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Stereochemistry of the Double Bond Saturation in the Formation in Baker's Yeast of 4-(4-hydroxyphenyl)-2-butanone (Raspberry Ketone)

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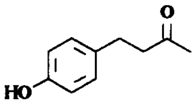
Abstract: Yeast extract-mediated reduction of 4-(4-hydroxyphenyl)-but-3-en-2-one **3** to raspberry ketone **1** occurs with transfer β to the carbonyl group of H_R from NADPH and of an hydrogen atom from the solvent at α position, as indicated by experiments with (4*R*) and (4*S*) [4-^{2}H]-NADPH and with deuterated solvent. The same extract converts **3** in **1** in the presence of NADH, but much less efficiently. In this latter case H_S and not H_R is transferred from the reduced cofactor to position 4 of **1**. Horse liver alcohol dehydrogenase-mediated reduction of **1**, bearing deuterium labelling at positions 1, 3 and 4 and obtained from **3** using the whole-cell system in the presence of D_2O, gave an *S* carbinol possessing 0.95 *ee*, shown by ^{2}H NMR studies onto the diacetate **7** to contain an excess of the (3*S*,4*S*) diastereoisomer, thus suggesting a prevalent β *re-face,syn* addition of hydrogen across the double bond of **3** in the whole-cell mediated conversion into **1**

4-(4-hydroxyphenyl)-2-butanone (Raspberry ketone) **1** occurs, free or bound as β-D-glucoside, in trace amounts in many plants, and is one of the components of the flavor of raspberry fruit.[1-4] In the synthetic modification[5] raspberry ketone **1** finds wide industrial applications in both flavor and fragrance formulations.[6] The *S* and *R* enantiomeric forms of carbinol **2** occur free or as glucosides (*epi*-rhododendrin and rhododendrin, respectively) in many plants, including *Rhododendrom maximum, Acer nikoense* MAXIM, *Taxus baccata* and *Betula alba.*[7]

Different hypotheses have been formulated on the biosynthesis of raspberry ketone.[8] However, recent experiments using cell-free extracts of raspberry fruits[9] indicate that the C-6---C-4 framework of raspberry ketone **1** is constructed by condensation of C-6---C-3 *p*-coumaryl-CoA with malonyl-CoA, followed by decarboxylation to the unsaturated ketone **3**, which eventually undergoes NADPH-assisted enzymic double bond saturation.



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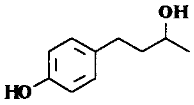

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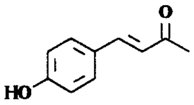

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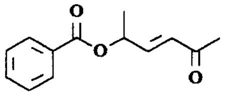
The mode of formation by natural procedures of raspberry ketone **1** recently became of interest as it is biogenerated from relatively abundant natural precursors. Indeed, there has been legislative discrimination[10] between chemically identical food aroma constituents of synthetic origin and those derived from natural sources. The latter products, labelled 'natural' are receiving consumer preferences, and, therefore, it has become desirable to obtain substantial quantities of these valuable materials either by extraction from botanical sources or by biotransformations of abundant natural precursors. The former approach is not applicable to **1**, which occurs in ripened raspberry fruits at the level of a few milligrams/kg.

In this context, the bioconversion of **3**, the natural precursor of **1** in the plant and available by condensation of the sodium salt of 4-hydroxybenzaldehyde with acetone, two materials readily available in the natural modification, into **1** using fermenting baker's yeast has been described as a viable entry to 'natural' raspberry ketone.[11] Similarly, 'natural' **1** is accessible from the *S* form of carbinol **2**, obtained by enzymic hydrolysis of the glucoside extracted from the bark of *Betula alba*, by oxidation with *Candida boidinii* and other microorganisms.[12]

For a few years we have been interested in the stereochemical aspects of the biogeneration of flavor materials through enzymic reduction of natural precursors containing carbonyl-activated *Z* double bonds. These studies were also useful to solve the problem of the 'authentication' of the pathway followed in the production of a 'natural' flavor. This goal is effectively achieved by site nuclear isotope fractionation (SNIF) NMR studies, which take advantage of knowledge on the origin and the stereochemistry of the hydrogen atoms eventually incorporated into the educts.[13-15] In the above connection we have investigated also the mode of saturation by baker's yeast of the E and *Z* double bonds of the acyclic unsaturated methyl ketones **4** and **5** in the conversion into **6**.[16] In the cases examined[13,14,16] the double bond reduction occurs in *anti* fashion with β *re-face* addition of hydrogen.



