Microbial Hydroxylation of Cedrol and Cedrene

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Microbial Hydroxylation. Cedrene, Cedrol, Sesquiterpenoid, 13C NMR

Biotransformation of cedrol with 5 different strains afforded previously undescribed hydroxy-
cedrols. The attack came from a hemisphere centered at C-12 approximately. At two positions
epimeric pairs were formed, which could be avoided by using different strains. Contrary to
previous studies of other groups we found the 2- and 12-hydroxylations as main reactions.
Cedrene needed prolonged fermentation and gave lower yields than cedrol. Apart from allylic
hydroxylations the oxidation pattern by Corynespora cassiicola DSM 62474 of cedrene and cedrol
was the same. The structures of the metabolites were elucidated by 2D NMR techniques.

Introduction

To date a wealth of natural products containing tricyclic sesquiterpene skeletons are known. Many of
them are biologically active, only to mention α-santalol, patchoulol, or the illudines. Total or partial
syntheses of these compounds are difficult, even chemical derivatization of the natural products is
often not possible. This is a challenge for biotransformation of compounds which are available in larger
counties to come to known or previously undescribed derivatives [1].

1972 Wang and coworker [2] made a biotransformation of cedrol with Aspergillus niger ATCC 9142
and isolated 3β-hydroxy-cedrol as the main product. Two years ago we reported on the biotransformation
of isolongifolene [3] and investigated later the trans-
formation of further sesquiterpene hydrocarbons. Microbial oxidation of humulene and caryophyllene
gave slowly many products in low to moderate yields whereas oxygen-bearing compounds which are more
soluble in water reacted faster and gave higher yields with fewer side products [4]. With this experience we
decided to use the alcohol cedrol (I) instead of the hydrocarbon cedrene (II).

Experimental

The microorganisms were precultivated at 27 °C
and 100 rpm in five 100 ml EM flasks containing
20 ml of the following medium: 1% of glucose, 1%
of universalpeptone (Merck), 2% of malt extract and
0.3% of yeast extract. After 48 h (C. cassiicola after
96 h) the cultures were passed into five 2 liter flasks
filled with 400 ml of the medium and incubated for
another period of 24 h (C. cassiicola 48 h). The sub-
strate (0.3 g flask solved in 0.4 ml DMF) was added
then aseptically. After 24, 48, and 72 h samples were
taken and analyzed as follows: To 1 ml of culture
broth 0.2 ml ethylacetate was added and shaken for
2 min prior to centrifugation. 10 μl of the extract
were developed on HPTLC plates with dichloro-
methane—acetone 9:1 [5]. The spots were made visi-
table by spraying with anisaldehyde-sulfuric acid in
acetic acid and heating to 110 °C for 1 min.

Extraction and purification: Culture medium and
mycelia were separated by filtration and both ex-
tacted three times with ethylacetate. The solvent
was evaporated and the crude extract separated on
Si-60 columns with a n-hexane/ethylacetate gradient
(changing from 19:1 to 1:1). When necessary the col-
lected fractions were purified further by preparative
TLC.

Instruments used: NMR: The 1H NMR spectra
were obtained at 400 MHz on a Bruker WM 400
spectrometer and the 13C NMR spectra at 75.5 MHz
on a Bruker AM 300 spectrometer. CDCl3 was the
solvent and TMS the internal standard. IR: spectra
were measured in chloroform on a IR Spectral-
Photometer 297, Perkin Elmer. Mass spectra were
recorded on a AE1 902S mass spectrometer with
70 eV. Melting points are uncorrected and were ob-
tained at Büchi 510 melting point apparatus. Optical
rotation: Perkin-Elmer Polarimeter 241.
Biotransformation of 0.9 g cedrol with *Rhizopus stolonifer* CBS 38252 gave after 24 h 203 mg 1, 173 mg 2, and 8 mg 9.

Biotransformation of 1.5 g cedrol with *Streptomyces bikiniiensis* IFO 13350 gave after 72 h 250 mg 1, 15 mg 5, 7 mg 3, and 4 mg 8.

Biotransformation of 1.5 g cedrol with *Verticillium tenerrum* DSM 63545 gave after 72 h 100 mg 2, 40 mg 7, 37 mg 1, 25 mg 6, 12 mg 5, and 3 mg 10.

