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Geosmin and Related Volatiles in Bioreactor-Cultured *Streptomyces citreus* CBS 109.60

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Streptomyces citreus **CBS 109.60 produced geosmin and a complex pattern of other volatile compounds during cultivation in a 2.5-liter laboratory bioreactor. Volatiles were isolated from disrupted cells, from the culture medium, and from the waste air of the bioreactor by adsorption on Lewatit OC 1064MD. Quantitative and qualitative analyses were carried out using capillary gas chromatography and coupled gas chromatography-mass spectroscopy. *S. citreus* produced 56 volatile compounds, which were mainly terpenoids but also included aliphatic ketones, alcohols, esters, pyrazines, furan(on)es, and aromatic types during the growth phase. The major components were geosmin and a germacradienol. A biosynthetic pathway for geosmin including eudesmanolides is proposed.**

Many streptomycetes are able to produce earthy or musty odors. In 1965 Gerber and Lechevalier identified geosmin and 2-methyl-isoborneol as the underlying chemical principles (11); meanwhile, other terpenoid compounds from geosmin-producing microorganisms, such as cadinane, selinane, or bicyclogermacrane, were identified (6, 10, 15, 21). Apart from streptomycetes, geosmin is produced by certain cyanobacteria, myxobacteria, liverworts, and higher fungi in soil and aquatic environments (6, 15, 17, 18, 21). Geosmin, with a low odor threshold of 10 ng · liter^{-1} (in water), has different effects on the palatability of diverse foods; it contributes a unique characteristic to red beet and whiskey aromas (3). On the other hand, a trace of geosmin can impart an off flavor to sugar beets, sweet corn, canned mushrooms, channel catfish, and drinking water (19). The biosynthesis of geosmin is still not understood, and methods to selectively control geosmin production are still not available.

Previous investigations indicated that geosmin is derived from sesquiterpene precursors (2). Feeding experiments with farnesyl PP_i, however, were unsuccessful, because most sesquiterpenes have an adverse effect on bacterial growth (8). A preceding study was carried out to screen for and to select a rapidly growing and high-yield geosmin producer (results not shown). A strain of *Streptomyces citreus* was identified as the most efficient producer of volatile metabolites. Analysis and identification of the volatile side products were expected to shed light on the structure of possible intermediates of the geosmin pathway. Similar work on the genesis of dehydrogeosmin in the family *Cactaceae* has shown recently that terpenoids of the eudesmane and selinane types were involved in plant metabolism (14). Because of the inherent drawbacks of plant experimental systems, a microbial model was preferred in this study.

MATERIALS AND METHODS

Bacterial culture. *S. citreus* CBS 109.60 was obtained from Centralbureau voor Schimmelcultures, Baarn, The Netherlands, and was used in this study.

Culture conditions. Cultures were grown in a 2.5-liter laboratory bioreactor (Meredos, Bovenden, Germany) at 25°C, 1,000 rpm, and 30% O_2 saturation. This O_2 level was maintained during the cultivation period (variable vvm) with a partial-O_2-pressure-measuring probe and PID controller (Meredos). The medium (V1) was developed during the preceding screening study (data not shown) to maximize geosmin formation. One liter contained 10 g of glucose, 10 g of malt extract, 3 g of yeast extract, 2 g of asparagine, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, and 2 ml of a trace element solution (22). The pH was adjusted to 7.5 with 1 M KOH. The inoculum was prepared from the combined spores and vegetative hyphae of a stationary-phase culture grown on agar plates (V1 medium with 15 g of agar · liter^{-1}, 72 h, 25°C). The cells harvested from one plate were transferred into a 500-ml Erlenmeyer flask with 150 ml of V1 medium and incubated for 24 h at 180 rpm and 25°C. A sample volume of 10 ml was centrifuged (5 min, 3,000 × *g*). The sediment was washed with sterile physiological NaCl solution until glucose was negative (test stick; Boehringer). The cells were resuspended in V1 medium and introduced into the bioreactor.

