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Diplogelasinospora grovesii IMI 171018, a new whole cell biocatalyst for the stereoselective reduction of ketones

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Abstract—A screening of 416 strains (71 bacterial strains, 45 actinomycetes, 59 yeast, 60 basidiomycetes, 33 marine fungi and 148 filamentous fungi) has been performed to look for microorganisms that display reductase activity in the absence of oxidase activity. A new microorganism, *Diplogelasinospora grovesii* IMI 171018 (a nonpathogen strain), was isolated and showed very high activity and stereoselectivity in the reduction of cyclic ketones. The fungus was selected due to its selectivity towards monocyclic and bicyclic ketones and its easy culture conditions, which allow an easy scale-up. *D. grovesii* is more active in the reduction of conventional ketones than *S. cerevisiae* type II (from Sigma) and can work in the presence of high ketone concentrations (<60 mM). Comparative molecular fields analysis (CoMFA) has been applied in order to explain the steric and electronic properties of the carbonylic compound to be substrate for the microbial alcohol reductase. The CoMFA model is highly predictive ($q^2 = 0.549$) and can be used to explain and to predict the structure of ketones that can or cannot be reduced by this microorganism.

1. Introduction

The number of biotransformation processes for the synthesis of organic compounds has increased recently including a rather large group of microorganisms or enzymes.[1] The main goal in many of these reactions is the formation of a new stereogenic centre in achiral molecules, with high stereoselectivity.[2] In this sense, bioreductions are a very useful tool in the synthesis of pharmaceuticals and fine chemicals.[3–6]

Until recently, the use of whole cells in organic chemistry laboratories has been limited to the well-known reduction of ketones to secondary alcohols using the baker's yeast alcohol dehydrogenase (YADH).[4,7,8] This yeast is suitable for use in organic chemistry laboratories, but secondary reactions and low ees due to the presence, of at least four reductases with opposite enantiopreference[4,9] are obtained. From a synthetic point of view, a high reductase activity without oxidase activity against the alcohol produced, is interesting, especially in regard to the reduction of large ketones. In this way, several yeasts[10,11] and bacteria[12] strains have been described as good producers of enantioselective reductases.[13] Nevertheless, only a small number of fungi have been described as good reductase producers.[10,14]

Alcohol dehydrogenases (ADH) are ubiquitous enzymes involved in many physiological processes. Some of these enzymes contain Fe(II) for the activation of the enzyme, as in the case of the ADH from *Zymomonas mobilis*.[15] Other ADH's do not present a cation in the enzyme, for example, the ADH from *Drosophila lebanoniensis*.[16] However, the majority of ADH's have several Zn(II) ions near the active site or in the dimerisation centre. This is the case with the horse liver dehydrogenase[17] and the baker's yeast ADH,[18] which contains four Zn(II) atoms. These cations could be substituted by Co(II) after Chelex treatment followed by cobalt insertion. These enzymes catalyse the reversible oxidation/reduction of alcohols/aldehydes or ketones using NAD^+/

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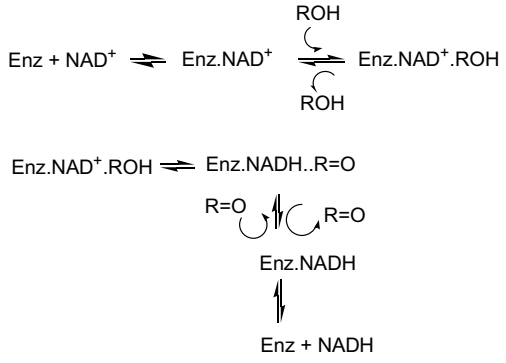
NADH as the hydride acceptor/donor.[16–18] Only in a few cases has NADP(H) been used as a co-factor, as with the ADH from *Clostridium beijerinckii*.[19] The reaction mechanism was described by Theorell and Chanwe in 1951.[20]



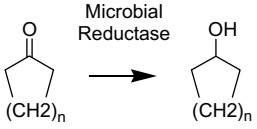
The rate controlling step is the hydride transfer in the ternary complex [enzyme+co-enzyme+organic compound]. In the active site, the presence of Ser, Tyr and Lys or Arg is generally recognised as a fundamental catalytic machinery for the hydride transfer step, as it has been proven by Svensson et al.[21] in the case of the ADH-2 from rat, using X-ray diffraction and molecular modeling.

Herein we report a new fungus, *Diplogelasinospora grovesii*, which shows high activity and enantioselectivity in the reduction of some acyclic or polycyclic hydrophobic ketones. The microorganism was isolated during a screening campaign for the identification of microbial strains with high reductase activity. Comparative fields analysis (CoMFA) was performed in order to show the steric and electronic characteristics that a ketone must show to be a substrate of the ADH from *D. grovesii*. This methodology is very interesting from a synthetic organic point of view because it allows us to obtain a predictive model of the recognition site without previous knowledge of the primary or 3D structure of the protein.

2. Results and discussion

2.1. Selection of strains in the primary screening

The primary screening was performed using 416 strains (71 bacteria strains, 45 actinomycetes, 59 yeast, 60 basidiomycetes, 33 marine fungi and 148 filamentous fungi) while looking for microorganisms selective in the reduction of simple cycloalkanones. The microbial collection was selected of maximising the biological diversity. After the first screening step, using cyclohexanone **1** as the substrate, the selected microorganisms were tested against different ring sized cycloalkanones: cyclobutanone **2**, cyclopentanone **3**, cycloheptanone **4**, cyclooctanone **5** and cyclododecanone **6**. In Table 1 we show the most interesting strains. These selected strains showed higher activity in the reduction of **1** than *S. cerevisiae* (13%) under the same experimental conditions. The selected strains did not show high oxidase activity using cyclohexanol as the substrate (yield in ketone <8%). *D. grovesii* displayed high selectivity with medium and small ketones, giving good alcohol yields (Table 1). No reduction of ketones larger than cyclooctanone was observed. Other interesting strains, such as *Echinosporangium transversale*, *Gongronella butleri*, *Actinoplanes* sp. and *Absidia glauca* were active in the reduction of large ketones such as cyclododecanone, but the culture conditions were not scalable and fully reproducible. Therefore they were rejected for further studies.



