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MICROBIAL TRANSFORMATION OF CEDROL

GALAL MAATOOQ,

College of Pharmacy, Faculty of Pharmacy, Department of Pharmacognosy, University of Mansoura, Mansoura, Egypt

SALEH EL-SHARKAWY, M.S. AFIFI, and J.P.N. ROSAZZA*

Division of Medicinal and Natural Products Chemistry, and Center for Biocatalysis and Bioprocessing, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

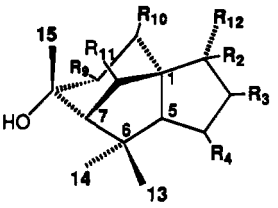
ABSTRACT.—(+)-Cedrol [**1**] was biotransformed by cultures of *Steptomyces griseus* ATCC 10137 and *Bacillus cereus* UI 1477 (NRRL B-14591). Metabolites isolated by solvent extraction and chromatography over Si gel and reversed-phase RP-C_{18} columns and by preparative tlc, were examined by ms and ^{1}H- and ^{13}C-nmr spectroscopy. *B. cereus* afforded 2*S*-hydroxycedrol [**2**] as the only metabolite. The metabolite **2** is apparently formed when cedrol is properly oriented at the cytochrome P-452 hydroxylase of *B. cereus*. *S. griseus* afforded 3*S*-hydroxycedrol [**3**], 4*R*-hydroxycedrol [**4**], 4-oxocedrol [**5**], 10*S*-hydroxycedrol [**7**], 4-oxo-3*S*-hydroxycedrol [**8**], 3*R*,4*S*-dihydroxycedrol [**9**], 10*S*,11*S*-dihydroxycedrol [**10**], and 11-oxo-10*S*-hydroxycedrol [**11**], 4*R*,10*S*-dihydroxycedrol [**12**], and 3*S*,10*S*-dihydroxycedrol [**13**]. Metabolites **5** and **8–13** are new oxygenated cedrol derivatives.

(+)-Cedrol [**1**] is an abundant monohydroxylated sesquiterpene, isolated from the volatile oil of *Juniperus phoenicea* (Cupressaceae). Biotransformation studies of cedrol were conducted to obtain new oxygenated derivatives and to explore the properties of two biocatalysts widely studied in our laboratory. *Streptomyces griseus* ATCC 10137 and *Bacillus cereus* UI 1477 were previously used for stereospecific hydroxylations of the bicyclic monoterpenes 1,4-cineole (1) and 1,8-cineole (2), and the labdane diterpene sclareol (3).

(+)-Cedrol possesses a rigid, tricyclic sesquiterpene structure. It is abundantly available, and it possesses properties which render it potentially valuable as a fragrance or as a synthon for organic chemistry. Previous biotransformation studies of cedrol were conducted using *Aspergillus niger* (ATCC 9142), which afforded 3*S*-hydroxycedrol (3β) [**3**] (4). Abraham *et al.* (5) studied the biotransformation of cedrol by *Rhizopus stolonifer* (CBS 38252), *Streptomyces bikiniensis* (IFO 13350), *Verticillium tenerum* (DSM 633545), *Streptoverticillium reticuli* (DSM 40776), and *Corynespora cassiicola* (DSM 62474). Nine microbial metabolites were isolated in this work and identified as 2*S*-hydroxycedrol [**2**], 3*S*-hydroxycedrol [**3**], 4*R*-hydroxycedrol [**4**], 10*S*-hydroxycedrol [**7**], 3*R*-hydroxycedrol [**14**], 9*S*-hydroxycedrol [**15**], 12-hydroxycedrol [**16**], 10*R*-hydroxycedrol [**17**], and 2*R*,10*R*-dihydroxycedrol [**18**]. In our laboratory, screening experiments demonstrated that *S. griseus* (ATCC 10137) extensively metabolized cedrol while *B. cereus* (UI 1477) produced only one metabolite in relatively low yield. In this report, we describe the structures of new mono- and dioxygenated cedrol metabolites and the major pathways by which oxygenated cedrol derivatives are obtained.

EXPERIMENTAL

INSTRUMENTAL METHODS.—^{1}H-nmr spectra were obtained at 360 MHz on a Bruker WM360 spectrometer while ^{13}C and selective INEPT spectra were obtained at 90.56 MHz. All spectra were obtained in $CDCl_3$ or C_5D_5N with TMS as internal standard. The selective INEPT technique (6,7) was used extensively in assigning the positions of hydroxyl groups and carbon atoms. Low resolution eims spectra were recorded on a Nermag R10-10C mass spectrometer, while hrms were obtained using a VG ZAB HF instrument at 70 eV. Melting points are uncorrected and were determined in open-ended capillary tubes with a Thomas-Hoover melting point apparatus.



	R_2	R_3	R_4	R_9	R_{10}	R_{11}	R_{12}
1	H	H	H	H	H	H	Me
2	βOH	H	H	H	H	H	Me
3	H	βOH	H	H	H	H	Me
4	H	H	βOH	H	H	H	Me
5	H	H	=O	H	H	H	Me
6	H	H	αOH	11	H	H	Me
7	H	H	H	H	αOH	H	Me
8	H	αOH	=O	H	H	H	Me
9	H	βOH	βOH	H	H	H	Me
10	H	H	H	H	αOH	βOH	Me
11	H	H	H	H	αOH	=O	Me
12	H	H	βOH	H	αOH	H	Me
13	H	βOH	H	H	αOH	H	Me
14	H	αOH	H	H	H	H	Me
15	H	H	H	βOH	H	H	Me
16	H	H	H	H	H	H	CH_2OH
17	H	H	H	H	βOH	H	Me
18	βOH	H	H	H	βOH	H	Me

(+)-Cedrol was purchased from Fluka Chemika, and its purity and structure were confirmed by tlc, gc, eims, and ^{1}H- and ^{13}C-nmr spectroscopy before use (5). $NaBH_4$ and PCC were obtained from Aldrich Chemical Company (Milwaukee, WI).