Sampling and isolation. The first sample was taken after 16 h of incubation. Subsequently samples were taken at 6-h intervals. Each sample (50 ml) was centrifuged at 3,000 × *g*, for 5 min at 4°C. The supernatant was removed and exposed to an excess of Lewatit OC 1064MD, a porous styrene-divinylbenzene resin from Bayer (16). As a rough outline for a mixed adsorption situation, Lewatit can bind solutes in an amount equivalent to 1/10 of its mass within 30 min; as a result, 100 mg of Lewatit was mixed with the 50-ml samples (16). Adsorbed substances were desorbed with pentane-dichloromethane (2:1). The pellet was divided into two equal parts, and one portion was used for dry weight determination. The other portion was stirred with 5 ml of methanol at 60°C for 3 h; all incubations were carried out with methanol in excess (>80%, vol/vol). Subsequently, the incubated sample (methanol plus about 1 ml of cell debris) was diluted with 200 ml of distilled water, and the substances were adsorbed and desorbed as described above. Finally, the waste air of the bioreactor was analyzed by direct adsorption on a glass tubing (length, 50 mm; inside diameter, 5 mm) filled with Lewatit. Gas-phase adsorption was faster but followed the same thermodynamics as liquid-phase adsorption. The various extracts were concentrated to 1 ml with a Vigreux column (40°C, no reflux) and subjected to further analysis. This procedure is known to induce the least changes in flavor extracts.

No corrections were made for changing aeration rates or distribution coefficients during the bioprocess, but these effects can be estimated to be minor. Cell-free media were recurrently analyzed by the same protocol to make sure that none of the volatiles reported was from the medium alone. A fairly constant chemical background was found. The medium contained trace volatiles introduced mainly by the malt and yeast extracts and some heterocycles produced during sterilization along Maillard routes. Terpenoids were not detected.

Biomass determination. Cells were separated from the medium by filtration with a cellulose nitrate membrane (0.45-μm pore size; Sartorius, Göttingen, Germany) and washed with physiological NaCl solution. Membranes were dried at 110°C.

Quantitative and qualitative analyses. Quantitative analysis was carried out by capillary gas chromatography (Carlo Erba Fractovap series 4160), with linalool as an internal standard and C_{10}-methyl ester as an external standard. Qualitative analysis was carried out by coupled gas chromatography-mass spectroscopy (Hewlett-Packard gas chromatograph HP 5890 series II coupled with a Hewlett-Packard mass spectrometer HP 5989 A). Gas chromatography conditions were as follows: capillary column, fused silica CW20M (25 m long; 0.32-mm inside diameter; 0.4-μm film thickness; Leupold, Freising-Weihenstephan, Germany); carrier gas, H_2; split injector, 1:15; flame ionization detector; temperature program, isothermal for 1 min at 40°C, change from 40 to 210°C at a rate of 3°C/min, and isothermal for 10 min at 210°C. Gas chromatography-mass spectroscopy conditions were as follows: capillary column, fused silica DB-WAX (30 m; 0.32-mm inside diameter; 0.5-μm film thickness; J&W, via Fisons); carrier gas,

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TABLE 1. Volatile terpenes produced by *S. citreus* CBS 109.60