1, n=3; **2**, n=1; **3**, n=2; **4**, n=4; **5**, n=5; **6**, n=9

Table 1. Reduction of cycloalkanones using different microbial strains

Microorganism	**2** (%)	**3** (%)	**1** (%)	**4** (%)	**5** (%)	**6** (%)
Diplogelasinospora grovesii	77	34	85	9	0	0
Pyrenochaeta oryzae	14	11	70	17	26	41
Echinosporangium transversale	10	27	55	94	0	30
Dactylospora haliotrepha	15	3	58	9	0	3
Neosartorya hiratsukae	0	44	68	7	14	0
Nocardia uniformis	13	13	37	26	0	0
Actinoplanes sp.	12	10	52	12	0	89
Gongronella butleri	67	33	100	26	18	26
Monascus kaoliang	29	6	86	15	11	0
Schizosaccharomyces octosporus	10	2	84	8	0	0
Absidia glauca	20	9	81	80	8	87

The reduction yield for each ketone is expressed as % substrate transformed into alcohol product. Reductions were carried out with cells grown for 48 h (yeasts and actinomycetes) and 72 h (filamentous fungi). $T = 28\,^{\circ}C$; shaking speed = 250 rpm; $V = 20$ mL in 100 mL Erlenmeyer flasks; [ketone] = 10 mM; reaction time 72 h; yield determined by GC.

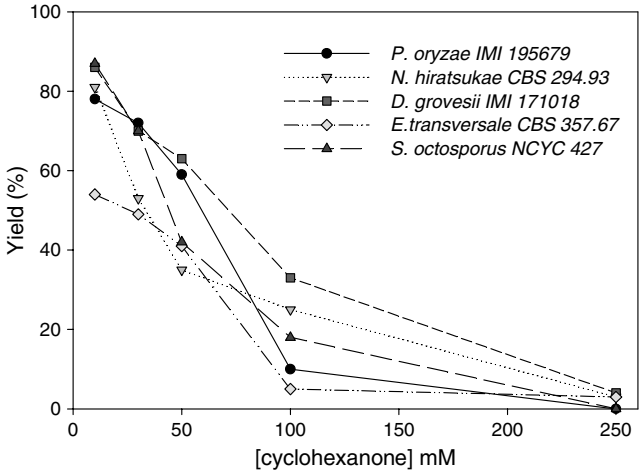
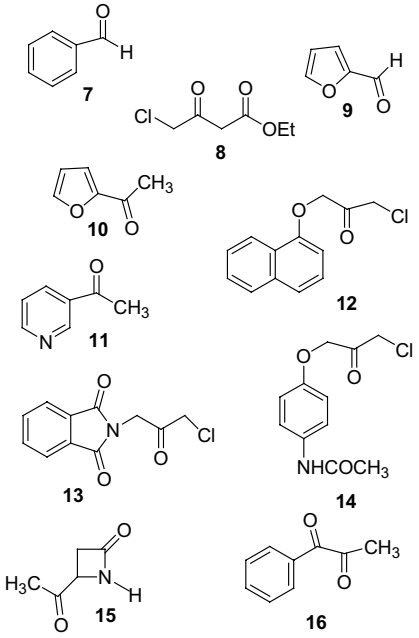


Figure 1. Toxicity of cyclohexanone against the microorganisms tested. Reaction conditions: $T = 28\,^{\circ}C$, shaking speed = 250 rpm, growing cells: yeast (48 h culture time) or filamentous fungi (72 h culture time), reaction time after the addition of substrate = 72 h.



Scheme 1.

The main part of the selected microorganisms cannot work (in our experimental conditions) with initial concentrations of cyclohexanone higher than 50 mM giving very low yield in alcohol. Only *D. grovesii* accepted higher concentrations as shown in Figure 1. Thus, at 50 mM cyclohexanone, we can see that the obtained yield with *D. grovesii* is nearly 70% in cyclohexanol while at 100 mM of substrate, the reaction yield is 35%. *D. grovesii* was able to tolerate concentrations of cyclohexanone higher than those traditionally described by other chemists such as Waagen et al. (1.6 mM)[22] or Molinari et al. (20 mM).[23] Therefore this fungus was selected for further biotransformations due to its selectivity, easy and scalable fermentation conditions and to the absence of ADH inhibition by the ketone until concentrations were higher than 50 mM.

2.2. Reduction of acyclic carbonylic compounds

Several simple acyclic carbonylic compounds were selected in order to give a profile of the reductase activity of the strain. For this work, we used a reagent concentration of 5 mM due to the fact that we did not know the intrinsic toxicity of all substrates tested against *D. grovesii* cells. This low concentration was selected as a compromise, assuming that the overall toxicity in these conditions should be low and would not affect the reduction yield of the fungus.

The structures in Scheme 1 show different steric hindrance around the C=O bond and different heteroatoms in the molecule. Therefore, they were considered as a useful primary test to explore the selectivity of the ADH from *D. grovesii* against other molecules different from **1**. The obtained results are shown in Table 2.

From the results we can deduce that the aldehyde group from compounds **7** and **9** is easily reduced, as expected. The conjugation of C=O with the pyridine ring **11** reduces the yield in alcohol but gives a high enantioselectivity. In the reduction of **10** and **15**, the substrates and the possible alcohols were not detected in the liquid medium, indicating that these compounds were probably fully degraded during the biotransformation and incorporated into the biomass serving as the carbon source. Finally the ketoester **8** gave (3*R*)-hydroxyester as the main stereomer but with a low ee. The stereopreference is the same as that described for baker's yeast but the ee was lower,[4,24,25] but opposite to that reported for ADH of *Candida magnoliae*.[13,26]

The (*S*)-alcohol from **11** and the (*R*)-alcohol from **8** were obtained as the main alcohols. The low ee obtained in the case of **8** was probably due to the small difference in

Table 2. Reduction of some acyclic carbonylic compounds by *D. grovesii*

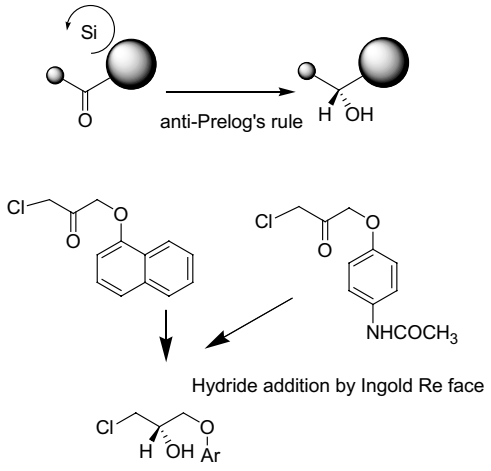
Compound	Yield (%)	Ee (%)	Stereopreference
7	94	—	—
8	88	12	*R* > *S*
9	98	—	—
10	—[a]	—	—
11	14	>99	*S*
12	99	>98	*S* > *R*
13	68	>99	*R* > *S*
14	99	87	*S* > *R*
15	—[a]	—	—
16	52	85	*S* > *R*

Reductions were carried out with cells grown for 72 h. $T = 28\,^{\circ}C$; shaking speed = 250 rpm; $V = 20$ mL in 100 mL Erlenmeyer type flasks; [ketone] = 2.5 mM; reaction time 72 h; yield determined by gas chromatography. Absolute configurations of the main isomer and ee determined by chiral HPLC and/or specific rotation $[\alpha]_D^{25}$.