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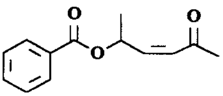

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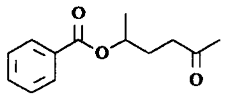

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The recent appearance of two reports on the obtainment of natural raspberry ketone **1**[11,12] using oxido-reductive enzymes prompted us to present the results of our studies in the field.

Thus, moist commercial baker's yeast effectively reduces the unsaturated ketone **3** to raspberry ketone **1** and to the carbinol **2**. The time-course of the transformation (Figure 1) clearly shows the modest capacity of baker's yeast to reduce the methyl ketone **1** to carbinol with respect to the ability to saturate the double bond of **3**.

No trace of the allylic alcohol formed by carbonyl reduction of **3** was observed.

The carbinol **2** was shown by HPLC studies on the diacetate, using a Chiralcel OD column, and comparison with an authentic sample,[7] to contain *ca.* 93% excess of the *S* enantiomer.

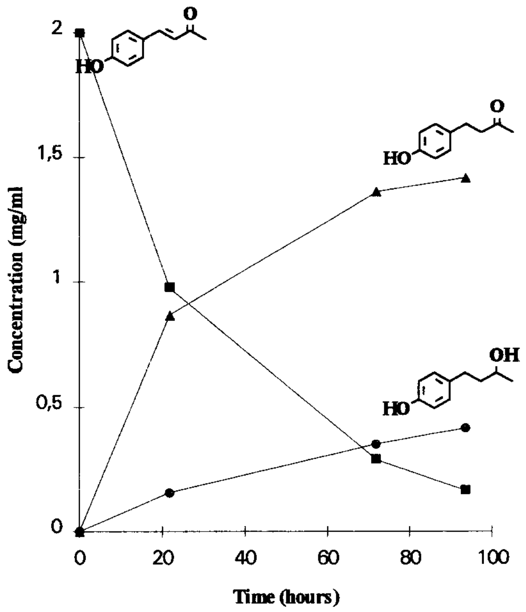


Figure 1. Time course of the baker's yeast reduction of **3**: 30 g b.y., 10 g glucose, 150 ml tap water and 300 mg of **3** in 1 ml of EtOH.

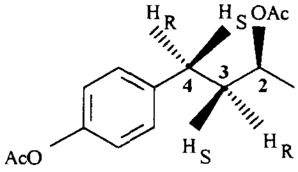
In order to determine the origin of the hydrogen atoms formally added on **3** during the reduction process, the yeast transformation was performed in the presence of deuterated water. The ^{2}H NMR spectra of materials **1** and **2** obtained from **3** under these conditions are reported in Figure 2, A and B, respectively. Thus, raspberry ketone **1** incorporates deuterium labelling at positions 1 (1.59 ppm), 3 (2.19 ppm) and 4 (2.60 ppm) in *ca.* 1.92, 1.58 and 1.00 ratios, respectively. Similarly, the *S* carbinol **2** shows the label at positions 1 (1.01 ppm), 2 (4.87 ppm), 3 (1.47 and 1.67 ppm) and 4 (2.34 and 2.41 ppm) in *ca.* 0.86, 1.00, 1.68 and 1.29 ratios, respectively.

The deuterium atoms present at the various positions of the framework of **1** and **2** derived from **3** are conceivably incorporated through different mechanisms. In particular, the labelling of the methyl group is expected to be due to the ketone enolization, whereas that at the remaining positions is a consequence of the enzymic reduction, and should occur as observed in previous experiments.[13-15] We would expect that the deuterium incorporated at position 3 of **1** and **2** is picked up from water during the bioreduction[13-17] and/or exchanged in **1** by ketone enolization. Conversely, the deuterium atoms located at position 4 of **1** and at positions 2 and 4 of **2** have been delivered as 'hydride' in the reduction step(s) from the reduced form of the nicotinamide cofactor(s), which exchanged with the solvent deuterium atoms the hydrogens at position 4 under the action of a diaphorase.[18]

This view is indeed supported by experiments of reduction of **3** performed using purified yeast extract

(Sigma type 2) in deuterated solvent, in the presence of NADPH. Indeed, ^2H NMR studies on the mixture of **1** and **3** obtained under these conditions indicated the incorporation in **1** of only one deuterium atom at position 3. This result thus indicates that in the enzymic reduction of the carbonyl activated double bond of **3** the hydrogen atom ending up α to the carbonyl is picked up from water as a proton, whereas that at position β is delivered from the reduced nicotine cofactor.[17] Moreover, the experiment indicates that the yeast extract is devoid of diaphorase activity since no label is present at position 4 of the reduced material.