Kovats index	Compound: *m/z* (%)[a]	Sample(s)[b]	Amt ($\mu g \cdot liter^{-1}$)[c]
1163	β-Myrcene	a	1
1193	Limonene	a	105
1465	δ-Elemene	a	4
1506	β-Bourbonene	a	2
1562	Sesquiterpene hydrocarbon: 161 (100), 120 (78), 105 (56), 91 (44), 41 (43), 119 (29), 81 (28), 79 (28), M^+ 204	m	10
1570	Sesquiterpene hydrocarbon: 43 (100), 99 (72), 161 (58), 55 (46), 29 (41), 41 (37), 56 (35), 69 (26), M^+ 204	a, m	36
1574	Sesquiterpene hydrocarbon: 43 (100), 99 (72), 161 (58), 55 (46), 29 (41), 41 (37), 56 (35), 69 (26), M^+ 204	a, m	4
1578	β-Elemene	a	7
1583	Calarene	a, m, c	112
1602	Sesquiterpene hydrocarbon: 82 (100), 93 (43), 41 (42), 79 (33), 105 (32), 91 (28), 77 (22), 107 (19), M^+ 204	a	9
1614	Sesquiterpene hydrocarbon: 131 (100), 187 (83), 202 (72), 173 (55), 159 (54), 108 (47), 145 (46), 91 (39), M^+ 204	a	5
1676	Lepidozene	a, m	18
1685	Sesquiterpene hydrocarbon: 80 (100), 134 (87), 105 (82), 133 (69), 91 (65), 119 (57), 79 (55), 81 (55), M^+ 204	m	
1696	Epi-bicyclosesquiphellandrene	a, m, c	484
1703	Germacrene d	m	22
1709	Bicyclogermacrene	a, m	17
1709	Dihydroagarofurane	a, m, c	23
1726	Cadinene	a	27
1745	δ-Cadinene	a	36
1810	Geosmin	a, m, c	1,960
1837	Geraniol	m	37
1979	Sesquiterpene alcohol: (100) 41, (76) 109, (73) 55, (67) 43, (63) 108, (61) 81, (53) 67, (52) 69, M^+ 222	m	70
2063	Sesquiterpene alcohol: 59 (100), 149 (98), 164 (73), 108 (70), 81 (53), 93 (38), 43 (34), 121 (26), M^+ 222	a, m	42
2088	Sesquiterpene alcohol: 82 (100), 43 (86), 41 (73), 67 (52), 55 (50), 81 (43), 93 (37), 161 (35), M^+ 222	a, m	28
2127	Germacradienol	a, m, c	1,029
2141	Sesquiterpene alcohol: 59 (100), 149 (54), 121 (53), 43 (29), 93 (29), 41 (25), 107 (22), 82 (18), M^+ 222	m	19
2160	Sesquiterpene alcohol: 161 (100), 59 (95), 204 (45), 43 (45), 81 (34), 41 (34), 105 (34), 93 (31), M^+ 222	m	130

[a] As suggested by mass spectral evidence; concentrations of unknowns were not sufficient for nuclear magnetic resonance analysis.
[b] a, waste air; m, medium; c, cytoplasm.
[c] Quantitative data represent average values from three consecutive experiments and total content of medium, cells, and waste air at maximum geosmin production after 40 h.

He (0.82 kPa on column injection); temperature program, isothermal for 1 min at 40°C, change from 40 to 210°C at a rate of 3°C/min, and isothermal for 25 min at 210°C; energy of ionization, 70 eV. Kovats index and authentic reference compounds from several essential oils were used for identification.

RESULTS AND DISCUSSION

In contrast to the production of antibiotics, investigations using agar plate-cultured actinomycetes frequently showed a positive correlation between growth and geosmin production (1, 9). In submerged culture, geosmin production by *S. citreus* was closely correlated with the linear growth phase (Fig. 1). During the first 20 h the intracellular geosmin content increased; concurrently geosmin started to pass through the cell membrane and was detected in the medium. The extracellular geosmin concentration, expressed as mass per milliliter of medium, was always higher than the intracellular concentration, expressed as mass per milliliter of cytosol. At the present, no transport mechanisms through the cell membrane are known. The accumulation against the gradient of concentration indicated the presence of an active transport mechanism. Geosmin concentration in the waste air increased continuously during cultivation. The pH dropped steadily during the first 48 h from 7.5 to 6 and then increased again towards the end of cultivation to the initial value.