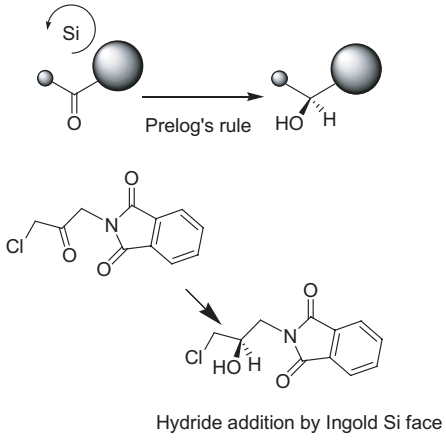
[a] Full degradation. No substrate or product was recovered from the culture medium.

size of both substituents at the α-position with respect to C=O.

The reduction of the haloketones **12**, **13** and **14** (adrenergic β-blocker precursors) took place with good ees and moderate to good yields (Table 2). The good ee values can be related to the large difference in size between the two groups linked to the C=O group, as described.[9] Nevertheless, the stereocontrol seems to change depending on the substrate. The attack was performed by the *Re* face in the case of **12** (Scheme 2) [giving the (*S*)-isomer] and **14** (giving $S > R$) and by the *Si* face in the case of **13** (Scheme 3) (giving $R > S$). The same stereobias for **12** has been described for the ADH from *S. cerevisiae* type II and from *Yarowia lipolytica* CECT 1240.[27] The opposite enantiopreference observed for **12** and **14** compared to **13** has been described in the case of *S. cerevisiae* type II.[27] This result was related to the different electronic properties and dipolar moment of **12** compared to **13** and **14** according to molecular orbital calculus (MO) calculations that could favour the different recognition of each molecule by the active site.[27]



Scheme 2.



Scheme 3.

Therefore, the substrate recognition by ADH from *D. grovesii* must be controlled by other factors other than steric ones, one of which could be the presence of polar amino acids on the active site that would control the interaction of the active site with the polar groups of **12**, **13** and **14**. In this sense, polar compound **8** gave the same stereopreference than the polar haloketone **13** (Table 2).

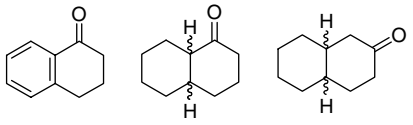
Finally, the 1,2-diketone **16** was reduced with moderate yield and acceptable ee giving the (2*S*)-hydroxypropiophenone. In this polar compound, the addition of the hydride follows the same stereobias described for **8** and **13**. The total reduction to the 1,2-dihydroxy compound was not observed, as it had been described for the other ADHs, for example, *Thrycospora cutaneum* MY1506 and 1,2-indanedione.[28,29]

2.3. Reduction of tetralone 17, and *cis*- or *trans*-1 or 2-decalones 18 and 19

Tetralone, **17**, *cis*- or *trans*-1-decalone, **18**, or *cis*- or *trans*-2-decalones, **19**, a mixture of isomers, were reduced as a model reaction for nonrigid bicyclic ketones. The reductions of **18** and **19** were carried out without previous stereomer separation. The yield in the reduction of each stereomer was obtained by the diminution of each peak (GC–MS). The stereobias was only assigned in the case of commercially available pure alcohols or with clear data from chiral-GC–MS (Table 3).

The stereochemistry of the alcohols produced in the reduction of **17** and of the *trans*-isomers of **18**, was $S > R$. However we do not have clear experimental evidence, except for *cis*-**18**. This stereochemistry would

Table 3. Reduction of tetralone **17** and decalones **18** and **19** by *D. grovesii*



17 **18** **19**

Compound	Alcohol yield (%)	Ee (%)	Stereopreference
17	16	83	$S > R$
trans-(4a*S*,8a*R*)-**18**	96	90	$S > R$
trans-(5a*R*,8a*S*)-**18**	96	>90	$S > R$
cis-(5a*S*,8a*S*)-**18**	86	n.d.	n.d.
cis-(5a*R*,8a*R*)-**18**	43	n.d.	n.d.
trans-(5a*S*,8a*S*)-**19**	72	>99	$R > S$
trans-(5a*R*,8a*R*)-**19**	78	48	$R > S$
cis-(5a*R*,8a*S*)-**19**	81	n.d.	n.d.
cis-(5a*S*,8a*R*)-**19**	24	n.d.	n.d.

Reductions were carried out with cells grown for 72 h. $T = 28\,^{\circ}C$; shaking speed = 250 rpm; $V = 20$ mL in 100 mL Erlenmeyer flasks; [ketone] = 2.5 mM; reaction time 72 h; yield determined by gas chromatography. Absolute configurations of the main isomer and ee determined by chiral HPLC and/or specific rotation $[\alpha]_D^{25}$.

agree with Prelog's rule as observed for the simple cyclic ketones such as **11**.

In the case of *cis*- or *trans*-2-decalones, **19**, the mixture of *trans*-ketones should lead to the (*R*)-alcohol as the main stereomer. As in the case of **18**, the stereochemistry of alcohols produced in the reduction of *cis*-2-decalones could not be determined. Three chiral columns were used and the resolution of the broad alcoholic peaks was not possible. The results in Table 3 indicate that the relative situation of both rings controls the recognition of this substrate by the microbial reductase.

2.4. Reduction of other cyclic compounds

Several polycyclic structures were reduced by using this microorganism to explore the scope of *D. grovesii* in the reduction of rigid bicyclic ketones. The results are shown in Table 4.

The aldehyde group of **25** was reduced but not the conjugated C=C bond. The accessible C=O groups, as in the cases of **21**, **23** and **24**, were reduced with moderate to good yields. The hindered C=O bonds in **26**, **27** and **28** were not accessible to NADH and were thus not reduced. Only **20** yielded a low percentage of alcohol. In these rigid or semi-rigid compounds, the stereopreference of the produced alcohols is in agreement with the geometry of the molecules as determined by H NMR using Eu(III)(fod)$_3$-d_{27}. The (*S*)-configuration of the alcohol agrees with the rigid structure and that the hydride from NADH will access via the less sterically hindered prochiral face.[13]

2.5. Reduction of carvones

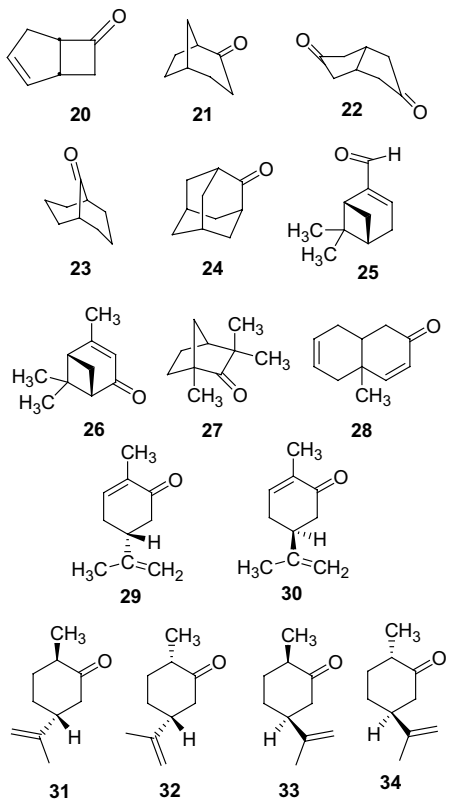
Carvones are natural commercially available ketones in both stereomers, (4*R*)- and (4*S*)-carvones **29** and **30**. Besides (+)- and (−)-dihydrocarvones—mixture of isomers—(**31**–**34**), were reduced (Scheme 4). The stereochemistry of the carbon with the isopropenyl group, dramatically alters the distribution and nature of the reduction products. The reduction process was followed by chiral GC coupled to MS, in order to analyse the structure of the main reaction products. The results are shown in Table 5.