FERMENTATION METHODS.—Cultures were grown according to our standard two-stage fermentation protocol (8) in 125 ml, stainless-steel-capped DeLong culture flasks holding one-fifth volumes of soybean meal/glucose medium. Cultures were incubated at 27° and 250 rpm. Screening and optimization experiments were conducted in 125-ml DeLong flasks, while preparative scale experiments were conducted using 1 liter flasks. For screening-scale experiments, after 24 h, 25 mg of the substrate cedrol in 0.1 ml DMF (1 mg cedrol/ml of culture medium) was added to Stage II culture flasks, which were incubated as described and sampled periodically for analysis. Preparative scale reactions received 100 mg of cedrol in 1 ml of DMF (0.5 mg cedrol per ml of culture medium). Resting cell cultures were prepared by growing Stage II cultures for 24 h, after which the cells were removed from the spent culture medium by centrifugation in an IEC HN-SII centrifuge at 3000×*g* for 10 min. Harvested cells were resuspended in equivalent volumes of pH 6.8 phosphate buffer, incubated as with growing cultures, and received 10 mg/0.1 ml of cedrol in DMF as before.

SAMPLING AND CHROMATOGRAPHY.—Culture samples of 1 ml were taken at 12, 24, 36, 48, 72, and 96 h following substrate addition. Samples were shaken for 1 min with 0.5 ml of EtOAc and spun at 3000×*g* for 1 min in an IEC HN-SII desk-top centrifuge. Samples of 10 ml of the extracts were spotted on Si gel GF_{254} tlc plates which were developed with CH_2Cl_2-Me_2CO (75:25), and spots were visualized by spraying developed plates with 0.5% vanillin/H_2SO_4 reagent followed by heating at 100° for 2 min to give rose-red to violet colors and the following R_f values: **1** 0.95, **2** 0.89, **3** 0.82, **4** and **5** 0.88, **7** 0.58, **8** 0.53, **9** 0.53, **10** 0.42, **11** 0.34, **12** 0.33, and **13** 0.15. Reversed-phase RP-C_{18} (Merck) tlc plates were developed with H_2O-MeCN-HCOOH (60:40:1) to give R_f values: **8** 0.21, **9** 0.30, **10** 0.24, **11** 0.37, **12** 0.40, and **13** 0.58.

SEMIQUANTITATIVE TLC DETERMINATION OF CEDROL METABOLITES IN FERMENTATION EXTRACTS.—MeOH stock solutions of cedrol were prepared such that 10 μl volumes gave spots on tlc plates containing the following amounts of cedrol and cedrol metabolites: 100 μg, 50 μg, 25 μg, 12.5 μg, 6.25 μg, 3.13 μg, 1.6 μg, 0.8 μg, 0.4 μg, 0.2 μg, 0.1 μg and 0.05 μg. Samples of culture extracts were spotted on the same tlc plates with the standards, and plates were developed in CH_2Cl_2-Me_2CO (80:20), sprayed with vanillin/H_2SO_4, and heated for 2 min at 100°. Yields of various compounds in extracts were estimated on the basis of the intensitites of colors obtained vs. amounts of metabolites spotted.

(+)-Cedrol (10 mg/0.1 ml DMF) was added to duplicate second-stage cultures which were incubated at 27° and 250 rpm for 48 h. Metabolites were exhaustively extracted from cultures with 10% MeOH/EtOAc (3×25 ml). Extracts were combined and dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum in a rotary evaporator. The residue was dissolved in 1 ml of MeOH, and the flask was washed twice with 1 ml MeOH to give a total extract volume of 3 ml. Color intensities and spot sizes in extracts from fermentations were matched to those of standards 1 h after visualization when maximum colors were observed. From *S. griseus* growing cultures, the following product yields were estimated: unreacted cedrol [**1**] 0.23 mg (2%), 3*S*-hydroxycedrol [**3**] 1.9 mg (20%), 4*R*-hydroxycedrol [**4**] 1.9 mg (20%), 4-oxocedrol [**5**] 0.06 (1%), 10*S*-hydroxycedrol [**7**] 0.94 mg (9%), 4-oxo-3*S*-hydroxycedrol [**8**] 0.12 mg (1%), 3*R*,4*S*-dihydroxycedrol [**9**] 0.059 (1%), 3*S*,10*S*-dihydroxycedrol [**13**] 1.9 mg (20%), and a mixture of 10*S*,11*S*-dihydroxycedrol [**10**], 11-oxo-10*S*-hydroxycedrol [**11**], and 4*R*,10*S*-dihydroxycedrol [**12**] 2.7 mg (30%). Resting cell incubations gave similar metabolite yields, except for the amount of cedrol recovered which was estimated to be 0.94 mg (9%) and 3*S*,10*S*-dihydroxycedrol [**13**] 0.94 mg (9%).

MICROBIAL TRANSFORMATION OF CEDROL BY *B. CEREUS*.—A 3-liter *B. cereus* culture containing 1.5 g of cedrol was incubated by the standard fermentation procedure. After 24 h the incubation mixture was harvested and extracted exhaustively as before to afford 1.6 gm of crude extract. The extract was purified by Si gel cc (2.5×50 cm/60 gm) and eluted with 0–30% Me_2CO/CH_2Cl_2, and fractions of 10 ml were collected. Fractions 311–388, eluted by 3–4% Me_2CO/CH_2Cl_2, contained 13 mg of **2**, R_f 0.31 [Si gel GF_{254}, Me_2CO-CH_2Cl_2 (1:19)] as colorless needles. Metabolite **2** gave the following results: mp 136–137° [lit. (5) 137°], ms *m/z* (% rel. abundance) 238 (5), 222 (6), 177 (10); 163 (100), 147 (30), 107 (20); ^{1}H-nmr see Table 1; ^{13}C nmr see Table 2; identical to 2*S*-hydroxycedrol (5).