S. citreus CBS 109.60 produced 56 different volatile compounds (Tables 1 and 2). Most of them were terpenes (two monoterpene hydrocarbons, one monoterpene alcohol, 17 sesquiterpene hydrocarbons, and six sesquiterpene alcohols). In contrast to other streptomycetes, *S. citreus* produced only a few monoterpenes and no 2-methylisoborneol (11). Furthermore, *S. citreus* produced 11 short-chain alcohols, six ketones, four aromatics, five furan(on)es, and three pyrazines. Pyrazines are typical volatile, odorous metabolites of streptomycetes (11). Six main compounds were produced by *S. citreus* in concentrations between 100 and 2,000 $\mu g \cdot liter^{-1}$. Apart from limonene, all main compounds were sesquiterpene hydrocarbons or alcohols. Twenty-six compounds in concentrations between 10 and 100 $\mu g \cdot liter^{-1}$ and 24 compounds in concentrations between 1 and 10 $\mu g \cdot liter^{-1}$ were found.

Tables 1 and 2 show the importance of analyzing samples of different origins. For example, most of the sesquiterpene hydrocarbons were detected in the waste air only. The more polar compounds frequently occurred in the medium sample. After

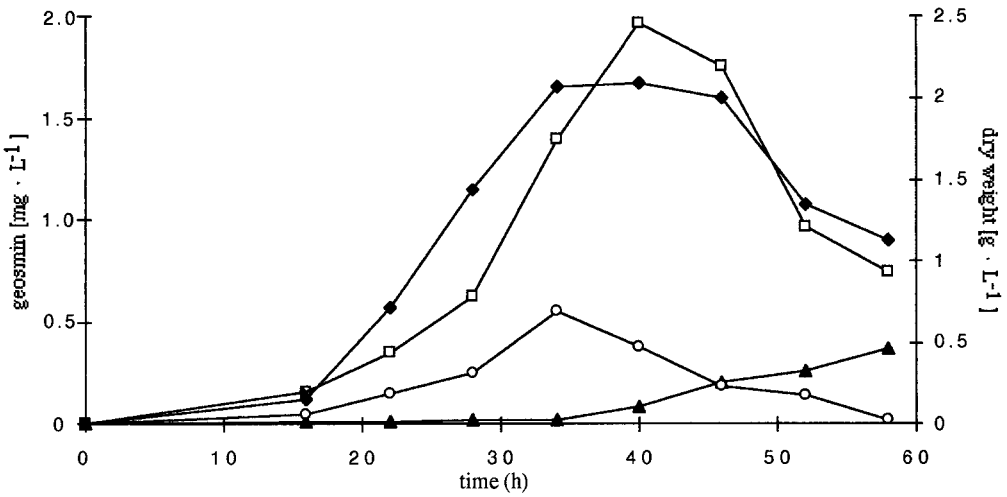

FIG. 1. Growth and geosmin production by a submerged culture of *S. citreus* CBS 109.60 in a 2.5-liter bioreactor (□, extracellular geosmin; ○, intracellular geosmin; ▲, geosmin in waste air; ◆, biomass).

TABLE 2. Nonterpenoid volatiles produced by *S. citreus* CBS 109.60

Type and Kovats index	Compound[a]	Sample(s)[b]	Amt (μg · liter^{-1})[c]
Ketones			
1113	4-Methyl-3-penten-2-one	m	6
1118	4-Heptanone	m	5
1167	2-Methyl-cyclopentanone	m	4
1176	Cyclopentanone	m	8
1275	3-Hydroxy-2-butanone	m	3
1799	2-Hydroxy-3-methyl-cyclopentenone	m	4
Alcohols and esters			
1071	Acetic acid butylester	a	24
1090	2-Methyl-1-propanol	a, m	3
1100	3-Methyl-2-butanol	m	4
1120	2-Pentanol	m	8
1140	1-Butanol	m	14
1153	1-Penten-3-ol	a, m	10
1200	3-Methyl-1-butanol	a, m	5
1248	3-Methyl-3-buten-1-ol	a, m	18
1317	3-Methyl-2-buten-1-ol	a, m	5
1326	3-Methyl-2-pentanol	m	17
1526	2,3-Butandiol	m	17
Pyrazines			
1258	Methylpyrazine	a, m	3
1317	2,5-Dimethylpyrazine	a, m	6
1317	2,6-Dimethylpyrazine	a, m	8
Furan(on)es			
1484	2-Acetylfurane	m	4
1555	3-Methyl-dihydrofuranone	m	11
1569	Dihydro-dimethyl-furanone	m	35
1647	2-Furane-methanol (furfurol)	m	16
1681	Ethyl-dihydro-methyl-furanone	m	19
Aromatic compounds			
1138	1,4-Dimethylbenzene	m	14
1605	Benzoic acid methylester	m	39
1849	Phenylmethanol	a, m	53
1883	2-Phenylethanol	a, m	66