Table 4. Reduction of some cyclic ketones by *D. grovesii*

Compound	Yield in alcohol (%)	Ee (%)
20	21	>98 (*S* > *R*)
21	74	>98 (*S* > *R*)
22	0	—
23	56	—
24	74	—
25	73[a]	—
26	0	—
27	0	—
28	0	—

Reductions were carried out with cells grown for 72 h. $T = 28$ °C; shaking speed = 250 rpm; $V = 20$ mL in 100 mL Erlenmeyer flasks; [ketone] = 2.5 mM; reaction time 72 h; yield determined by gas chromatography. Absolute configurations of the main isomer and ee determined by chiral HPLC and/or specific rotation $[\alpha]_D^{25}$.

[a] Myrtenol was the only reaction product detected.



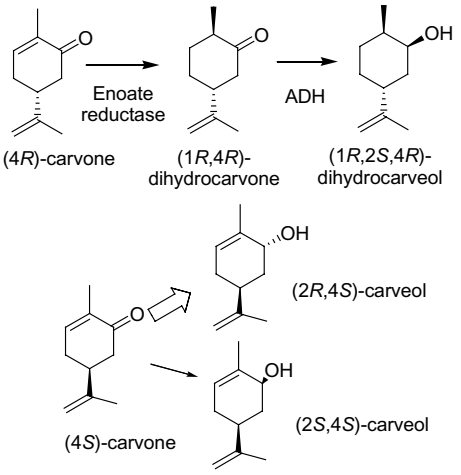
Scheme 4.

Table 5. Reduction of carvones and dihydrocarvones using *D. grovesii*

Compound	Reaction product (%)
(4*R*)-Carvone, **29**	(1*R*,2*S*,4*R*)-Dihydrocarveol (90%) and **31** (9%)
(1*R*,4*R*)-Dihydrocarvone, **31**	(1*R*,2*S*,4*R*)-Dihydrocarveol (98%)
(1*S*,4*R*)-Dihydrocarvone, **32**	No reaction
(4*S*)-Carvone, **30**	(2*R*,4*S*)-carveol (83%) and (2*S*,4*S*)-carveol (7%)
(1*R*,4*S*)-Dihydrocarvone, **33**	(1*R*,2*R*,4*S*)-Dihydrocarveol (17%) and (1*R*,2*S*,4*S*)-dihydrocarveol (3%)
(1*S*,4*S*)-Dihydrocarvone, **34**	(1*S*,2*S*,4*S*)-Dihydrocarveol (48%) and (1*S*,2*R*,4*S*)-dihydrocarveol (40%)

Reductions were carried out with cells grown for 72 h. $T = 28$ °C; stirring speed = 250 rpm; $V = 20$ mL in 100 mL Erlenmeyer flasks; [ketone] = 2.5 mM; reaction time 72 h; yield determined by gas chromatography. Absolute configurations of the main isomer and ee determined by chiral HPLC and/or specific rotation $[\alpha]_D^{25}$.

From the results in Table 5, we can deduce that (4*R*)-carvone, **29**, carries to the total reduction of the C=C–C=O bonds giving (1*R*,2*S*,4*R*)-dihydrocarveol as the main reaction product. In this process, (1*R*,4*R*)-dihydrocarvone **31**, seems to be the intermediary product. This was confirmed because of the mixture of **31** and **32** led to (1*R*,2*S*,4*R*)-dihydrocarveol leaving **32**, unaltered. This behaviour seems to indicate that an enoate reduc-



Scheme 5. Reduction pathway of carvones.

tase[28] is present in the microorganism. The enzyme could be very sensitive to the steric hindrance because it cannot recognise the C=C–C=O bond in the case of myrtenal **25**. The (*R*)-configuration produced in C-1 is in accordance with the stereopreference described for this kind of enzyme[28] (Scheme 5).

In the case of (*S*)-carvone, **30**, the reduction of C=O to a mixture of (*R* > *S*)-alcohols is the main reaction pathway, with the C=C bond remaining unaffected. The different behaviours observed with **30** compared to **29** is due to the stereochemistry of isopropylene group in **30**; similar to the situation of *gem*-dimethyl groups in **25** that avoids the catalytic activity of the enoate reductase. In fact (4*R*)-carvone, **29**, gives **31** and the dihydrocarveol with a (2*S*)-configuration in the alcohol while (4*S*)-carvone, **30**, gives the (2*R*)-carveol as the main alcohol. When the mixture of **33** and **34** was reduced, a very complex mixture was achieved according to GC and MS data. The analysis of the relative proportion of each compound shows low stereocontrol in the process. From this analysis we can say that (1*S*,4*S*)-dihydrocarvone, **34**, is preferentially reduced compared to (1*R*,4*S*)-dihydrocarvone, **33**.

2.6. Comparative molecular field analysis (CoMFA)

The main drawback of biocatalysis for synthetic organic chemists is that it is very difficult to predict if a new unnatural substrate (i.e., one not reported in the literature) would be reduced by a biocatalyst and what the stereobias of the process would be. Therefore, in spite of the high potential of asymmetric bioreduction of ketones, synthetic organic chemists traditionally discard it favouring a reduction by a transition metal complex, which are generally more expensive, less stereoselective and have larger environmental and downstream problems than biocatalysts. Comparative molecular field analysis (CoMFA), a QSAR-3D methodology, can be used to overcome this drawback because it can picture a semi-quantitative active site structure. CoMFA has proven its ability to predict biological properties of systems not amenable to direct analysis, as docking of enzyme–substrate, because of the lack of protein structure data.

Although the fundamentals of this methodology have been well described in literature,[30–35] CoMFA has only been applied a few times in biocatalysis.[36] In the present study 2-adamantanone, **24**, was selected as the template for the molecule fitting. Several fittings were used but the optimal results were achieved when the carbon of the C=O was in the coordinate origin, the largest group was fitted to C_3 and C_4 and the small one over C_1. The criterion used for the alignment of the conformers of the molecules in the CoMFA analysis is shown in Figure 2. In Figure 3 we show the fitting of the minimum energy conformers of all molecules that are reduced by the ADH of *D. grovesii*. The surface area that includes this fitting would be the active site of this unknown ADH.

