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Resolution of Allylic Alcohols by Cholesterol Oxidase Isolated from *Rhodococcus Erythropolis*

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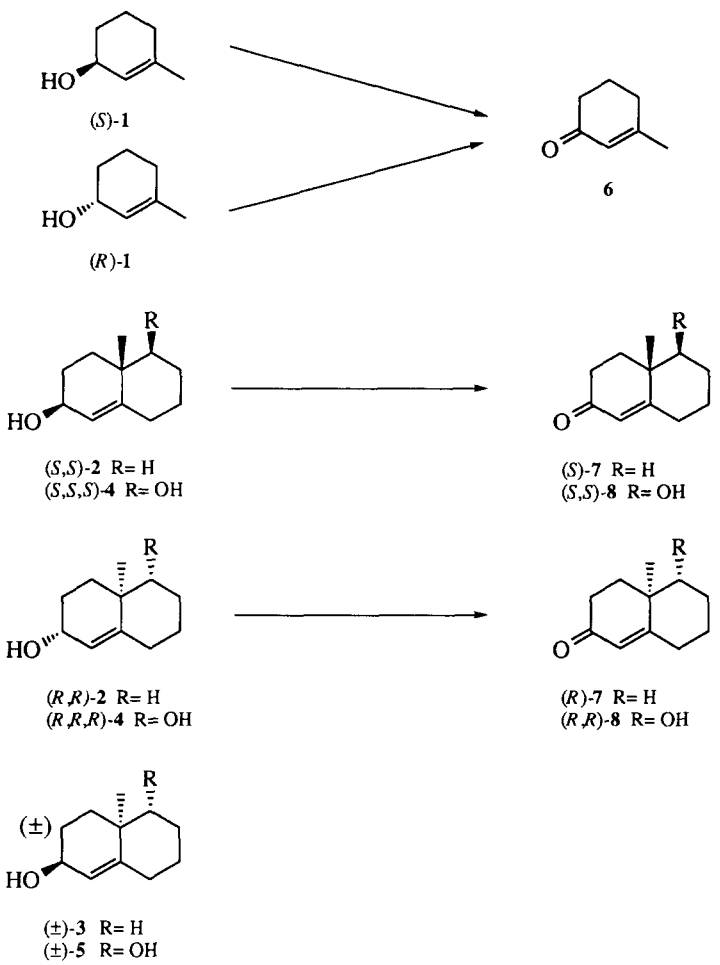
Abstract: The oxidation of non-steroidal compounds by cholesterol oxidase isolated from *Rhodococcus erythropolis* is reported for the first time. It was regio-, stereo- and enantio-selective. The enzyme oxidized preferentially the 2-cyclohexenyl-1-alcohols whose configuration is (*S*) at the reaction center.

The flavoenzyme cholesterol oxidase (EC 1.1.3.6) catalyses the oxidation and isomerization of 3β-hydroxy-5-ene-steroids to 3-keto-4-ene-steroids. The cofactor is regenerated by oxygen with production of hydrogen peroxide.[1,2] Cholesterol oxidase specifically oxidizes the 3β-hydroxy group of steroids. Cholest-4-en-3β-ol is a substrate of the enzyme.[3] As an extension, we have studied the oxidation of the following allylic alcohols: (±) 3-methyl-2-cyclohexen-1-ol **1**,[4] (±) (1α,4aα)-2,3,4,4a,5,6,7,8-octahydro-4a-methyl-2-naphthalenol **2**,[5] and (±) (1α,6α,8aα)-1,2,3,4,6,7,8,8a-octahydro-8a-methyl-1,6-naphthalenediol **4**.[6] Their oxidation was regio-, stereo- and enantio-selective.