[a] Stereochemistry as suggested by library data of mass spectrometry.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

cell disruption, only those compounds that were most abundant in medium samples, for example, geosmin and germacradienol, were detected. This was merely an analytical effect reflecting the low absolute concentrations of intracellular volatiles.

According to Bentley and Meganathan, geosmin should be derived from the group of eudesmanes (2). Figure 2 shows sesquiterpenes and sesquiterpene alcohols with different basic skeletons that were produced by *S. citreus*: germacrane (diagrams 1 and 2), eudesmane (diagrams 3 and 4), elemane (diagram 5), bicyclogermacrane (diagram 6), cadinane (diagram 7), and aristolane (diagram 8) types were identified. Ruzicka developed a biosynthetic scheme for sesquiterpenoids (20) relating the first five groups by their origin from the cyclization of (*E*2,*E*6)-farnesyl PP_i. The cadinane skeleton should arise from the cyclization of (*Z*2,*E*2)-farnesyl PP_i.

Until now, no enzyme in the biosynthesis of geosmin could

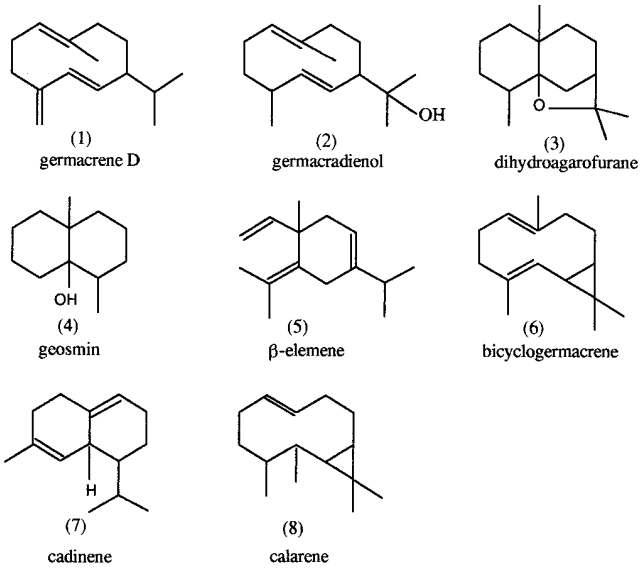

FIG. 2. Sesquiterpenes with germacrane, eudesmane, elemane, bicyclogermacrane, cadinane, and aristolane skeletons produced by *S. citreus*.