The optimisation of the geometry of the molecules and the conformational analysis are described in the experimental. The statistic CoMFA parameters are shown in Table 6.

We can see in Figure 4b that a yellow zone associated with the high steric hindrance is at the back of the structure of the ketone. This zone could be related to the presence of either bulky amino acids or an α-helix as in

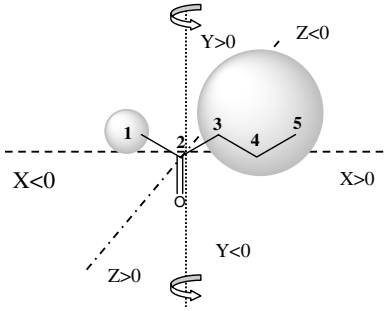


Figure 2. Fitting criterion used for alignment of the conformers of the molecules in CoMFA analysis.

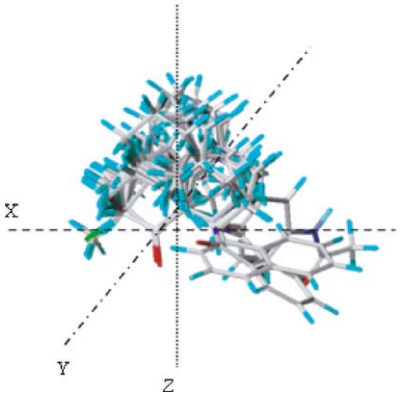


Figure 3. Fitting of the minimum energy conformers of all molecules that are reduced by the ADH of *D. grovesii*.

Table 6. Statistics parameters of CoMFA for *D. grovesii*

Parameters	Analysis	Value
q^2	Leave one out (LOO)	0.549
Number of components	Leave one out (LOO)	7
R^2	No validation	0.991
F values ($n_1 = 7$, $n_2 = 17$)	No validation	280.575
Prob. of $R^2 = 0$ ($n_1 = 7$, $n_2 = 17$)	No validation	0.000

The q^2 value, $0.549 > 0.5$, indicates a good prediction level for the model. The CoMFA structures are shown in Figure 3.

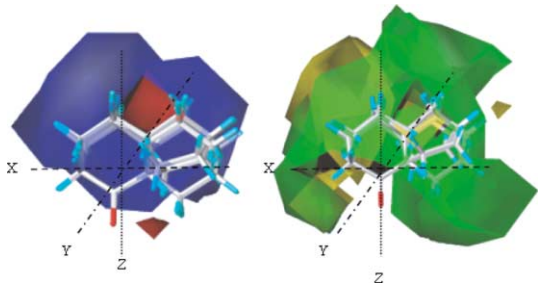


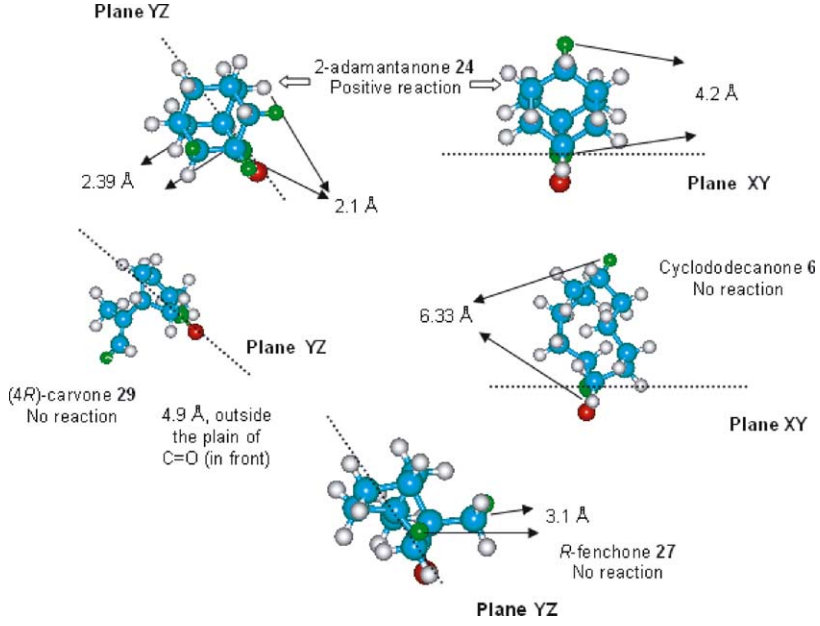
Figure 4. Electrostatic (a) and steric (b) surfaces in CoMFA for *D. grovesii* with the fitting of 1-decalones, **18** in the model.

the case of ADH from *Drosophila lebanoniensis*.[37] Evidently, the presence of substituents in the yellow zones causes very low enzymatic activity as in the case of (*R*)-fenchone, **27** and (−)-verbenone, **26** (Fig. 4). In Figure 4b we observe that **18** is perfectly located inside the green zone meaning that high yields and ees are obtained (Table 3). This yellow zone would explain the different stereochemistries and yields obtained in the reduction of *cis*- and *trans*-decalones, **18** and **19** (Table 3). The location of the second cyclohexane ring of **19** in this zone would determine the yield and the main stereoisomer of the 2-decanol produced in the reduction.

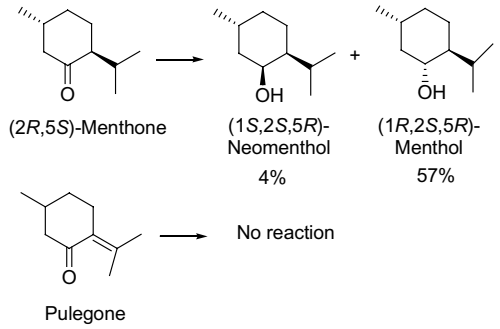
In Scheme 4 we show a schematic picture of the molecules illustrating that the presence of methyl groups in **26** and **27** produces molecules with larger distances than those of 2-adamantanone, **24**, used as a template in CoMFA analysis (Scheme 6) and as a result, no reduction being observed. In fact, we can see in Scheme 4 that the hydrogen atoms of the methyl groups in **27** are at 3.1 Å from the *YZ*-plane. This distance is greater than in the adamantanone (used as the template), which is 2.1–2.4 Å. This is also true for the hydrogen atoms of (4*R*)-carvone is at 4.9 Å from the *YZ*-plane with no reduction of the C=O to carveol being observed (Table 5).

Similarly dodecanone, **6** (Table 1) is not reduced by the *D. grovesii* ADH because the distance between the C=O (origin of coordinates in the CoMFA fitting) and the H of C_7 (the largest distance) 6.33 Å in the *YZ*-plane is too great. This distance is larger than the one between C=O and the opposite hydrogen, as in the case of **24** (4.2 Å in Scheme 4). The same explanation can be obtained from CoMFA steric surfaces to justify the low yield achieved with **20** (Table 4), with the rigid structure justifying the high ee obtained.