Biotransformation of 0.9 g cedrol with *Streptoverticillium reticuli* DSM 40776 gave after 72 h 155 mg 1 and 5 mg 5.

Biotransformation of 1.5 g cedrol with *Corynespora cassiicola* DSM 62474 gave after 72 h 105 mg 9, 70 mg 1, 30 mg 3, and 10 mg 4.

Biotransformation of 1.8 g cedrene with *Corynespora cassiicola* DSM 62474 gave after 72 h 83 mg 11, 25 mg 13, 5 mg 14, and 3 mg 12.

2-Hydroxy-cedrol (2): Colorless crystals, m.p. 137 °C. 1H NMR: s 1.29, s 1.28, s 1.18, s 1.04 (12-H, 13-H, 14-H, 15-H). 13C NMR data are listed in Table I.

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} (c = 1.00).
\]

3β-Hydroxy-cedrol (3): 1H NMR: ddd 3.61 (J = 10, 10, 5, 3-H), d 0.96 (J = 7, 12-H), s 1.26 (13-H), s 1.02 (14-H), s 1.34 (15-H).

Irradiation at δ = 0.96 gave an NOE enhancement at δ = 3.61. 13C NMR data are listed in Table I.

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} (c = 1.00).
\]

3α-Hydroxy-cedrol (4): Colorless crystals, m.p. 158 °C. 1H NMR: qd 1.76 (J = 7.5, 4.5; 2-H), dt 4.28 (J = 5, 4; 3-H), m 1.62 (4-H), t 2.15 (J = 9; 5-H), d 0.91 (J = 7.5; 12-H), s 1.34 (13-H), s 1.01 (14-H), s 1.26 (15-H). 13C NMR data are listed in Table I. MS (m/e): M* - CH3 223.169 (223.169 calculated for C13H23O2).

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} (c = 1.00).
\]

4β-Hydroxy-cedrol (5): Colorless crystals, m.p. 118 °C. 1H NMR: m 2.15 (2-H), ddd 1.90 (J = 12, 6, 3, 3-H), m 1.52 (3'-H), ddd 4.43 (J = 6, 4, 3; 4-H), dbbr (br) 1.75 (J = 6; 5-H), dbbr (br) 1.64 (J = 5; 7-H), m 1.86 (9α-H), m 1.38 (9β-H), m 1.8 (10-H), dbbr (br) 1.51 (J = 13; 11-H), m 1.76 (11'-H), d 0.90 (J = 7; 12-H), s 1.42 (13-H), s 1.34 (14-H), s 1.27 (15-H), long-range couplings between 5-H and 11-H, 9α-H and 15-H, 3-H and 12-H, 3'-H and 12-H from COSY. 13C NMR data are listed in Table I. MS (m/e): M* 238.1928 (238.1933 calculated for C13H23O2).

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} + 5.9° + 6.0° + 7.2° + 14.0° + 26.0° (c = 1.00).
\]

9β-Hydroxy-cedrol (6): 1H NMR: m 1.72 (2-H), m 1.87 (3-H), m 1.28 (3'-H), m 1.39 (4-H), m 1.54 (4'-H), t 1.75 (J = 6; 5-H), d 1.67 (J = 3.5; 7-H), dd 4.08 (J = 11, 7; 9-H), m 1.85 (10-H), dd 1.21 (J = 11, 7; 10'-H), dd 1.62 (J = 9, 2; 11-H), d 1.38 (J = 9; 11'-H), d 0.86 (J = 6; 12-H), s 1.28 (13-H), s 0.99 (14-H), s 1.22 (15-H), long-range couplings between 5α-H and 11α-H, 10α-H and 11β-H, 13-H and 14-H from COSY. 13C NMR data are listed in Table I. MS (m/e): M* 238.1928 (238.1933 calc. for C13H23O2).

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} + 0.2° + 0.4° + 0.9° + 1.6° (c = 1.00).
\]

10β-Hydroxy-cedrol (7): Colorless crystals, m.p. 88 °C. 1H NMR: m 1.87 (2-H), m 1.9 (3-H), m 1.6 (4-H), d (br) 1.60 (J = 4; 7-H), dd 2.00 (J = 14, 4; 9α-H), d 1.77 (J = 14, 2; 9β-H), dd 3.96 (J = 4, 2, 2; 10-H), d (br) 1.92 (J = 12; 11-H), dd 1.52 (J = 12, 5, 2; 11'-H), d 0.92 (J = 7; 12-H), s 1.03 (13-H), s 1.28 (14-H), s 1.45 (15-H), long-range couplings between 5-H and 11-H, 9α-H and 15-H, 7-H and 14-H, 7-H and 9β-H, 11α-H and 14-H, 13-H and 14-H from COSY. 13C NMR data are listed in Table I. MS (m/e): M* 238.1928 (238.1933 calculated for C13H23O2).