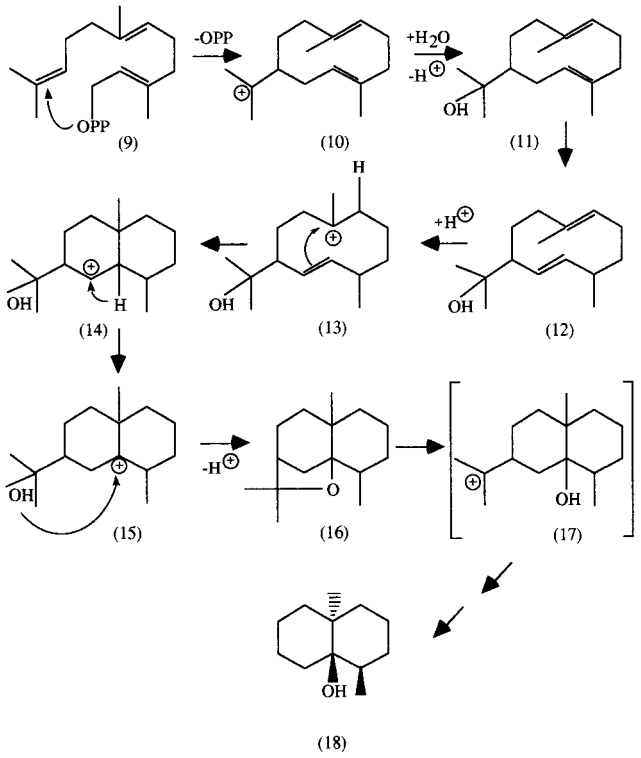

FIG. 3. Postulated route for the biosynthesis of geosmin.

be isolated or identified. Dionigi et al. postulated the participation of a mixed-function oxidase (7). Cyclization of sesquiterpenes frequently leads to different types of germacranes. Croteau postulated that different germacranes arise from (*E*2,*E*6)-farnesyl PP_i (4). In contrast to the function of monoterpene cyclases (5), the behavior of sesquiterpene cyclases has been the subject of few investigations. The best known, farnesyl PP_i cyclase, was isolated from *Penicillium roqueforti*; this enzyme was able to catalyze the complete reaction sequence from the cyclization of farnesyl PP_i to the synthesis of aristolochene (13). Figure 3 shows two constituents closely related to the structure of geosmin (diagram 18): germacradienol (diagram 12) and dihydroagarofurane (diagram 16). Based on the occurrence of these compounds, current knowledge of the cyclization of (*E*2,*E*6)-farnesyl PP_i, and the formation of germacranes (4), a pathway for the biosynthesis of geosmin is proposed with the key intermediates numbered in consecutive order (12).

The pathway starts with two prenyl transferase-mediated condensations of five carbon units yielding (*E*2,*E*6)-farnesyl PP_i (intermediate 9). Replacement of PP_i from C-1 by the distal double bond, as indicated by the arrow, would lead to a germacradienyl cation (intermediate 10). The following step is receipt of a hydroxy ion from water and conversion to hedycariol (intermediate 11). After isomerization of the double bond, germacradienol (intermediate 12) is formed. Hydrogenation of the double bond between C-1 and C-10 then occurs (intermediate 13) and is followed by a mechanistically reasonable cyclization to the bicyclic eudesmanol (intermediate 14). The positive charge of C-6 in intermediate 14 is moved to C-5 by a 1,2-hydride shift (intermediate 15). Dihydroagarofurane and eudesmole are frequently associated in essential oils, implicating an intramolecular electrophilic attack on the positively charged C-5 of intermediate 15 to yield dihydroagarofurane (intermediate 16). No compound corresponding to the hypothetical eudesmol intermediate (intermediate 17) in the biosynthesis of dehydrogeosmin (14) could be isolated. It would have to lose the isopropyl side chain for the final formation of geosmin (intermediate 18).

The sesquiterpenes described were produced in different concentrations. Germacrene D and dihydroagarofurane were found in small amounts of about 20 $\mu g \cdot liter^{-1}$, which may be interpreted as an indication of rapid metabolic turnover. The concentration of germacradienol was $>1{,}000$ $\mu g \cdot liter^{-1}$. Probably different enzymes with different activities are involved in the biosynthesis of geosmin. The occurrence of other sesquiterpenes, such as lepidozene or elemene, indicates the presence of some other farnesyl PP_i cyclases. Because of the ability of *S. citreus* to produce a wide variety of different sesquiterpenes, this strain could represent a valuable tool for future metabolic studies on geosmin and microbial terpene pathways in general. Direct feeding of farnesols, however, caused immediate growth inhibition and must, as a result, be replaced by more-refined methodology.

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