A small red zone (Fig. 4a) where the presence of negative charges favours the reduction of halohydrin β-blocker precursors explains because **12**, **13** and **14** with polar heteroatoms—with negative charge—will be located there. In Figure 3 we show the fitting of the minimum energy conformers of these molecules. Unfortunately the model does not allow us to predict why the stereopreference in the reduction of **12** and **14** is different than that obtained for **13** because only these three structures were tested. To explain this behaviour



Scheme 6.



Scheme 7.

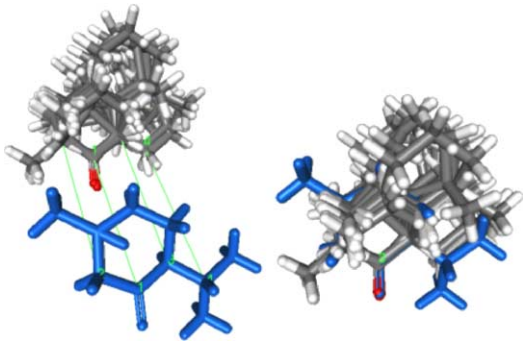


Figure 5. Fitting of (2*R*,5*S*)-menthone to the molecule structure that could be reduced by the ADH of *D. grovesii*.

the tertiary structure of the protein would be necessary. The low reduction yield obtained with **11** (14%, Table 2) and the large one obtained with **8** are explained because the nitrogen of pyridine ring **11** is outside the red zone, but the ester group **8** is in the red zone. In the same way, the benzene of **17** is in this zone with a low yield being obtained (Table 3).

The large blue zone where the positive charge favours the process (Fig. 4a) is not important because all the ketone bonds can be located in this area. Finally the empty zone near the C=O bond should be occupied by NADH that transfer the hydride ion.

Additionally, the CoMFA model can be used to predict if new ketones can be reduced using *D. grovesii*. In fact, (−)-menthone can be reduced to (−)-menthol (57% yields in 72 h) with only 4% of neo-menthol (Scheme 7). This is due to the *i*-Pr group being located in the green zone (Fig. 5) where the steric hindrance favours the reduction yield. Awkwardly, pulegone cannot be reduced because the C=C bond is in the blue zone (Fig. 3) where the negative charge of the C=C bond disfavours the reduction process (Scheme 7).

3. Experimental

Microorganism *D. grovesii* IMI 171018, was isolated from a soil sample from Japan. The sample was collected in an environment contaminated with waste water rich in phenol compounds.

Culture conditions: The cultures were developed using a rich culture medium suitable for each type of microorganism, as described in the ATTC catalogue. The selected culture medium for *D. grovesii* IMI 171018 was: glycine 2 g/L, soybean flour 6 g/L, starch 20 g/L, glycerol 10 g/L, nutrient solution 10 mL/L ($FeSO_4 \cdot 7H_2O$ 1 g/L, $MnSO_4 \cdot 4H_2O$ 1 g/L, $CuCl_2$ 0.025 g/L, $CaCl_2$ 0.1 g/L, H_3BO_3 0.056 g/L, $ZnSO_4 \cdot 7H_2O$ 0.2 g/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.1 g/L).

3.1. General reaction conditions for the reduction of cycloalkanones

Screening for the activity of each microorganism in the reduction of cycloalkanones (**1**, **2**, **3**, **4**, **5**, **6**) was performed according to the same experimental procedure.

As an example, we describe the methodology followed in the case of cyclohexanone, **1**. Cultures were performed in an orbital shaker at 250 rpm and 28 °C (48 h for yeasts and bacteria, and 72 h for fungi and actinomycetes). After this time, 20 μL (10 mM) of cyclohexanone, **1** (or the corresponding cycloalkanone) were added to the culture flasks. The contact time between the microorganism and the substrate was selected to be 72 h for all the microbial groups. The biotransformation was quenched by the addition of HCl solution until acidic pH value.

Then, the cell culture medium, after removal of cells, was extracted with ethyl acetate (5 mL ethyl acetate/20 mL cell culture medium) for 5 min. The organic phase was analysed by GC. Gas chromatographic analysis was performed using a column SGL-1000 (60 m, 0.25 mm and 0.25 μm) (Sugelabor, Spain). Varian T_i 155 °C; t_i 1 min; rate: 4 °C/min; T_f: 175 °C; t_f: 5 min. Flow(He) 40 psi and split 100 mL/min. Injector and detector temperature 250 °C. The oven temperature was programmed from 155 °C (isotherm 1 min) to 175 °C at 4.0 °C/min and then held at the upper limit for 6.5 min, with the chromatogram final time being 12.5 min. The column pressure was 40 psi, the flow rate of the carrier gas (helium) was 2.1 mL/min (auxiliary gas was not necessary), and the split flow ratio was 20:1. Using these conditions, the retention times of substrate and product of each reaction were: Cyclohexanone, **1** = 4.36 min; cyclohexanol = 4.83 min. Cyclobutanone, **2** = 4.0 min; cyclobutanol = 4.2 min. Cyclopentanone, **3** = 3.86 min; cyclopentanol = 4.05 min. Cycloheptanone, **4** = 5.22 min; cycloheptanol = 6.93 min. Cycloctanone, **5** = 6.71 min; cyclooctanol = 8.508 min. Cyclododecanone, **6** = 14.51 min; cyclododecanol = 21.78 min.

3.2. Toxicity assays

The toxicity of cyclohexanone, as a substrate model was analysed by adding it at different concentrations: 5, 10,

30, 50, 100, 250 and 500 mM to the culture medium of each microorganism in the reactions conditions described above. The decrease in yield of cyclohexanol with time was followed by GC as described above.

3.3. Preparation of 12, 13 and 14

The haloketones, **12**, **13** and **14** were prepared by the oxidation of (*RS*)-alcohols as previously described.[11]

3.4. Microbial reduction of haloketones

The reduction of **12** (for example) using whole cells was accomplished according to this methodology. A solution of **12** (40 mg) in benzene (200 μL) was added to a 100 mL flask containing 20 mL of culture cells (48 h fermentation time). After 120 h ethyl acetate (5 mL) was added to extract the organic compounds. The mixture was centrifuged and decanted. The organic phase was dried over anhydrous $MgSO_4$ and analysed by HPLC.