PREPARATIVE SCALE CONVERSION OF CEDROL BY *S. GRISEUS*—Fifteen 1-liter DeLong flasks received a total of 1.5 gm of cedrol. After incubating for 48 h under the usual conditions, cultures were combined and exhaustively extracted with three half volumes of 10% MeOH in EtOAc. The solvent was evaporated under vacuum, the crude extract (1.7 gm) was purified by flash cc on Si gel (Baker, 40 mm flash chromatography packing, J.T. Baker Inc., Phillisburg, NJ, 2.5×50 cm) with Me_2CO in CH_2Cl_2 in a stepwise gradient ranging from 0% to 50% Me_2CO. Fractions (solvent composition) of 10 ml were collected and spotted on Si gel GF_{254} and developed with 25% Me_2CO/CH_2Cl_2. Fractions 123–137 (1% Me_2CO/CH_2Cl_2) R_f 0.95; fractions 138–216 (1%–2% Me_2CO/CH_2Cl_2); fractions 270–329 (2%–3% Me_2CO/CH_2Cl_2); fractions 342–401 (4% Me_2CO/CH_2Cl_2); fractions 402–527 (4%–5% Me_2CO/CH_2Cl_2); fractions 540–632 (5%–7% Me_2CO/CH_2Cl_2); fractions 729–803 (7%–10% Me_2CO/CH_2Cl_2).

PURIFICATION AND PROPERTIES OF CEDROL METABOLITES FROM COLUMN FRACTIONS.—The results of ^{1}H- and ^{13}C-nmr and Selective INEPT experiments are presented in Tables 1, 2, and 3, respectively.

Preparative tlc of fractions 270–329 (48 mg, R_f 0.58) on Si gel GF_{254} [CH_2Cl_2-Me_2CO (9:1)] afforded 27 mg of **3** as colorless needles: mp 158–159°; eims *m/z* (% rel. abundance) 238 (8), 222 (10), 202 (10), 167 (100), 149 (30), 121 (35), 107 (60), 93 (50); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2; identical to 3*S*-hydroxycedrol (5).

Fractions 138–216 (218 mg, R_f 0.86) were concentrated to give 161 mg of colorless needle-shaped crystals, mp 80–82°. The ^{13}C-nmr spectrum revealed more than 20 carbon signals, indicating that the crystals actually consisted of a mixture of one alcohol **4** and one ketone cedrol metabolite **5**. Since it was impossible to separate this mixture of metabolites directly by chromatography, 160 mg of crystals were dissolved in 10 ml of MeOH, and 400 mg of $NaBH_4$ (9) was added. The reduction was complete after 1 h to give a mixture of two compounds at R_f 0.26 and R_f 0.64. The reaction mixture was concentrated to dryness under vacuum and dissolved in 1 ml of MeOH, and the two components were isolated by preparative tlc over Si gel GF_{254}, using Me_2CO-CH_2Cl_2 (1:9) as solvent, to afford 84 mg of 4*R*-hydroxycedrol [**4**] and 23 mg of 4*S*-hydroxycedrol [**6**], each as colorless needles.

Metabolite **4** gave mp 113–114° [lit. (5) 118°], eims *m/z* (% rel. abundance) $[M]^+$ 238 (1), 223 (13), 187 (14), 165 (100), 123 (58), 93 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2; properties of **4** were identical to 4*R*-hydroxycedrol (5). Oxidation of 20 mg of **4** in 3 ml of CH_2Cl_2 with 40 mg of pyridinium chlorochromate for 2 h afforded quantitative yields of **5**. The ketone product **5** gave mp 108–110°; hreims *m/z* $[M]^+$ 236.1773 (100%) (calcd for $C_{15}H_{24}O_2$, 236.1770); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2.

The alcohol **6** gave mp 144–145°; hrms *m/z* $[M]^+$ 238.1928 (100%) (calcd for $C_{15}H_{26}O_2$, 238.1933); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2. Compound **6** (20 mg) was dissolved in 3 ml of CH_2Cl_2 containing 40 mg of PCC (10) and stirred at room temperature for 2 h to give quantitative yields of **5**. The ketone **5** obtained in this way was identical to **5** described above, and carbon signals recorded for **5** (Table 2) were identical to those found in the ^{13}C-nmr spectrum of the original crystalline mixture of **4** and **5** from fraction A.

Fractions 342–401 (152 mg, R_f 0.53) were purified by preparative tlc on Si gel GF_{254} [CH_2Cl_2-Me_2CO (85:15)], and the bands at R_f 0.42 and R_f 0.37 were obtained by extraction from Si gel with Me_2CO. Further

TABLE 1. ^{1}H-nmr Spectral Data for Cedrol [**1**] and Microbial Transformation Products.[a]