Due to the poor solubility of these alcohols in aqueous media, the enzymatic tests were performed in a biphasic medium (butyl acetate / 0.1 M sodium phosphate buffer pH 7.5, volume ratio 2:1) at 30°C with constant stirring, under air.[7] In all experiments, the concentration of cholesterol oxidase from *Rhodococcus erythropolis* was 1.5 mg/mL buffer.[8] Catalase (0.16 mg/mL buffer) was added to destroy hydrogen peroxide which inactivates cholesterol oxidase.[9] The progress of the reaction was followed by ultraviolet spectrophotometry by monitoring the increase of the absorbance at 240 nm corresponding to the formation of the α,β-unsaturated ketone. The stereoselectivity of the enzymatic oxidation was monitored by gas chromatography (GC) on a chiral column.[10] The enantiomers, except **5**, were well separated and their retention time determined by injecting the optically pure or enriched compounds.[4,5,11] By addition of an internal standard (naphthalene or diphenyl) to the medium the reaction progress and the enantiomeric excess (ee) were followed during the reaction. Blanks were run in the same conditions without enzyme, and no oxidation was detected.



Scheme 1

The alcohol (*S*)-**1**, having the hydroxyl group like in cholest-4-en-3β-ol, was oxidized faster. The enzymatic resolution of a racemic mixture of **1** was followed (figure 1) and at 70% oxidation the ee of (*R*)-**1** was 90%. The enantioselectivity (*E*), as defined by Chen *et al*, was 7.[13] Therefore, even for such a small substrate compared to the steroids, the resolution with cholesterol oxidase was significant. If the hydroxyl group and the hydrogen atom at C1 have, for both enantiomers, the same position with respect to the amino acid acting as the general base and to the cofactor FAD, the enzyme is able to distinguish the faces of the 3-methyl-cyclohexene ring. Resolutions of compound **1** by transesterification with lipases and cholesterol esterase were reported with limited enantioselectivity.[14-17] With horse liver alcohol dehydrogenase an oxidation limited to 50% of a racemic mixture of **1** has been reported but the stereochemical course of that oxidation has not been determined.[18]

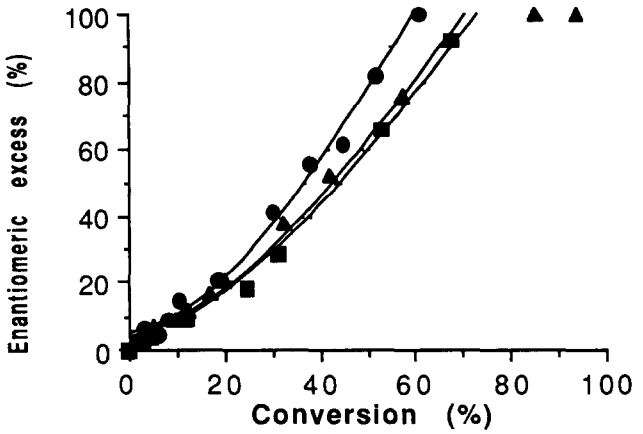


Figure 1. Plot of percent enantiomeric excess of the residual alcohols (*R*)-**1** (■), (*R*,*R*)-**2** (▲) and (*R*,*R*,*R*)-**4** (•) as a function of the percent conversion. Each racemate (±)-**1**, (±)-**2**, or (±)-**4** (2 mg in 1 mL butyl acetate) was added to 0.5 mL of a cholesterol oxidase from *Rhodococcus erythropolis* solution (1.5 mg/mL of 0.1 M sodium phosphate buffer pH 7.5) containing catalase (0.16-mg/mL). The biphasic medium was incubated at 30°C with vigorous stirring. At different times, aliquots of the organic phase were analyzed by gas chromatography on a chiral column to determine the percent enantiomeric excess and the percent conversion owing to the presence of an internal standard (naphthalene or diphenyl) in the medium. The data fitted well with theoretical curves calculated for enantioselectivity (*E*)[13] of 7, 8 and 20 respectively.