3.5. Characterisation of the reaction products (halohydrin)

The halohydrins obtained were previously characterised.[11] The absolute configurations of **12** and **14** were determined from the $[\alpha]_D^{25}$ value of the (*R*)-halohydrin. The preparation in the case of **12** was according to the following procedure. Under nitrogen atmosphere, 6 mL of DMF solution and 41 mg (1.05 mmol) of NaH were added to 112 mg (0.918 mmol) of 1-naphthol in 6 mL of dry DMF. The mixture was stirred at room temperature for 25 min. 2*S*-Glycidyl tosylate [200 mg (0.857 mmol)] in dry DMF were then added. The mixture was stirred for 4.5 h at room temperature. It was washed with a saturated aqueous solution of NaCl and the organic phase dried over anhydrous $CaCl_2$ and concentrated at vacuum. The obtained compound was purified in a SiO_2 column using CH_2Cl_2 as mobile phase. The spectroscopic data of (2*S*)-3-(naphthyloxy)-1,2-epoxipropane was compared to that of racemic mixture. The hydrolysis in $CHCl_3$ with HCl at 5 °C gave the (*R*)-halohydrin with 98% ee, $[\alpha]_D^{25}$ +9.9 (*c* 0.5 g/100 mL, EtOH). A similar experimental procedure was followed in the preparation of the *R*-halohydrin of **14** was obtained $[\alpha]_D^{25}$ +7.7 (*c* 1 g/100 mL, EtOH). The highly purified *R*-halohydrins were analysed by chiral HPLC using a ConstaMetric 4100 system, equipped with a chiral column (Chiralcel OD), UV–vis detector. Mobile phase was hexane/isopropanol/diethylamine [70:30:0.1 (v/v/v)], flux 0.5 mL/min. The retention times for the halohydrins were assigned as follows: the main peak to the (*R*)-halohydrin and the low peak to (*S*)-halohydrin.

In the case of **13**, the absolute configuration of the halohydrins was determined by a similar synthetic procedure. The (*R*)-halohydrin was prepared as follows: To a solution of 62 mg (0.875 mmol) of potassium phthalimide in 10 mL of dry DMF, 200 mg (0.875 mmol) of (2*S*)-glycidyl tosylate in 5 mL of DMF were mixed. The solution was stirred for 10 h at room temperature and washed with an aqueous saturated solution of NaCl. It was then dried and concentrated at vacuum. The obtained compound was purified in a SiO_2 column using CH_2Cl_2 as the mobile phase. The H NMR and C NMR data of (2*S*)-3-phthalamidyl-1,2-epoxipropane were compared to that of the racemic mixture. The hydrolysis in $CHCl_3$ with HCl at 5 °C gave the *R*-halohydrin with 98% ee, $[\alpha]_D^{25}$ +16.25 (*c* = 0.48 g/100 mL, EtOH).

The enantiomeric excess (ee) of the reaction product from **12** (1-chloro-3(1-naphthoxy)-2-propanol) was determined by HPLC using a ConstaMetric 4100 system equipped with a chiral column (Chiralcel OD), UV–vis detector. The mobile phase was hexane/isopropanol/diethylamine [70:30:0.1 (v/v/v)]. Flux 0.5 mL/min. Retention time of the (*R*)-halohydrin = 12.5 min; (*S*)-halohydrin = 14.2 min; **12** = 10.1 min.

The reduction of **13** was followed by HPLC. Mobile phase hexane/isopropanol/diethylamine (80:20:0.1 v/v/v). Flux 1.0 mL/min. Retention time of **13** = 25.0 min; (*R*)-halohydrin = 18.0 min; (*S*)-halohydrin = 21.1 min.

The reduction of **14** was followed by HPLC. Mobile phase hexane/isopropanol/diethylamine (80:20:0.1 v/v/v). Flux 1 mL/min. Retention time of **14** = 28.3 min; (*R*)-halohydrin = 13.1 min; (*S*)-halohydrin = 15.5 min

3.6. Reduction conditions for polycyclic ketones

The reduction of the ketones was performed as described for cycloalkanones, [ketone] = 2.5 mM. The reactions products were isolated in EtOAc as described before. GC analysis was carried out using two different columns and conditions. This proved interesting for the identification of the products:

CG-I: Column *Sugelabor carbowax SGL-1000* (60 m, 0.25 mm and 0.25 μm). Varian T_i: 155 °C; t_i: 1 min; rate: 4 °C/min; T_f: 175 °C; t_f: 5 min. Flow(He) 40 psi and split 100 mL/min. Injector and detector temperature 250 °C.

CG-II: Chiral capillary column *Sugelabor carbowax CP 7502* (25 m, 0.39 mm, 0.25 μm). The chromatographic conditions were T_i: 90 °C; t_i: 5 min; rate: 5 °C/min; T_f: 175 °C; t_f: 7 min. Flow(He) 25 psi and split 100 mL/min. Injector and detector temperature 250 °C.

The retention times of the reaction products were compared, if they were not commercial, with those of reduction products obtained by chemical reduction. The chromatographic conditions and retention times of the compounds were:

7: CG-I conditions were used: benzaldehyde = 6.0 min; benzyl alcohol = 11.5 min.

8: CG-I conditions: ethyl 4-chloro-acetoacetate = 4.1 min; ethyl 4-chloro-3-hydroxy-acetoacetate = 13.5 min. CG-II conditions: ethyl 4-chloro-acetoace-

tate = 8.2 min; ethyl (3*R*)-4-chloro-3-hydroxy-acetoacetate = 16.1 min; ethyl (3*S*)-4-chloro-3-hydroxy-acetoacetate = 16.8 min.

9: CG-I conditions: 2-furaldehyde = 4.0 min; (2-furyl)-methanol = 4.8 min.

10: CG-I conditions: 2-acetyl-furane = 4.1 min; 1-furyl-ethanol = 4.7 min. CG-II conditions: 2-acetyl-furane = 4.5 min; (*R*)-furyl-ethanol = 7.4 min; (*S*)-furyl-ethanol = 7.6 min.

11: CG-I conditions: 2-acetyl pyridine = 4.7 min; 2-pyridinyl-1-ethanol = 6.2 min. CG-II conditions: 2-acetyl-pyridine = 7.3 min; (*R*)-1-(2-pyridinyl)-1-ethanol = 11.5 min; (*S*)-1-(2-pyridinyl)-1-ethanol = 11.3 min.

15: CG-I conditions: 4-acetoxy-azetidyn-2-one = 5.8 min; 4-hydroxyazetidinyl acetate = 6.7 min (racemic mixture).

16: CG-I conditions: 1-phenyl-propane-1,2-dione = 14.2 min (alcohol not detected), (2*R*)-hydroxypropiophenone = 16.4 min; (2*S*)-hydroxypropiophenone = 16.6 min.

17: CG-I conditions: α-tetralone = 11.2 min; α-tetralol = 14.0 min. CG-II conditions: α-tetralone = 16.7 min; (*R*)-α-tetralol = 18.9 min; (*S*)-α-tetralol = 18.8 min.

18: CG-I conditions: *trans*-1-decalone 6 min; *cis*-1-decalone = 6.3 min; 1-decalol = 6.7 min.