This observation allowed us to establish the origin of the hydrogen atom delivered at position 4 of **1** in the enzymic reduction of **3** by means of incubation experiments with the commercial yeast extract using [4(*R*)-^2H]- and [4(*S*)-^2H]-NADPH,[19] without the danger of washout of the label during the incubation. Again, ^2H NMR studies on the mixture of **1** and **3** obtained from the incubation of **3** with the two forms of stereospecifically deuterated cofactor indicated that H_R of the latter and not H_S is delivered at position 4 of **1** enzymically produced from **3**. The precise definition of the origin of the hydrogen atoms delivered onto the double bond of **3** during the enzymic reduction to **1**, *i.e.*: the α hydrogen from water, the β hydrogen from H_R of NADPH (Scheme 1), stimulated studies designed to define the absolute stereochemistry of the double bond saturation. This was achieved by means of ^2H NMR studies on the *S* diacetate **7** obtained from the *S* form of carbinol **2** enzymically produced from **3**.



7

To this end the complete assignment of the ^1H spectrum of **7** is a prerequisite, which implies some knowledge of the conformational properties of the molecule. A proton spectrum with sufficient signal separation to allow a first order analysis was obtained in deuterated benzene at 600 MHz (Table 1). Although the values of the vicinal coupling constants reported in Table 1 show that the molecule is conformationally rather flexible, some couplings (as J(2,3) of 7.9 Hz and J(3,4) of 10.2 Hz) suggest that it should have a slightly preferred conformation. In particular hydrogen H-2 displays a vicinal coupling constant of 7.9 Hz with one of the protons at C-3 indicating that the two nuclei are preferentially *anti* oriented.

To identify the C-3 methylene hydrogen showing the major coupling constant with H-2, we have acquired the undecoupled carbon spectrum of **7** to determine the long-range proton-carbon coupling constants. The methyl group CH_3-1 (20.7 ppm, C6D6) has three long-range coupling constants by 1.6, 3.2 and 3.2 Hz (triplet of doublets) due to the coupling of carbon C-1 with protons H-2, H_S-3 and H_R-3 respectively. The values of $^3J(^{13}C,^1H)$ follow the Karplus equation, reaching the maximum (ca. 7-9 Hz) for dihedral angles near 0° and 180° and the minimum (ca. 0-2 Hz) for dihedral angles near 90°.[20] For compound **7** the methyl group is oriented preferentially *gauche* to H_R-3 and H_S-3 (*i.e. anti* to C-4) since carbon C-1 shows small coupling constants (3.2 Hz) to both hydrogens.

From all these observations it can be concluded that the coupling constant by 7.9 Hz occurs between protons

H-2 and H$_S$-3. Once recognized H$_S$-3 the other protons can be easily assigned, being H$_S$-3 (1.68 ppm) and H$_R$-3 (1.46 ppm) *anti* to H$_R$-4 (2.35 ppm) and H$_S$-4 (2.43 ppm) respectively as indicated by the value of 10.2 Hz of ^{3}J(H$_S$-3,H$_R$-4) and ^{3}J(H$_R$-3,H$_S$-4).

Table 1 ^{1}H chemical shifts and coupling constants for compound **7**[a,b]