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} - 9.8° -10.1° -11.3° -17.8° -24.5° (c = 1.00).
\]

10α-Hydroxy-cedrol (8): 1H NMR: d 3.78 (J = 10, 6; 10-H), d 1.14 (J = 7; 12-H), s 1.04 (13-H), s 1.26 (14-H), s 1.34 (15-H). 13C NMR data are listed in Table I. MS (m/e): M* - CH3 223.1691 (223.1698 calculated for C13H23O2).

\[
\alpha = \frac{589 \text{nm} + 578 \text{nm} + 546 \text{nm} + 365 \text{nm}}{4} + 0.7° + 0.8° + 0.5° + 9.9° (c = 1.00).
\]

12-Hydroxy-cedrol (9): Colorless crystals, m.p. 126 °C. 1H NMR: m 1.77 (2-H), m 1.89 (3-H), m 1.39 (3'-H), m 1.52 (4-H), m 1.41 (4'-H), t 1.82 (J = 8; 5-H), d 1.58 (J = 5; 7-H), m 1.84 (9-H), m 1.68 (9'-H), ddd 1.53 (J = 12, 12, 5; 10-H), m 1.44 (10'-H), m 1.69 (11-H), d 1.46 (J = 13; 11'-H), dd 3.67 (J = 10, 7; 12-H), dd 3.48 (J = 10, 8; 12'-H),...
s 1.31 (13-H), s 1.00 (14-H), s 1.25 (15-H), long-range couplings between 7-H and 13-H, 9-H and 15-H, 11'-H and 13-H, 13-H and 14-H from COSY. ¹³C NMR data are listed in Table I.

\[ \alpha = \frac{589\text{nm} - 578\text{nm} + 463\text{nm} + 365\text{nm}}{+7.3^\circ +7.6^\circ +8.4^\circ +14.1^\circ +22.2^\circ (c=1.00)}. \]

2,10-β-Dihydroxy-cedrol (10): ¹H NMR: m 4.05 (10-H), s 1.28 (12-H), s 1.08 (13-H), s 1.31 (14-H), s 1.45 (15-H). ¹³C NMR data are listed in Table I. MS (m/e): M⁺ 254.1877 (254.1882 calculated for C₁₅H₂₆O₃).

\[ a = +7.3^\circ +7.6^\circ +8.4^\circ +14.1^\circ +22.2^\circ (c=1.00). \]

3α,15-Dihydroxy-cedrene (12): ¹H NMR: q 1.87 (7 = 7.5, 4.5; 2-H), ddd 4.35 (7 = 5, 5, 5; 3-H), m 1.62 (4-H), m 1.64 (4'-H), d(br) 1.99 (7 = 4; 7-H), s(br) 5.52 (9-H), d(br) 2.29 (7 = 11; 10-H), m 1.96 (11'-H), d 0.92 (J = 7.5; 12-H), s 1.02 (13-H), s 0.98 (14-H), d 4.06 (J = 13; 15-H), d 3.96 (J = 13; 15'-H). MS (m/e): M⁺ 236.1775 (236.1776 calculated for C₁₅H₂₆O₂).

\[ a = -6.3^\circ -6.7^\circ -7.6^\circ -12.0^\circ (c = 0.25). \]

10,12,15-Trihydroxy-cedrene (14): ¹H NMR: m 2.32 (2-H), ddd 1.88 (J = 12, 5.5, 5.5; 3-H), ddd 1.58 (J = 12, 5.5, 1.5; 5-H), d(br) 2.06 (J = 4; 7-H), dt 5.84 (J = 3, 1.5; 9-H), dt 3.64 (J = 3, 1.5; 10-H), dd 1.66 (J = 11, 4; 11-H), d 1.34 (J = 11; 11'-H), dd 4.26 (J = 9.5, 8.5; 12-H), dd 3.50 (J = 9.5, 7.5; 12'-H), s 1.05 (13-H), s 0.99 (14-H), dt 4.12 (J = 14.5, 1.5; 15-H), dt 4.06 (J = 14.5, 1.5; 15'-H).

\[ a = -60.3^\circ -63.2^\circ -72.8^\circ -137.0^\circ (c=1.00). \]

Table. ¹³C NMR data of 1—10 and 13 (75.5 MHz, CDCl₃, TMS as internal standard).