Proton	Compound												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
H-12	0.85	1.29	0.96	0.92	0.89	0.89	1.14	1.17	0.98	1.16	1.20	1.15	1.70
	(7.50)	(*S*)	(7.20)	(7.30)	(7.20)	(6.80)	(7.10)	(6.50)	(7.10)	(7.80)	(7.20)	(7.00)	(7.00)
H-13	1.00	1.28	1.26	1.44	1.48	1.40	1.04	1.49	1.43	1.23	1.46	1.42	1.50
H-14	1.32	1.18	1.03	1.35	1.33	1.32	1.26	0.97	1.33	1.29	1.32	1.35	1.65
H-15	1.25	1.05	1.34	1.28	1.03	1.05	1.34	1.33	1.26	1.43	1.11	1.24	1.15
HCOH	—	—	3.60	4.34	—	3.50	3.78	3.89	3.50	3.26	3.99	3.78	4.06
			(H-3)	(H-4)		(H-4)	(H-10)	(H-3)	(H-3)	(H-11)	(H-10)	(H-10)	(H-10)
			dt	dd		m	dd	d	dd	d	dd	dd	dd
			(9.8, 5.1)	(8.6, 4.3)			(9.2, 6.1)	(11.3)	(10.7, 3.2)	(2.6)	(9.8, 6.3)	(9.9, 6.3)	(10.1, 6.5)
HCOH	—	—	—	—	—	—	—	—	4.06	3.78	—	4.36	4.47
									(H-4)	(H-10)		(H-4)	(H-3)
									t	dd		m	dt
									(3.6)	(9.8, 6.4)			(9.8, 5.2)

[a]Spectra were obtained at 360 MHz in $CDCl_3$ with TMS as internal standard. Chemical shifts are given in ppm. Metabolite **13** was determined in C_5D_5N. Values in parentheses are *J* in Hz.

TABLE 2. ^{13}C-nmr Chemical Shifts (ppm) for Cedrol [**1**] and its Microbial Transformation Products.[a]

Carbon	Compound												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
C-1	54.1	57.6	50.8	52.9	53.6	53.0	58.2	49.6	50.3	58.7	57.6	57.5	52.9
C-2	41.5	79.5	50.2	37.8	35.4	38.8	44.6	42.8	49.1	52.2	49.5	39.5	47.1
C-3	37.0	36.5	81.4	46.2	47.7	46.8	40.1	80.8	82.1	46.7	36.4	48.7	81.1
C-4	25.3	21.5	32.1	73.0	220	73.5	26.3	218	73.3	44.5	40.8	73.0	36.2
C-5	56.5	59.2	52.7	60.9	59.7	65.2	52.2	61.9	57.3	60.8	57.1	56.2	56.8
C-6	34.4	44.9	42.5	46.0	46.2	42.8	43.0	45.6	44.6	42.3	46.1	44.8	46.1
C-7	61.1	53.7	61.0	62.7	60.9	61.6	60.9	59.4	61.5	60.2	58.7	61.5	60.6
C-8	75.0	74.8	74.8	74.8	74.4	74.9	74.5	74.4	74.6	72.6	74.1	74.1	73.4
C-9	35.3	35.6	35.3	34.7	33.2	34.8	45.4	34.7	34.2	41.6	46.1	44.6	41.3
C-10	31.3	30.1	34.2	31.3	31.3	31.8	72.0	34.3	31.7	71.0	71.5	71.6	71.2
C-11	42.0	41.3	43.1	42.7	41.4	42.5	40.1	36.1	43.0	65.8	219	40.5	43.5
C-12	15.5	24.3	12.3	15.1	18.3	15.3	14.7	12.9	11.8	15.5	19.0	14.1	12.2
C-13	27.6	28.4	29.5	30.1	28.4	28.3	29.3	28.2	28.7	29.1	28.0	28.9	29.7
C-14	28.9	27.4	27.3	28.9	28.2	28.6	27.4	28.1	30.1	31.3	28.1	31.1	28.4
C-15	30.2	30.4	30.2	30.6	30.4	30.3	31.5	30.5	31.2	32.3	31.1	31.2	32.5

[a]Obtained at 360 MHz in $CDCl_3$ except for metabolite **13** which was obtained in C_5D_5N with TMS as internal standard.

TABLE 3. The Results of Selective INEPT Nmr Experiments with Cedrol Metabolites.

Compound	Irradiated proton (ppm)		Enhanced carbon signal (ppm)												
			1	2	3	4	5	6	7	8	9	11	12	13	14
8	H-14	(0.97)	—	—	—	—	61.9	45.6	59.4	—	—	—	—	28.2	—
	H-12	(1.17)	49.6	42.8	80.8	—	—	—	—	—	—	—	—	—	—
	H-15	(1.33)	—	—	—	—	—	—	59.4	74.4	34.7	—	—	—	—
	H-13	(1.49)	—	—	—	—	61.9	45.6	59.4	—	—	—	—	—	28.1
	H-3	(3.89)	—	—	—	218.1	—	—	—	—	—	—	12.9	—	—
9	H-14	(1.33)	—	—	—	—	—	44.6	61.5	74.6	—	—	—	—	—
	H-3	(3.48)	50.3	—	—	—	—	—	—	—	—	—	11.8	—	—
	H-4	(4.06)	50.3	—	—	—	—	44.6	—	—	—	—	—	—	—
10	H-14	(1.29)	—	—	—	—	—	42.3	60.2	72.6	—	65.8	—	—	—
	H-11	(3.26)	—	—	—	—	—	42.3	60.2	—	—	—	—	—	—
	H-10	(3.78)	—	52.2	—	—	60.8	—	—	—	—	—	—	—	—
11	H-13	(1.10)	—	—	—	—	57.1	46.1	58.7	—	—	—	—	—	—
	H-12	(1.19)	57.6	—	36.4	—	—	—	—	—	—	—	—	—	—
	H-15	(1.31)	—	—	—	—	—	—	58.7	74.1	46.1	—	—	—	—
	H-14	(1.45)	—	—	—	—	57.1	46.2	58.7	—	—	—	—	28.0	—
	H-10	(3.99)	57.6	—	—	—	—	—	—	—	—	218.9	—	—	—
12	H-12	(1.15)	57.5	39.5	48.7	—	—	—	—	—	—	—	—	—	—
	H-15	(1.24)	—	—	—	—	—	—	61.5	74.1	44.6	—	—	—	—
	H-13	(1.35)	—	—	—	—	—	44.8	61.5	—	—	—	—	—	28.9
	H-14	(1.42)	—	—	—	—	56.5	44.8	—	74.1	—	—	—	31.1	—
	H-10	(3.78)	57.5	—	—	—	56.5	—	—	—	—	—	—	—	—
	H-4	(4.36)	57.5	39.5	—	—	56.5	—	—	—	—	—	—	—	—
13	H-13	(1.50)	—	—	—	—	—	46.1	60.6	73.3	—	—	—	—	28.4
	H-12	(1.70)	52.9	—	81.1	—	—	—	—	—	—	—	—	—	—
	H-10	(4.06)	—	47.1	—	—	56.8	—	—	—	—	43.5	—	—	—
	H-3	(4.46)	52.9	—	—	—	56.8	—	—	—	—	—	—	—	—