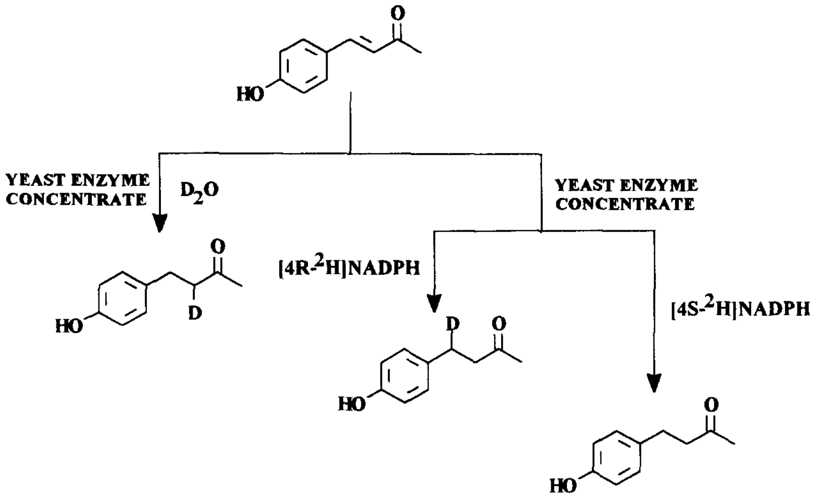
Proton	δ(ppm)	J(Hz)	
CH$_3$	1.04	J(H-2, CH$_3$)	6.3
H-2	4.93	J(H-2,H$_R$-3)	4.7
H$_R$-3	1.46	J(H-2,H$_S$-3)	7.9
H$_S$-3	1.68	J(H$_R$-3,H$_S$-3)	13.8
H$_R$-4	2.35	J(H$_R$-3,H$_R$-4)	6.1
H$_S$-4	2.43	J(H$_R$-3,H$_S$-4)	10.2
COCH$_3$	1.71	J(H$_S$-3,H$_R$-4)	10.2
COCH$_3$	1.77	J(H$_S$-3,H$_S$-4)	5.8
		J(H$_S$-4,H$_R$-4)	13.9

[a] Solvent C$_6$D$_6$ [b] Spectrum taken at 600 MHz

This stereochemical assignment based on the NMR spectra has been partially confirmed experimentally by performing a catalytic reduction of **3** with deuterium gas. This reduction leads to the obtainment of carbinol **2** deuterated in positions 2, 3 and 4. The two diastereotopic deuterium atoms at C-3 give rise to signals at 1.44 and 1.66 ppm in a ratio ca. 6:4 respectively. Analogously the deuterium atoms at C-4 give rise to signals at 2.33 and 2.41 ppm respectively in the same 6:4 ratio. Since the catalytic reduction occurs *via* cis addition of D$_2$ to the double bond the couple of signals of major intensity (and also that of minor intensity) belongs to deuterium atoms which are *syn* on C$_3$-C$_4$ fragment (H$_R$-3 and H$_R$-4, major signals, or H$_S$-3 and H$_S$-4, minor signals), in agreement with the NMR findings.

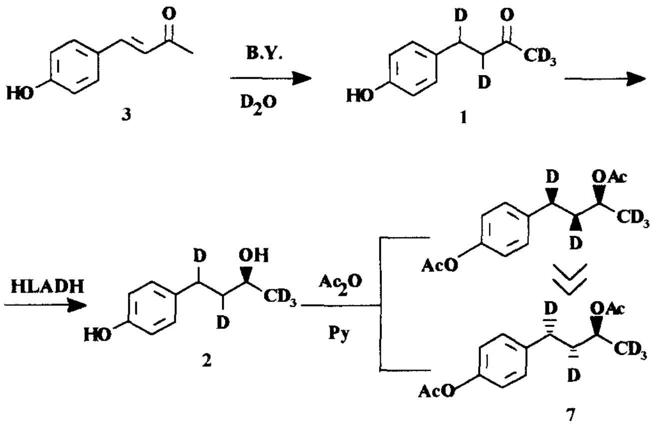
Thus, the assignment of the stereochemistry of the operation occurring in the production of the saturated ketone **1** from **3** (Scheme 1) can be achieved by NMR studies on enantiomerically pure **7**.

Unfortunately, baker's yeast reduction of **1** affords carbinol **2** of *S* absolute configuration, but with *ee* values not exceeding 0.87. The presence of the *R* enantiomer in the material so obtained renders the calculations more complicated. Accordingly, the transformation of **1** into enantiomerically pure (*S*) **2** using isolated enzymes was studied. Horse liver alcohol dehydrogenase in the presence of formic acid/formate dehydrogenase effectively reduces **1** to (*S*) **2**, but the *ee* value of the product, determined on the diacetate **7** by HPLC and ^{1}H NMR studies in the presence of a chiral shift reagent resulted to be 0.94-0.95. We were unable to produce from **3** the fairly large amount of deuterated **7** required for the ^{2}H NMR studies using in the first enzymic step [(4*R*)-^{2}H]-NADPH for cost reasons. Accordingly, we reduced to **2** with HLADH/NADH the ketone **1** obtained from **3** upon baker's yeast reduction in deuterated water. In the material obtained in this way, as indicated above (Figure 2, C), the deuterium located at position 4 (2.34 and 2.41 ppm) arises indirectly from water through the diaphorase-mediated exchange of the hydrogen atom(s) at position 4 of the reduced nicotinamide cofactor.