CG-II conditions: *trans*-(4a*R*,8a*S*)-1-decalone = 14,9 min; *trans*-(4a*S*,8a*R*)-1-decalone = 15.2 min; *cis*-(4a*R*,8a*R*)-1-decalone = 15.7 min; *cis*-(4a*S*,8a*S*)-1-decalone = 15.8 min; 1-decalol 16.6 min (stereoisomers not identified). Calculus performed by diminution of ketone peaks.

19: CG-I conditions: *cis*-2-decalone = 6.4 min; *cis*-2-decalol = 6.7 min; *trans*-2-decalone = 7.3 min; *trans*-2-decalol = 8.2 min. CG-II conditions: *trans*-(4a*R*,8a*R*)-2-decalone = 17.3 min; *trans*-(4a*S*,8a*S*)-2-decalone = 17.4 min; *cis*-2-decalone (unresolved) = 16.1 min; (2*,4a*R*,-8a*S*)-2-decalol = 16.9 min; (2*,4a*S*,8a*R*)-2-decalol = 17.2 min; *trans*-(2*R*,4a*R*,8a*R*)-2-decalol = 18.6 min; *trans*-(2*S*,4a*R*,8a*R*)-2-decalol = 18.4 min; *trans*-(2*R*,-4a*S*,8a*S*)-2-decalol = 19 min; *trans*-(2*R*,4a*R*,8a*R*)-2-decalol = 18.8 min.

20: CG-I conditions: *cis*-bicyclo[3.2.0]hept-2-en-6-one = 4 min; *cis*-bicyclo[3.2.0]hept-2-en-6-ol 4.7 min.

21: CG-I conditions: bicyclo[3.2.1]octan-2-one = 5.3 min; bicyclo[3.2.1]octan-2-ol = 5.6 min.

22: CG-I conditions: *cis*-bicyclo[3.3.0]octan-3,7-dione = 16.5 min. Alcohol not isolated.

23: CG-I conditions: bicyclo[3.3.1]nonan-9-one = 5.8 min; bicyclo[3.3.1]nonan-9-ol = 6.7 min. CG-II conditions: bicyclo[3.3.1]nonan-9-one = 14.7 min; bicyclo-[3.3.1]nonan-9-ol = 18.7 min.

24: CG-I conditions: 2-adamantanone = 7.3 min; 2-adamantanol = 8.5 min.

25: CG-I conditions: (1*R*,5*S*)-6,6-dimethyl-bicyclo-[3.3.1]hept-2-en-2-carbaldehyde (*R*)-mirtenal = 4.9 min; ((1*R*,5*S*)-6,6-dimethyl-bicyclo[3.3.1]hept-2-en-2-yl)-methanol (*R*)-mirtenol = 5.9 min; ((1*R*,2(*RS*),5*S*)-6,6-dimethyl-bicyclo[3.3.1]hept-2-yl)-methanol (*R*)-mirtanol = 6.3 min. CG-II conditions: (1*R*, 5*S*)-6,6-dimethyl-bicyclo-[3.3.1]hept-2-en-2-carbaldehyde (*R*)-myrtenal = 12.5 min; *R*-mytrnol = 15.0 min; *R*-myrtanol = 16.8 min.

26: CG-I condition: 4,6,6-trimethyl-bicyclo[3.1.1]hept-3-en-2-one (verbenone) = 4.8 min; 4,6,6 trimethyl-bicyclo[3.1.1]hept-3-en-2-ol = 5.6 min.

27: CG-I conditions: *R*-fenchone = 3.8 min; fenchyl alcohol = 4.5 min.

28: CG-I conditions: (4a*R*)-methyl-4,4a,5,6,7,8-hexahydro-3*H*-naphthalen-2-one = 11.8 min; (4a*R*)-methyl-4,4a,5,6,7,8-hexahydro-3*H*-naphthalen-2-ol = 15.4 min.

29: CG-I conditions: dihydrocarvone = 4.8 min; dihydrocarveol = 5.2 min; carvone = 5.6 min; carveol = 6.0 min. CG-II conditions: (4*R*)-carvone = 11.2 min **29**; (1*R*,4*R*)-dihydrocarvone = 10.1 min **31**; (1*R*,2*S*,4*R*)-dihydrocarveol = 11.8 min; (1*S*,2*S*,4*R*)-dihydrocarveol = 12.2 min; (1*S*,4*R*)-dihydrocarvone = 10.3 min **32**.

30: CG-I conditions: dihydrocarvone = 4.8 min; dihydrocarveol = 5.2 min; carvone = 5.6 min; carveol = 6.0 min. CG-II conditions: (4*S*)-carvone = 11.1 min **30**; (2*R*,4*S*)-carveol = 13.2 min; (2*S*,4*S*)-carveol = 12.7 min; (1*R*,4*S*)-dihydrocarvone = 10.1 min **33**; (1*R*,2*R*,4*S*)-dihydrocarveol = 12.2 min; (1*R*,2*S*,4*S*)-dihydrocarveol = 12.1 min; (1*S*,4*S*)-dihydrocarvone = 10.3 min **34**; (1*S*,2*R*,4*S*)-dihydrocarveol = 11.8 min; (1*S*,2*S*,4*S*)-dihydrocarveol = 11.7 min.

In the case of noncommercial enantiomerically pure compounds **20** and **21**, the enantiopreference was evaluated by means of H NMR of Eu(fod)$_3$-d_{27}.

3.7. CoMFA analysis

The SYBYL/CoMFA® analysis was performed in a Silicon Graphics Octane-2 workstation and in the Computer service of Universidad Autonoma of Barcelona. The template molecule was 2-adamantanone, **24**. The database molecules were aligned by overlapping the C=O and the neighbouring carbons. The carbon of the C=O was the origin of coordinates (0,0,0). The substituents of the C=O were fitted with the small one on position 1 and the large one on the 3 and 4 positions. If the molecule had more substituents or several cycles, these groups would be located on position 4 (Fig. 2).

The optimisation of the geometry of the data base molecules in the postulated active conformation was performed using a 6-31G* basis set. The charges of each atom were calculated from the molecular electrostatic

potential[30] using a 6-31G* basis set. The solvating energies of the substrates were calculated with a continuos polarised model[30,31] using 6-31G* basis set. The QSAR table for CoMFA included the steric and electronic field values, the stabilisation energy of enzyme substrate complex, the solvating energy and the yield in alcohol obtained for each carbonylic group. The electrostatic and steric fields were calculated at each lattice intersection grid of 2 Å.

The partial least squares (PLS) analysis[32,33] was used to obtain straight lines from the matrix. Leave one out (LOO) cross-validation method was used to select the number of main components of QSAR-3D analysis and to calculate the statistic parameters (q^2).

The CoMFA model was generated using a no cross-validation method and the number of components indicated by the LOO validation. QSAR-3D/CoMFA analysis was performed with the QSAR module of SYBYL 6.5 program.[33] The calculations were performed using GAUSSIAN-98.[34]

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