purification by RP-C_{18} flash cc (1.5×40 cm/25 g) using H_2O-MeCN (70:30) as solvent afforded 11 mg of **7** and 22 mg of **8**, both as colorless needles.

Metabolite **7** gave mp 68–70°; eims *m/z* (% rel. abundance) 238 (2), 224 (25), 205 (5), 177 (12), 150 (35), 135 (40), 107 (30), 95 (30), 87 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2; identical to 10α-hydroxycedrol (5).

Metabolite **8** gave mp 107–109°; hrms *m/z* (% rel. abundance) $[M]^+$ 252.1728 (14) (calcd for $C_{15}H_{24}O_3$, 252.1719), 234 (7), 219 (3), 181 (100), 177 (17), 165 (35), 151 (7), 123 (20), 121 (27), 95 (19); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2.

Fractions 402–527 (41 mg, R_f 0.33) were subjected to RP-C_{18} cc (1.5×40 cm/25 gm), using H_2O-MeCN (70:30), to afford 4 mg of **9** and 18 mg of **10**, both as colorless needles. Metabolite **9** gave mp 139–140°; hrms *m/z* $[M]^+$ 254.1861 (2%) (calcd for $C_{15}H_{26}O_3$, 254.1927), 181 (5), 121 (68), 95 (7), 81 (9), 69 (18). Metabolite **10** gave mp 136–137°; hrms *m/z* $[M]^+$ 254.1874 (2%) (calcd for $C_{15}H_{26}O_3$, 254.1927), 237 (20), 219 (14), 190 (12), 164 (67), 151 (47), 135 (26), 87 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2.

Fractions 540–632 were resolved by RP-C_{18} flash cc (1.5×40 cm/25 gm) using H_2O-MeCN (80:20) to afford 16 mg of **11** and 26 mg of **12**, both as colorless amorphous solids. Compound **11** gave mp 144–146°; hrms *m/z* $[M]^+$ 252.17281 (1%) (calcd for $C_{15}H_{24}O_3$, 252.1719), 235 (70), 220 (73), 217 (65), 166 (65), 121 (38), 119 (45), 110 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2. Metabolite **12** gave mp 115–116°; hrms *m/z* $[M]^+$ 254.1861 (36%) (calcd for $C_{15}H_{26}O_3$, 254.1927), 237 (46), 219 (100), 201 (37), 183 (19), 163 (16), 119 (25).

Preparative tlc of fractions 729–803 (82 mg) (R_f 0.15) on Si gel GF_{254}, using Me_2CO-CH_2Cl_2 (1:4) gave 13 mg of metabolite **13**, R_f 0.15, as a colorless amorphous solid, mp 140–142°, hrms *m/z* 254.1893 (1%) (calcd for $C_{15}H_{26}O_3$, 254.1927), 252 (5), 236(9), 203 (15), 181 (26), 165 (21), 121 (36), 107 (39), 87 (100).

BIOTRANSFORMATION OF **3**, **4**, AND **7** BY *S. GRISEUS*.—Samples of 5 mg of **3**, **4**, and **7** were added separately as substrates to 25-ml volumes of 24-h-old second-stage cultures, and incubations were continued at 27° and 250 rpm. After 24 h, 4-ml samples were withdrawn analyzed by tlc [Si gel GF_{254}, CH_2Cl_2-Me_2CO (75:25)]; and compared with previously isolated oxo-, dihydroxy-, and oxo-hydroxy-cedrol metabolites. It was observed that spots at R_f values 0.53 and 0.15 corresponding to metabolites **8**+**9** and **13** were obtained from **3**; at R_f values 0.53 and 0.33 corresponding to metabolites **8**+**9** and **12** from **4**; and at R_f 0.42, 0.34, 0.33, and 0.15 corresponding to metabolites **10**, **11**, **12**, and **13** from **7**.

RESULTS AND DISCUSSION

S. griseus cultures and *B. cereus* have been used in our laboratory as biocatalysts capable of achieving many useful types of bioconversion reactions (1,2,11,13,14). These have included *O*- and *N*-dealkylations, alicyclic hydroxylation, benzylic and aromatic hydroxylations, epoxidation and epoxide cleavage, and intramolecular etherification. The enzyme system responsible for these reactions has been clearly identified as a soluble cytochrome P450 system in *S. griseus* (12), and studies with inhibitors and CO-difference visible spectroscopy have confirmed the presence of a soluble cytochrome P452 enzyme system in *B. cereus*. We became interested in exploring the possiblity of obtaining oxygenated cedrol metabolites with these biocatalysts.