Scheme 1. Mode of labelling of **1** obtained by yeast-extract mediated reduction of **3**.

Thus, the sample of **7** of 0.95 *ee*, produced from **3** through the operations of Scheme 2, displays the ^{2}H NMR spectrum reported in Figure 2 C. This indicates 78:22 relative intensities of the deuterium signals at positions (4*S*) (2.41 ppm) and (4*R*) (2.34 ppm), whereas the ratio relative to (3*S*) (1.67 ppm) and (3*R*) (1.47 ppm) is 6:4, respectively (Figure 2, C).



Scheme 2. Mode of production of deuterated **7**, whose ^{2}H NMR spectrum is in Figure 2, C.

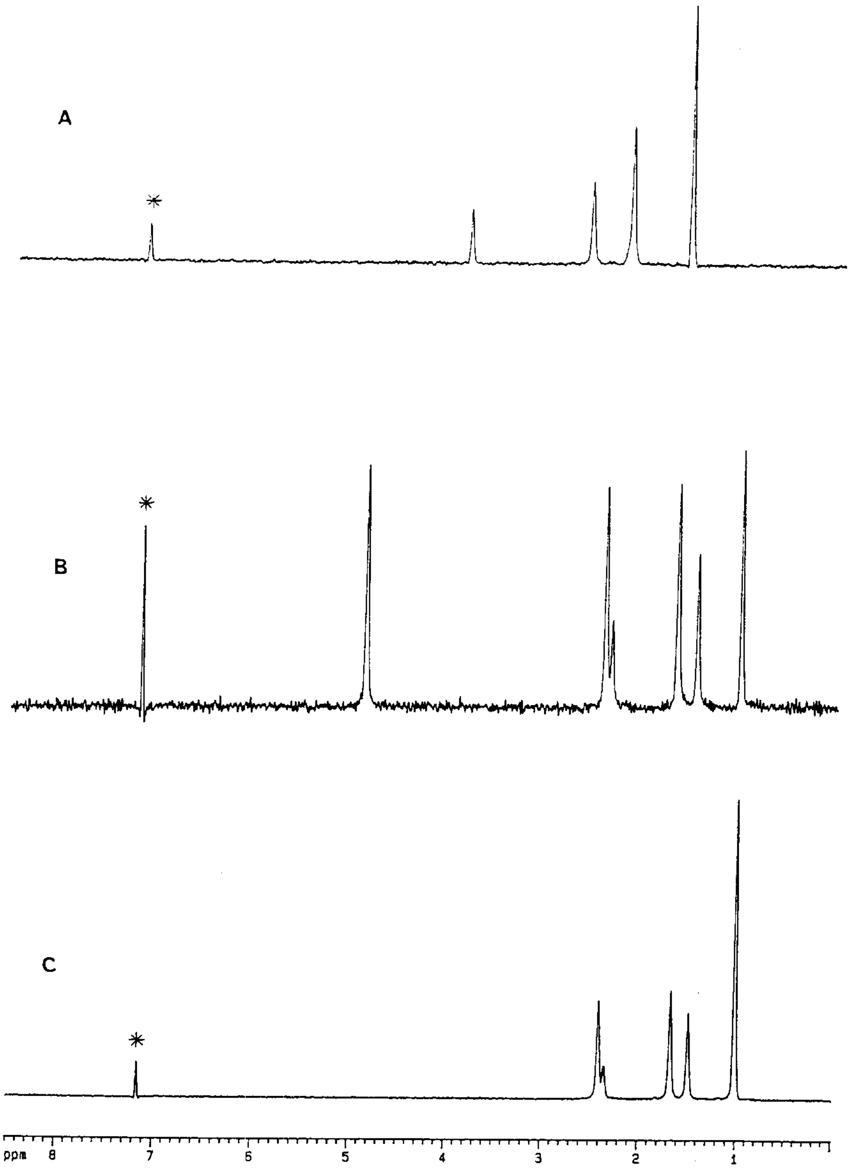


Figure 2. ^2H NMR spectra in C_6H_6 of A) compound **1** obtained in baker's yeast/D_2O reduction of **3**; B) diacetyl derivative of **2** obtained from **3** in baker's yeast/D_2O reduction and C) compound **6** obtained by HLADH reduction of **1**, produced from **3** in baker's yeast/D_2O. Asterisks denote the natural abundance deuterium signal of the solvent.