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* Assignments may be interchanged.
solved protons so their $^{13}$C NMR spectra were used to elucidate the structure. For this purpose 2D NMR techniques were applied for the assignments of the carbons [6]. The protons of 12-hydroxy-cedrol (9) were identified via homonuclear double resonance or 2D chemical shift correlation ("COSY"). Important informations concerning the relative configuration of the molecule were obtained from the long-range couplings, especially "W"-couplings (Fig. 1).

Once the $^1$H NMR data had been assigned, the carbons can be identified by heteronuclear double resonance ($^1$H/$^{13}$C) (Fig. 2). This procedure was also applied for 4β- and 10β-hydroxy-cedrol (5) and (7). These data were used to assign the $^{13}$C NMR data of the remaining compounds. By comparison of the $^{13}$C shifts the relative configuration of the additional hydroxy group could also be deduced. So 10α- and 10β-hydroxy-cedrol (8) and (7) were distinguished by different shifts caused by the γ-effect at C-11 and the δ-effect at C-3 and C-15. The differences of these effects were not so pronounced with 3α- and 3β-hydroxy-cedrol (4) and (3), so nuclear Overhauser enhancement difference $^1$H NMR spectra were also used to elucidate the configurations.

The biotransformation products are shown in Fig. 3. The different yields reveal that the main attack occurred at C-2 and C-12. 3- and 10-hydroxy-cedrols were formed in epimeric pairs. Their formation could be avoided by using different strains. So *Streptomyces bikiniensis* formed 10α-hydroxy-cedrol (8), while *Verticillium tenerum* gave only 10β-hydroxy-cedrol (7).

For comparison cedrene was transformed by *Corynespora cassicola* DSM 62474, too. Even prolonged fermentation gave a much lower yield than that of cedrol. Beside hydroxylation at allylic positions the 12-hydroxylation was again the main reaction (Fig. 4). Because the overall yield was very low, it must be assumed that the total degradation is the favoured pathway.
Fig. 2. $^1$H/$^1$C-2D NMR spectrum of 12-hydroxy-cedrol (9). (Resonances of C-8 and 12-H not shown.)

Fig. 3. Biotransformation products of cedrol (1).

Fig. 4. Hydroxylation products of cedrene (11) formed by Corynespora cassicola DSM 62474.

Fig. 3. Biotransformation products of cedrol (1).
Conclusions

Biotransformation of cedrol with 5 different strains afforded 8 previously undescribed hydroxycedrols. The attack came from one side of the molecule in a hemisphere centered at C-12 approximately. At two positions epimeric pairs were formed, which could be avoided by using different strains. Outside of this hemisphere no attack was observed, the geminal methyl groups were also not oxidized. Contrary to Wang and coworkers we found the 2- and 12-hydroxylations as the main reactions, the 3β-oxidation led only to a side product. Cedrene which was also used as substrate needed prolonged fermentation and gave lower yields than cedrol. Apart from allylic hydroxylations the oxidation pattern by Corynespora cassiicola DMS 62474 of cedrene and cedrol was the same.

Beside of the production of previously unknown sesquiterpenes and the study of the selectivity of the strains used, the biotransformation presented here produced valuable data for NMR spectroscopy. To date the prediction of $^{13}$C NMR shifts of these complex molecules is relatively uncertain. Now the effects of additional hydroxy groups in the cedrol molecule can be deduced from the data of the cedrols described here. Thus additional data are now available to predict the shifts of related substances.

12-Hydroxy-cedrol (9) can easily be oxidized to give isocedrolic acid, a natural product isolated in 1976 by Kuo et al. [7] from Juniperus squamata Lamb. Some of these biotransformation products were formed in sufficient amounts for tests of their biological activity. These investigations are still in progress.

Acknowledgement

We thank Mrs. Schwab-Hanisch for her very skilful technical assistance with the separation of the compounds, Miss Mull for screening, and Dr. Luder Ernst for the 2D NMR spectra.