Screening scale experiments were intially conducted with 10 cultures previously used for the biotransformation of alkaloids, terpenes, antibiotics, and aromatic substrates (11). Screening was made simple by the appearance of brilliant red to violet spots for cedrol and all of its metabolites when developed chromatograms were sprayed with vanillin H_2SO_4 and warmed, either with a heat gun or in an oven at 100°. Of the cultures examined, *S. griseus* could reproducibly convert cedrol into numerous metabolites in apparently good yield. A semiquantitative assessment of metabolite yields suggested that there were five metabolites obtained in 10–20% yield, and that additional minor ones were also present in fermentation extracts. Although initial chromatographic evidence suggested that four or five major metabolites were being produced, it became clear during preparative scale isolation work that major spots consisted of more than one overlapping metabolite.

Quantitative isolation of metabolites was rendered difficult because of substrate and product volatility. However, from a preparative scale biotransformation reaction with *S. griseus*, ten different metabolites were isolated. The successful resolution of cedrol

metabolites ultimately required both normal and reversed-phase cc. There was only one metabolite produced in an estimated 2% yield in *B. cereus* incubations. Although the yields of the compound were low, it was of interest to determine whether the previously suggested enzyme-active site model for the cineole-hydroxylase system of *B. cereus* was suitable in predicting oxygenation sites with cedrol.

The *B. cereus* metabolite was readily identified as 2*S*-hydroxycedrol [**2**] based on spectral comparisons with **2** previously obtained by Abraham *et al.* by microbial transformation (5). The low yield of 2*S*-hydroxycedrol compared to those obtained earlier with 1,4-cineole and 1,8-cineole (1,2) suggests that cedrol binds poorly with the hydroxylating enzyme. In Figure 1 the structure of cedrol is superimposed on the previously proposed model hydroxylating site for *B. cereus* (1). When oriented in such a way that the 2 position is brought in closest proximity to the putative oxygenating site, the oxygen atom on position 8 is above the hydroxylated position, just as the bridgehead oxygen atom of 1,4- and 1,8-cineoles is situated during cineole hydroxylations. The added bulk of the tricyclic sesquiterpene cedrol is likely to preclude the best fit for hydroxylation to occur, resulting in lower yields of **2** vs. the cineoles. A similar argument was used to explain relatively low yields of 2-*exo*-hydroxy-1,4-cineole from 1,4-cineole substrate and the complete lack of 2-*exo*-hydroxylation with 1,8-cineole (1,2).

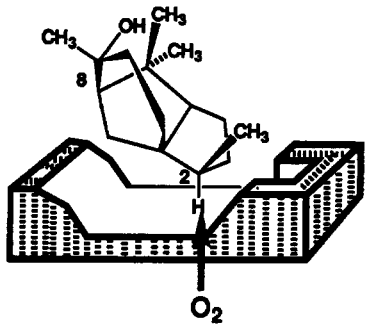


FIGURE 1. The proposed orientation of cedrol on the hydroxylase enzyme active site of *Bacillus cereus*.

The assignment of several isolated cedrol metabolite structures, from *S. griseus*, was made possible by ^{1}H- and ^{13}C-nmr spectra, mass spectra, and melting points, and by comparing these data with the physical and spectral properties of previously isolated microbial transformation products (5). Positions of introduced oxygen atoms and assignments of the four methyl groups (15) were confirmed by the use of the selective INEPT nmr spectral technique. Mass, proton, and carbon spectral properties of 2*S*-hydroxycedrol [**2**], 3*S*-hydroxycedrol [**3**], 4*R*-hydroxycedrol [**4**], and 10*S*-hydroxycedrol [**7**] reported by Abraham *et al.* were nearly identical to those for the same metabolites isolated in our laboratory. The structure of the known metabolite **7**, which was also isolated from *S. griseus* incubations, was established as follows. The mol wt of *m/z* 238 for $C_{15}H_{26}O_2$ indicated the introduction of a single oxygen atom as an alcohol functional group. This was confirmed by both ^{13}C (72 ppm) and ^{1}H (3.78 ppm, dd, J=9, 6 Hz) nmr. Signals in the ^{13}C-nmr spectrum, particularly those for C-9 and C-10 and C-5 and C-6 were significantly different than those found in the starting material cedrol and were in direct agreement for those reported for 10*S*-hydroxycedrol (5). The 10*S* (α)-orientation of the hydroxyl group signal was inferred from the observed large anisotropic effect exerted on the methyl group at position 12 which resonated at 1.14 ppm (J=7.1 Hz

coupled to H-2), or at 0.32 ppm lower field than the same signal in cedrol. In 10*R* (β)-hydroxycedrol [**17**], the Me-12 signal resonates much higher, at 0.92 ppm (5).

A chromatographically inseparable mixture of **4** and **5** was first noticed in the ^{13}C-nmr spectrum where more than the expected 15 carbon signals were observed. The nature of the overlapping carbon spectra clearly indicated that the mixture consisted of an alcohol and a ketone metabolite. Separation of the mixture relied on the facile reduction of the ketone in mixture with $NaBH_4$ and the subsequent resolution of alcohols **4** and **6**. Both alcohols exhibited similar ^{13}C-nmr spectra, indicating the likely existence of isomers containing hydroxyl groups on the same position. Since **4** was the known 4*R*- hydroxycedrol isomer, **6** was assigned as the 4*S*-hydroxycedrol isomer. PCC oxidation of **4** and **6** to the same ketone product **5** confirmed these identities.

Relative stereochemistries of hydroxyl groups of the remaining compounds were inferred from comparisons of ^{13}C-nmr shifts of the literature values, from observed ^{1}H-nmr anisotropic effects, and from coupling constants.