Clearly, deuterated (2*S*) **7** is a mixture of diastereoisomers in which the (2*S*,3*S*,4*S*) material prevails. However, from a mechanistic point of view, particularly relevant is the stereochemical situation at position 4. Indeed, one could expect that the methylene group at position 3 of **7**, produced from the methyne at position 3 of **3** by pick-up of a $^2H^+$ from the solvent during the double bond saturation, could have lost the configurational integrity through enolization. The same is seemingly not possible for the methylene group at position 4 of **7**, generated by hydride transfer from the reduced cofactor during the reduction.

An economic interpretation of these stereochemical results suggests that in baker's yeast the saturation of the double bond of **3** occurs through the intervention of at least two enzymic systems acting with opposite stereochemistry. If we judge from the above data, *i.e.*: the prevalence in **7** of the (2*S*,3*S*,4*S*) diastereoisomer, ketone **1** is mostly produced with the participation of an enzyme which determinates *syn* addition of hydrogen atoms across the double bond, from the β-*re* face of the α,β unsaturated ketone. The latter particular feature has been observed in previous baker's yeast mediated reductions of substrates containing a carbonyl-activated double bond,[13,14] including **4** and **5**, structurally related to **3**.[16] However, in these and other instances,[21] the overall process occurs by formal *trans* addition, in line with the stereochemical outcome of enoyl-CoA reductase-mediated reductions.[22] Particularly significant to this purpose is the result of incubation of **3** with the yeast extract in the presence of (4*R*) and (4*S*) [4-^{2}H] NADH. Indeed there is a modest but definite conversion of **3** in **1** which resulted at ^{2}H NMR studies to incorporate at position 4 the deuterium atom originally located in position (4*S*) of the reduced cofactor. Thus, in yeast extract the reduction of **3** to **1** is mediated by enzymic activities transferring at position 4 H_R from NADPH and H_S from NADH. As far as the observed *syn* stereochemistry is concerned a precedent occurred in *Nicotiana tabacum*, where the saturation of the *Z* hindered double bond of verbenone leading to verbanone is taking place by *re-re*, *syn* hydrogen addition.[23]

It is likely that the production of **1** in deuterated water in the whole cell system labelled with deterium in positions (4*R*) and (4*S*) in a 78:22 ratio, as shown by NMR studies on **7** (Figure 2, C) is the consequence of this dual pathway of reduction. Further studies to elucidate the nature of the two enzymic activities are in progress.

Experimental

All NMR spectra were acquired on a Bruker ARX 400 spectrometer. The ^{2}H spectra were run in the gated ^{1}H broad-band decoupling mode to eliminate all small proton-deuterium couplings and obtain sharp deuterium signals.

HPLC analyses were performed on a Merck-Hitachi L-6200 with a UV detector using a Merck Hitachi D-2500 integrator employing two different types of columns: *i*) a Chiralcel OD (Daicel) column, eluted with n-Hexane/i-PrOH 9/1 with 0.6 ml/min flow, to separate the acetate derivatives of **2** (couple of enantiomers) **1** and **3**; readings were made at 220 nm. The retention times were 9.9 and 11.2 min for the *R* and *S* diacetates of **2** , 16.7 and 23.6 min for the acetates of **1** and **3** respectively. *ii*) a 10-μm Nucleosil C_{18} column (4.6 x 250 mm), eluted isocratically with 20 mM phosphate buffer (pH 7) and 5% methanol with 1 ml/min flow, to separate NAD from NADH and to follow the course of NADPH; readings were made at 340 and 260 nm. The retention times were 12.2 and 14.1 min for NAD and NADH, while NADPH and NADP were both eluted at 11.3 min.