For the bicyclic alcohols, the position of the hydroxyl group with respect to the methyl group was essential. Indeed, when they are trans, as in compounds **3** and **5**, no significant oxidation took place. Alcohols **2** and **4**, having the methyl and the hydroxyl groups cis, were oxidized and the enantiomers were oxidized at different rates. The presence of the additional hydroxyl group in diol **4** increased significantly the enantioselectivity of the enzyme, as illustrated by the oxidation of racemate **4**, which furnished optically pure diol (*R*,*R*,*R*)-**4**.[11] The enantioselectivity (*E*) increased from 8 for alcohol **2** to 20 for diol **4** (figure 1). Concerning the ketone (*S*)-**7**, the ee was higher than 90% up to 20% conversion, then decreased to 50% after 50% conversion (*data not shown*). For hydroxy-ketone (*S*,*S*)-**8**, the ee was higher than 90% at 50% conversion (*data not shown*).

The oxidation of the allylic alcohols by cholesterol oxidase from *Rhodococcus erythropolis* is stereoselective and enantioselective. The hydroxyl group in the equatorial position and the (*S*)-alcohol were preferentially oxidized. The enzyme is regioselective. In the case of diol **4**, only the hydroxyl group in position 6 was oxidized. Surprisingly, the enzyme isolated from *Brevibacterium sterolicum* which seems to be less substrate specific,[1,2] did not oxidize any of the alcohols reported herein. The oxidation of other allylic alcohols and the determination of optimal conditions are under investigation for the use of that enzyme in preparative chemistry. Oxidases are non-polluting oxidation agents. They use oxygen for the regeneration of the flavinic prosthetic group and hydrogen peroxide, the second product of the enzymatic reaction, is easily disposed off by catalase.

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References and notes.

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4. Racemic compound **1** was from Aldrich. Optically enriched (*R*)- and (*S*)-3-methyl-2-cyclohexenol **1** were generous gifts from Dr K. Mori.[16] They showed an enantiomeric excess of 77% and over 95% respectively, as determined by gas chromatography. See reference (9).
5. Compounds **2** and **3** were prepared by reduction of (±) 4,4a,5,6,7,8-hexahydro-4a-methyl-2(3H)-naphthalenone **7** with $NaBH_4$ and $CeCl_3$ according to Gemal, A.L.; Luche, J.L. *J. Am. Chem. Soc.* **1981**, *103*, 5454-5459 . A mixture of two epimers, **2** and **3**, was obtained in a 3/1 ratio. The mixture was used as such. The optically pure (*R*) and (*S*) ketones were purchased from Fluka and reduced in the same conditions to afford mixtures of (*R,R*)-**2** and (*S,R*)-**3** and (*S,S*)-**2** and (*R,S*)-**3** respectively.
6. The reduction of the Wieland-Miescher ketone 3,4,8,8a-tetrahydro-8a-methyl-1,6-(2H,7H)-naphthalenedione (Ramachandran, S.; Newman, S. *Org. Synth.* **1973**, *coll. vol. 5*, 486-489) gave compounds **4** and **5** which were separated by chromatography on silicagel with diethylether as the eluant.
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8. Cholesterol oxidase from *Rhodococcus erythropolis* was obtained as a 3M NaCl solution from Boehringer Mannheim. It was dialyzed against 0.1M sodium phosphate buffer pH=7.5. Specific activity towards cholesterol was 25 U/mg at 25°C as specified by the supplier.
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10. We used a SGE Cydex-B 25QC2 (25 m, 0.25 mm) column on a Chrompack CP 9001, with He as the carrier gas (flow : 0.325 m/s). The injector was a split/splitless with 150 mL/min split flow.
11. The (*R,R,R*)-(-) enantiomer of diol **4** was obtained after oxidation of the racemate (100 mg) with cholesterol oxidase in the same conditions. The reaction was stopped when the increase of the absorbance at 240 nm reached a plateau (about 60 % oxidation of the racemic mixture of the diol to be sure that the (*S,S,S*)-(+) diol was totally oxidized). The resulting hydroxy-ketone and the residual (*R,R,R*)-(-) diol **4** were purified by chromatography on a silicagel column (eluant: dichloromethane/ diethylether, gradient 25 to 100%). Recrystallization of the diol afforded optically pure (*R,R,R*)-enantiomer **4** $[\alpha]_D$ = -52, (c = 1.27, EtOH). Litt[12] $[\alpha]_D$ = -50.3 (c = 1.025, EtOH). The yield (not optimized) was 15 %.
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