Metabolite **8** was a new cedrol derivative which was ultimately identified as 4-oxo-3*S*-hydroxycedrol. The hrms indicated a molecular ion of 252.1728 for $C_{15}H_{24}O_3$, a metabolite containing new ketone and alcohol functional groups. The ^{1}H-nmr spectrum showed a clear doublet at 3.89 ppm (J=11.3 Hz), due to the coupling of H-3β with H-2β, and the absence of protons at the 4 position. Signals at 80.8 ppm and 218 ppm in the ^{13}C-nmr spectrum confirmed the presence of carbinol methine and ketone functional groups. The positions of the oxygenated carbons were confirmed by selective INEPT (Table 3). Irradiation of the proton signal for Me-12 at 1.17 ppm caused signal enhancement for C-1, -2, and -3, while irradiation of the carbinol methine doublet at 3.89 ppm caused signal enhancement for C-4 and C-12. Selective irradiations of protons at positions 13, 14, and 15 confirmed the structure of the metabolite.

The ^{1}H-nmr spectrum of 3*R*,4*S*-dihydroxycedrol [**9**] gave two carbinol methine signals for H-3 at 3.5 ppm (dd, J=10.7 and 3.2 Hz) and H-4 at 4.06 ppm (t, J=3.6 Hz). The ^{13}C signals at 82.1 and 73.3 ppm were assigned to hydroxylated C-3 and C-4, respectively (5). Selective INEPT experiments confirmed the positions of the hydroxyl groups on the adjacent C-3 and C-4, since irradiation of the signal at 3.5 ppm caused enhancement of the C-1 and C-12 signals (Table 3), while irradiation of the signal at 4.06 ppm caused selective enhancement of the C-1 and C-6 signals. The stereochemistries of the hydroxyl groups were assigned as 3*R*(β) and 4*S*(β) based upon chemical shift and coupling constant values. A β orientation of the hydroxyl group at position 3 was inferred from the ^{13}C chemical shift of C-3 (82.1 ppm) (5) and the coupling constant of 3.2 Hz, due to the 110° dihedral angle between the β- hydrogen at position 2 and the α-hydrogen at position 3. The 10.7 Hz coupling constant for H-3 can be explained by the existence of adjacent 3α and 4α protons with a dihedral angle of 0°.

Metabolite **10** gave hrms *m/z* 254.1874 for $C_{15}H_{26}O_3$, or a metabolite with two additional oxygen atoms. The ^{1}H-nmr spectrum showed a doublet at 3.26 ppm, J=2.6 Hz, assigned for H-11, while the double-doublet at 3.78 ppm, J=9.8, 6.4 Hz, was assigned to H-10β. The signals at 71.0 ppm and 65.8 ppm, in the ^{13}C spectrum, were assigned to an α-hydroxylated C-10 (*S* absolute stereochemistry) and β-hydroxylated (*R* absolute stereochemistry) C-11 position. Signals at 58.7 ppm and 52.2 ppm were assigned to downfield shifted C-1 and C-2, respectively, due to a γ-gauche effect from 10- and 11-position hydroxylations (15). In the selective INEPT spectrum, irradiation of the methyl group signal at position 14 caused enhancement of signals for C-6, -7, -8, and -11. Position 10 was assigned as 10*S*(α)-hydroxy due to the downfield shift of the Me-12 signal at 1.16 ppm.

Assignment of the stereochemistry at C-11 was more difficult, because the carbinol methine protons of the two possible alcohol isomers at position 11 possess nearly

identical dihedral angles with the proton at position 7. However, the *S* absolute stereochemistry of the hydroxyl group could be deduced from the large anisotropic effect exerted on the Me-15. An OH group of the *S* configuration at C-11 is in close proximity to the Me-15, while the corresponding *R* isomer is opposite and away from C-15. Thus, the 1.43 ppm resonance for C-15 (Table 1) in metabolite **10** is consistent with the presence of an 11*S*-OH. The structure of **10** is 10*S*, 11*S*-dihydroxycedrol.

Metabolite **11** gave a hrms *m/z* $[M]^+$ 252.1728 for $C_{15}H_{24}O_3$ a cedrol derivative containing two additional oxygen functional groups. The ^{1}H-nmr spectrum indicated a single carbinol methine proton at 3.99 ppm (dd, J=9.8, 6.3 Hz). ^{13}C-nmr signals at 71.5 (CHOH) and 219 ppm (C=O) were assigned to positions 10 and 11, respectively, by selective INEPT. Irradiation of the carbinol methine proton signal at 3.99 ppm caused enhancement of signals for C-1 and C=O. This fixed both the positions of the alcohol at 10 and the ketone at 11. The 10*S*(α)-hydroxy stereochemistry was inferred from the anisotropic effect on the Me-12 signal, which was shifted downfield by 0.35 ppm relative to cedrol.

The structure of metabolite **12** was established as 4*R*,10*S*-dihydroxycedrol. The hrms of **12** gave *m/z* $[M]^+$ 254.1861 for $C_{15}H_{26}O_3$ a structure containing two additional oxygen atoms. The ^{1}H-nmr spectrum indicated the presence of two new carbinol methine signals at 3.78 ppm (d,d, J=9.9, 6.3 Hz) assigned to H-10, and a multiplet at 4.36 ppm assigned to H-4. Signals at 73.0 ppm and 71.6 ppm in the ^{13}C-nmr spectrum confirmed the presence of two carbinol methine carbons, and the positions were established by selective INEPT (Table 3). Irradiation of the signal at 4.36 ppm caused enhancement of signals for C-1, -2, and -5; irradiation of the carbinol methine proton at 3.78 ppm caused enhanced in signals for C-1 and C-5. Irradiations of proton signals for methyl groups at positions 12, 13, 14, and 15 enabled the direct assignments of ten of the carbons of **12** (Table 3). The relative stereochemistries of these positions were inferred from the chemical shifts and the coupling constants. As shown with metabolites **4** and **6**, 4-hydroxyl groups exert little or no shielding or deshielding effects on Me-12 proton signals. Since the Me-12 signal in **12** resonates at 1.15 ppm downfield compared to cedrol [**1**], the hydroxyl group at position 10 must be *S* or alpha. The assignment of the stereochemistry of the 4 position is more difficult. However, in compounds containing a 4β-OH (**4**, **9**, and **12**), the carbinol methine resonances range between 4.06 and 4.36 ppm, and the Me-15 signal occurs between 1.24 and 1.28 ppm. In the 4*S*(α)-OH isomer **6**, the carbinol methine signal was at 3.5 ppm, and the Me-15 resonance was at 1.05 ppm. In the ^{1}H-nmr spectrum of compound **12** the Me-15 singlet occurred at 1.24 ppm, and homonuclear decoupling of the H-4 signal at 4.36 ppm collapsed the H-5 doublet at 2.31 ppm (J=5 Hz) to a singlet. This result indicates that H-5 is coupled to an H-4α, with a dihedral angle of 0°. The structure of **12** is thus confirmed as 4*R*, 10*S*-dihydroxycedrol.