GLC analyses were performed on a Dani HS 86.10 gas chromatograph equipped with a fused silica capillary column 30m x 0.249 mm i.d. DB-5 d.f. 0.25 μm and a FID detector; in order to separate **3**, **2** and **1** the analysis conditions adopted were: 80 °C(1 min), 10 °C min^{-1} up to 155 °C(1 min), 1 °C min^{-1} up to 165 °C(2 min), 15 °C min^{-1} up to 250 °C(3 min). The retention times were 16, 16.5 and 23 min for **1**, **2** and **3** respectively.

General Procedure for the Baker's Yeast Reduction of the Unsaturated Methyl Ketone 3.

A suspension of 30 g of baker's yeast and 10 g of D-glucose in 150 ml of tap water was stirred at 36°C. A solution of the unsaturated ketone **3**, 300 mg (1.85 mmol), in 2 ml of ethanol was then added. After 92 hours **3** had been completely consumed to give **2**; $\delta_H(CDCl_3)$ 1.33 (3H, d, J 6.3, 1-Me), 1.74 (2H, m, 3-CH_2), 2.64 (2H, m, 4-CH_2), 3.84 (1H, , J 6.3, 2-H), 5.37 (1H, br, OH), 6.75 (2H, m, aromatic protons *ortho* to OH), 7.05 (2H, m, aromatic protons *meta* to OH) and **1**; δ_H (C_6D_6) 1.54 (3H, s, 1-Me), 2.13 (2H, t, J 7.3, 3-CH_2), 2.66 (2H, t, J 7.3, 4-CH_2), 4.74 (1H, br, OH), 6.59 (2H, m, aromatic protons *ortho* to OH), 6.85 (2H, m, aromatic protons *meta* to OH). The course of the reaction was monitored through HPLC (Fig. 1): to this end , a sample of reaction mixture was extracted with AcOEt and the residue of the organic phase was acetylated with Ac_2O/pyridine, evaporated and used for the analysis.

Baker's Yeast Reduction of 3 in D_2O. To a stirred mixture of baker's yeast (200 g) and glucose (50 g) in 450 ml of tap water and 150 ml of D_2O, a solution of **3**,800 mg (4.94 mmol) in ethanol (3 ml) was dripped in at 35-38°C. After 24 hours 100 g of baker's yeast and 25 g of glucose were added. After 48 hours the mixture was diluted with AcOEt (200 ml), stirred for 10 min and filtered on a Buchner funnel through a Celite pad. This was washed twice with AcOEt (200 ml) and the combined organic phases were used to extract the filtrate. The washed and dried organic phase was evaporated to give the crude product mixture. Product **1** was then separated from the carbinol **2** by a flash column chromatography (n-Hexane/AcOEt=7/3) of the crude extract. Both products were obtained with 45% yield each. The carbinol **2** underwent acetylation to give **7**, shown to possess by HPLC an *e.e.*=86.87%; δ_H (C_6D_6) (see Table 1), δ_D (C_6H_6 see Figure 2, B) 1.01 (1-Me), 1.47 (3-H_R), 1.67 (3-H_S), 2.34 (4-H_R), 2.41 (4-H_S)

General Procedure for the Acetylation of 2.

To a stirred solution of **2**, 340 mg (2 mmol), in pyridine (6 ml), 1 ml of Ac_2O (10 mol) was added; the solution was left overnight at room temperature. The reaction mixture was poured into ice-water and extracted with AcOEt; the organic phase was then washed with diluted HCl to remove the pyridine, aqueous $NaHCO_3$ and water. The oily residue obtained upon evaporation of the dried solution was chromatographed over a SiO_2 column with n-Hexane/AcOEt 7/3 to give **7**, 480 mg of an oil , in 95% yield of **2**.

Yeast Enzyme Concentrate Reduction (Type II Yeast Extract Sigma, Y-2875 Lot 19C7230)

a) Experiment in D_2O.

60 ml of a solution, made up of 30 ml tap water and 30 ml D_2O, containing 1.045 g (6 mmol) potassium phosphate, 60 mg (0,37 mmol) substrate **3**, 240 mg (0,288 mmol) NADPH and 300 mg of Yeast Enzyme Concentrate was titrated to pH 7 and left to react at room temperature. After 6 hours 150 mg of Enzyme

