as to obtain 2.6 g (86% yield) of pure **6**, colourless oil, $[\alpha]_D$ = +40.68 (c 5, benzene, lit [20] +41). Anal calcd for $C_9H_{12}O$: C, 79.37; H, 8.88. Found: C, 79.35; H, 8.74. ^1H NMR δ ($CHCl_3$) 1.22 (3H, CH_3, d), 1.71 (1H, OH, s), 2.72 (2H, CH_2, m), 3.99 (1H, CH, m) and 7.25 (5H, Ph, m).

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