Metabolite **13** was identified as 3*S*,10*S*-dihydroxycedrol. The hrms gave a molecular ion at 254.1893 for $C_{15}H_{26}O_3$, a cedrol metabolite containing two additional oxygen atoms. The ^{1}H-nmr spectrum gave two carbinol methine signals at 4.06 ppm (dd, J=10.1, 6.5 Hz), assigned to H-10, and 4.47 ppm (dt, J=9.8, 5.2 Hz), assigned to H-3. The Me-12 signal was significantly deshielded at 1.7 ppm, suggesting a 10*S*(α)-OH. The signals at 81.1 ppm and 71.2 ppm in the ^{13}C-nmr spectrum of **13** were assigned to 3*S*- and 10*S*- hydroxylated positions, respectively. The carbinol methine signals for 3*S*-hydroxycedrol and 3*R*-hydroxycedrol resonate at 81.5 ppm and 79.5 ppm, respectively (5). The location of hydroxylated carbons was confirmed by selective INEPT, since irradiation of proton signals for Me-12 selectively enhanced signals for C-1 and C-3; irradiation of the proton signal for position 3 selectively enhanced signals for C-1 and C-5; and irradiation of the proton signal for position 10 selectively enhanced carbon

signals for C-2, -5, and -11. The relative stereochemistries at these positions can be inferred from the coupling constant values. The dihedral angles between the protons at C-2 and C-3 are 110° for H-3α (4 Hz) or 0° for H-3β (11 Hz) (Dreiding model). Coupling of 3.2–5.1 Hz between H-3α (110°) occurs with both H-2β and H-4β, while 9.8–10.7 Hz coupling occurs with H-4α.

Extensive mono- and dioxygenation of cedrol was observed with *S. griseus* as biocatalyst. From the structures of metabolites, it is possible to suggest that the organism likely achieves 3β-, 10α-, and 4β-hydroxylation as the major first steps. All of the other metabolites can derive from these three initial oxidation reactions. Incubations of monohydroxylated substrates revealed that the second oxygenation steps are interlinked, and one metabolite can be derived from different pathways. We observed that 3*R*,4*S*-dihydroxycedrol [**9**] and 4-oxo-3*S*-hydroxycedrol [**8**] were obtained from incubations with both 3*S*-hydroxycedrol [**3**] and 4*R*-hydroxycedrol [**4**]. Furthermore, 3*S*,10*S*-dihydroxycedrol [**13**] was obtained from incubations of 3*S*-hydroxycedrol [**3**] and 10*S*-hydroxycedrol [**7**], and 4*R*,10*S*-dihydroxycedrol [**12**] was obtained from incubations of both 4*R*-hydroxycedrol [**4**] and 10*S*-hydroxycedrol [**7**]. These results permit the description of biotransformation pathways of cedrol as shown in Figure 2.

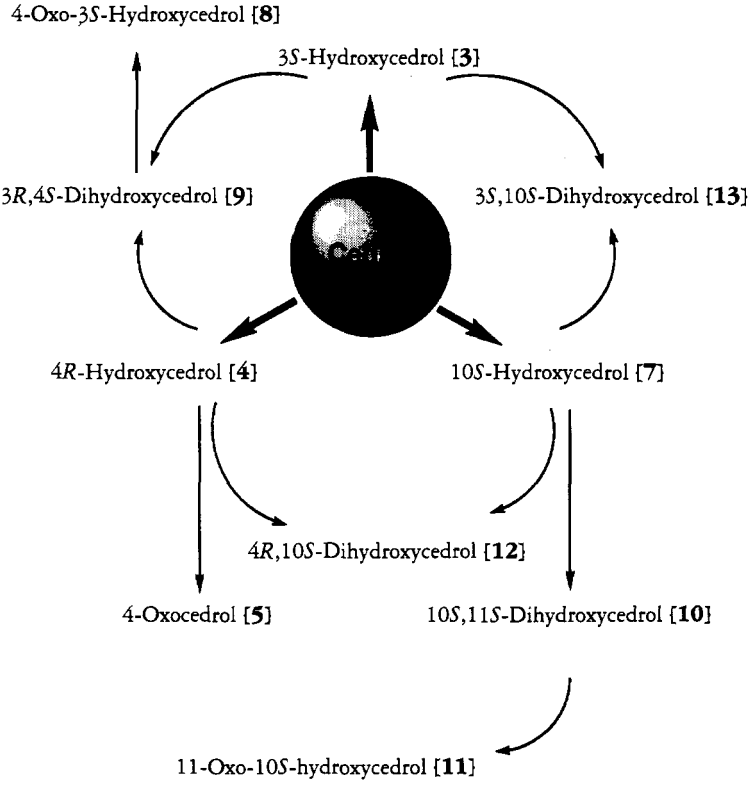


FIGURE 2. Pathway for the formation of mono- and dioxygenated cedrol metabolites by *Streptomyces griseus*.

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