Concentrate were added. The course of the reaction was monitored through GC, thus separating the unsaturated ketone **3** from the main product **1** (no alcohol was formed under these conditions); at the same time it was possible to follow the behaviour of NADPH with HPLC at 340 nm: the reduced coenzyme appears to be partially degraded by the action of a probable pyrophosphatase actually present in the extract.[24] After 24 hours the ketone formed was 10% of the precursor **3** and the reduced cofactor added to the solution had been completely consumed. The recovery of the reaction was quantitative: the solution was extracted with AcOEt and filtered on a Buchner funnel through a Celite pad to destroy emulsions eventually formed. The washed and dried organic phase was evaporated to give the crude product mixture containing 90% substrate **3** and 10% of ketone **1**, δ_D (C_6H_6 + $CHCl_3$) 2.47 (3-H of **1**), 2.17 ($COCH_3$ of **3**), 1.88 ($COCH_3$ of **1**), (no signal for deuterium atoms in position 4 of **1** (expected at 2.71 ppm) has been detected).

b) Experiments with [4R-^{2}H] and [4S-^{2}H] NAD(P)H.

Reduced nicotinamide cofactors were prepared and purified according to the described procedure.[25]

b1) Experiments with [4R-^{2}H] and [4S-^{2}H] NADPH.

100 ml of a 0.1 M potassium phosphate solution,containing 100 mg (0.62 mmol) substrate **3**, 500 mg of Yeast Enzyme Concentrate, 2.23 g (5 mmol)$Na_4P_2O_7$ (sodium pyrophosphate was added in order to inhibit the degradative action of the pyrophosphatase on the coenzyme[26]) and 400 mg (0.492 mmol) deuterated reduced coenzyme (as ammonium salt), was titrated to pH 7 and left to react at room temperature. The course of the reduction of **3** to **1** was monitored through GC, while the oxidation of NADP^{2}H was followed through HPLC. After 24 hours the deuterated coenzyme had been completely consumed and the conversion was 10%. The solution was then processed as described above. The recovery of the crude products was quantitative. The ^{2}H spectrum of **1** recovered from the reduction with [4*S*-^{2}H] NADPH does not show any deuterium signal, while the one obtained with [4*R*-^{2}H] NADPH displays a signal at 2.68 ppm (solvent C_6H_6 + $CHCl_3$ ca. 50%) indicating the presence of deuterium atoms in position 4.

b2) Experiments with [4R-^{2}H] and [4S-^{2}H] NADH.

100 ml of a 0.1 M potassium phosphate solution,containing 100 mg (0.62 mmol) substrate **3**, 500 mg of Yeast Enzyme Concentrate, 2.23 g (5 mmol)$Na_4P_2O_7$ and 400 mg (0.57 mmol) deuterated reduced coenzyme (as ammonium salt), was titrated to pH 7 and left to react at room temperature. The course of the reduction of **3** to **1** was monitored through GC, while the oxidation of NAD^{2}H was followed through HPLC. After 24 hours the deuterated coenzyme had been completely consumed and the conversion was 3%. The solution was then processed as described above. The recovery of the crude products was quantitative. Only the ^{2}H spectrum of **1** obtained from the reduction carried out with [4*S*-^{2}H] NADH shows a small deuterium signal at *ca.* 2.8 ppm (solvent $CHCl_3$) corresponding to labelled C-4.

HLADH mediated reduction of deuterated 1.

The deuterated ketone **1** obtained from the baker's yeast reduction of **3** in D_2O was reduced to **2** with HLADH. 122 ml of a 0.1 M potassium phosphate solution,containing 200 µl (5.3 mmol) formic acid, 60 mg (84.58 µmol)NADH, 200 mg (1.22 mmol) substrate, 50 U of formate dehydrogenase and 50 U of horse liver alcohol dehydrogenase was titrated to pH 7.6 and left to react at room temperature. In this experiment the auxiliary enzyme system FDH-HCOOH is used to regenerate the coenzyme: 13 enzyme recyclings are obtained in this way.[27]

After about 24 hours the reaction was complete and 90% of the ketone had been reduced to carbinol **2**. The aqueous solution was extracted with AcOEt. The washed and dried organic phase was evaporated to give the crude mixture. Purification of the reduced alcohol was performed through a flash chromatography with n-Hexane/AcOEt = 7/3. The carbinol **2**, recovered with 85% yield, subsequently underwent acetylation to give the diacetate **7** with an *e.e.* = 95.6% (HPLC); δ_D (C_6H_6,see also figure 2, C) 1.02 (1-Me), 1.48 (3-H_R), 1.67 (3-H_S), 2.35 (4-H_R), 2.42 (4-H_S).

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